Elevated levels of Bcl-3 inhibits Treg development and function resulting in spontaneous colitis

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Bcl-3 is an atypical NF-κB family member that regulates NF-κB-dependent gene expression in effector T cells, but a cell-intrinsic function in regulatory T (Treg) cells and colitis is not clear. Here we show that Bcl-3 expression levels in colonic T cells correlate with disease manifestation in patients with inflammatory bowel disease. Mice with T-cell-specific overexpression of Bcl-3 develop severe colitis that can be attributed to defective Treg cell development and function, leading to the infiltration of immune cells such as pro-inflammatory γδ T cells, but not αβ T cells. In Treg cells, Bcl-3 associates directly with NF-κB p50 to inhibit DNA binding of p50/p50 and p50/p65 NF-κB dimers, thereby regulating NF-κB-mediated gene expression. This study thus reveals intrinsic functions of Bcl-3 in Treg cells, identifies Bcl-3 as a potential prognostic marker for colitis and illustrates the mechanism by which Bcl-3 regulates NF-κB activity in Tregs to prevent colitis.
The mucosal immune system of the gastrointestinal tract mediates immune protection against foreign pathogens and simultaneously conveys tolerance to microbes in the gut. Failure to tolerate microbial antigens can result in inflammatory bowel disease (IBD), which includes Crohn’s disease (CD) and ulcerative colitis (UC). The pathological process of both CD and UC involves cycles of inflammation, ulceration and subsequent regeneration of the intestinal mucosa. CD is classically considered as a Th1-mediated disease, due to the predominance of interferon-γ (IFN-γ)-producing CD4+ T cells in the mucosa, whereas UC is characterized by infiltrating T12 cells and the production of interleukin (IL)-5 (ref. 3). γδ T cells, which can secrete high levels of the pro-inflammatory cytokine IL-17A in the gut, have important functions in the pathogenesis of IBD7–7.

Regulatory T cells (Tregs) are essential for the maintenance of gut immune homeostasis, owing to their function as suppressors of cytokine production in T12 and T112 cells. Moreover, Treg cells are important mediators of tolerance in the intestine and various studies have linked defects in Treg cell development or function to the onset of IBD. Even though the contribution of T cells in the prevention of IBD is well-appreciated, the molecular factors regulating the functionality of Treg cells during IBD are still not entirely characterized.

The nuclear factor-kB (NF-kB) transcription factor family is composed of five members: RelA (p65), RelB, c-Rel, p50 (NF-kB1) and p52 (NF-kB2). These factors have been implicated in the development and function of natural Treg (nTreg) cells, which develop in the thymus, as well as inductive Treg (iTreg) cells, which are derived from naïve CD4+ T cells after antigenic stimulation in peripheral tissues such as the gut. Indeed, mice lacking NF-kB members such as p50, c-Rel and p65 have impaired Treg cell development. Furthermore, in mice with T-cell-specific transgenic expression of an inhibitor of kB (IkB) super-repressor, the number of CD4+ Foxp3+ T cells correlates with NF-kB activity. Nevertheless, although mice lacking p50, c-Rel and p65 have defective Treg cell development, only mice lacking p65 develop signs of autoimmunity, leaving an open question as to how NF-kB activity modulates Treg cell functionality to prevent the development of autoimmunity.

NF-kB activity is regulated by members of the classical IkB protein family, including IkBα, IkBβ and IkBe, as well as p105/NF-kB1 and p100/NF-kB2 precursors, whereas the atypical IkB proteins, including IkBε, IkBNS and Bcl-3 (ref. 18), bind directly to NF-kB members in the nucleus and modulate NF-kB-mediated gene expression. Bcl-3, originally identified as a proto-oncogene in a subgroup of B-cell leukaemia, enters the nucleus and associates selectively with DNA-bound NF-kB p50 or p52 homodimers to regulate NF-kB-dependent gene transcription. Bcl-3 was shown to enhance NF-kB-mediated transactivation by acting as a coactivator for p50 and p52 dimers. Further studies have shown that Bcl-3 is also able to inhibit NF-kB-mediated transactivation by binding to p50 homodimers. The mode of Bcl-3 action, whether inhibitory or activating, further depends on the cell type investigated. Studies using Bcl-3-deficient mice underline the importance of Bcl-3 in effective adaptive and innate immune responses against pathogens, in central tolerance and the prevention of autoimmune diseases, as well as in effector T-cell plasticity. Moreover, Bcl-3 regulates intestinal epithelial cell proliferation and was shown to be essential for the induction of dextran sulfate sodium-induced colitis. Although these studies indicate a possible involvement of Bcl-3 in the regulation of effector T cells and gut immune homeostasis, the exact functions of Bcl-3 in Treg cells and IBD have not been reported.

In this study, we demonstrate that Bcl-3 is important for the maintenance of Treg cell function and the prevention of spontaneous colitis. Patient data show that Bcl-3 expression levels correlate with disease severity. In line with this, mice that overexpress Bcl-3 in T cells develop severe spontaneous colitis, which is not mediated by effector T cells but instead is caused by impaired Treg cell function resulting from altered NF-kB activity via cell-intrinsic regulation by Bcl-3. In Treg cells, Bcl-3 interacts with p50 and inhibits p50 DNA binding, and thereby alters the NF-kB-mediated genetic programmes that are required for Treg cell development and function. Thus, our study highlights the necessity to monitor Bcl-3 expression in both CD and UC, and implicates Bcl-3 as a potential therapeutic target in IBD.

Results

Bcl-3 expression levels are increased in patients with IBD. To study a potential role of Bcl-3 in the pathogenesis of human IBD, we used immunohistochemistry to examine Bcl-3 expression levels in colons of patients suffering from either CD or UC. This staining revealed that CD and UC patients had massive infiltration of Bcl-3+ cells in the lamina propria (LP), whereas control groups displayed only few Bcl-3 expressing cells within this area (Fig. 1a). Accordingly, quantitative reverse transcriptase–PCR (RT–PCR) analysis using RNA isolated from colons of patients with active CD or UC showed significantly increased levels of Bcl-3 expression compared with controls (Fig. 1b). To investigate specifically which cells express Bcl-3, immunohistochemistry from colon cross-sections of patients with CD and UC was performed. This analysis revealed increased numbers of CD4+ T cells in the inflamed colon of both CD, as well as UC patients compared with healthy patients (Fig. 1c). Indeed, we could show that in most regions, the majority of infiltrating CD4+ T cells was positive for Bcl-3 expression (Fig. 1c). Furthermore, we isolated LP T cells from UC and control patients and analysed the expression of Bcl-3 by western blotting. We observed that patients with UC expressed increased protein levels of Bcl-3 compared with control patients (Fig. 1d). Together, these data illustrate a direct correlation between Bcl-3-expressing CD4+ T cells and the pathogenesis of IBD.

Bcl-3TOE mice develop intestinal inflammation. To evaluate the functional role of increased Bcl-3 expression in T cells in the pathogenesis of IBD, we used a mouse model in which Bcl-3 and enhanced green fluorescent protein (eGFP) are expressed upon Cre-mediated recombination of a loxP flanked transcriptional STOP cassette. These mice were crossed to the CD4-Cre mouse strain to obtain mice, termed Bcl-3TOE, that specifically overexpress Bcl-3 in all mature γδ T cells including Treg cells. Western blot analysis of purified CD4+ T cells of Bcl-3TOE mice confirmed higher expression of Bcl-3 compared with control T cells (Fig. 2a). Starting at 8 weeks of age, Bcl-3TOE mice suffered from severe diarrhoea and rectal prolapse (Fig. 2b, left). The development of a rectal prolapse is a predisposition for colonic inflammation, those mice were examined by mini-endoscopy. We found that Bcl-3TOE mice spontaneously developed severe intestinal inflammation, as indicated by a significantly higher clinical score of colitis (Fig. 2b right and Fig. 2c), with an incidence of >90%. In addition, macroscopic examination of the intestine showed severe pancolitis affecting all parts of the colon distal from the caecum (Fig. 2d). Furthermore, histological haematoxylin and eosin analysis revealed a dramatic infiltration of immune cells into the colon of Bcl-3TOE mice (Fig. 2e). As T-cell-specific Bcl-3 overexpression drives the development of a strong colonic inflammation similar to the histopathology observed in patients with IBD, we further characterized the colitis...
Colitis in Bcl-3TOE mice is not mediated by naïve T cells. To understand how T-cell-specific overexpression of Bcl-3 drives the initiation of intestinal inflammation, we analysed the effect of Bcl-3 overexpression on T-cell pathogenicity. Therefore, we used Bcl-3-overexpressing CD4⁺ T cells to induce intestinal inflammation using the T cell transfer model of colitis. However, transfer of Bcl-3TOE CD4⁺CD25⁻ T cells failed to induce colitis in RAG1⁻/⁻ recipients, whereas control CD4⁺CD25⁻ T cells induced colitis as measured by significantly increased clinical scores of intestinal inflammation (Fig. 3a) and weight loss (Fig. 3b). As Bcl-3TOE CD4⁺ T cells failed to mediate colitis, we assessed the proliferative capacity of these cells, as it was shown that the transfer of naïve T cells into lymphopenic mice initiates their homeostatic proliferation. This analysis revealed an impaired proliferative capacity of Bcl-3-overexpressing CD4⁺ T cells compared with control CD4⁺ T cells upon stimulation (Fig. 3c), an impairment that can explain the failure of these cells to induce transferred colitis. Previously, it has been demonstrated that Bcl-3 overexpression promotes T-cell survival. To evaluate whether this holds true also in our system, we performed Annexin V and 7-aminoactinomycin D staining of purified CD4⁺ T cells cultured for 4 days without stimulation. Indeed, we also found a survival advantage of Bcl-3-overexpressing CD4⁺ T cells compared with control T cells (Supplementary Fig. 2), similar to the data published by Marck et al. To examine whether Bcl-3 has an impact on T-cell differentiation, we analysed naïve versus memory/effector phenotype in these mice and examined gene expression of different pro-inflammatory cytokines. Along with increased levels of Bcl3 transcript, expression of Il6, Il17a, Tnfα and Ifng were significantly elevated in the colons of Bcl-3TOE mice compared with littermate controls (Fig. 2f), whereas expression of Il10 was unaffected (Fig. 2f). Next, we performed immunohistochemistry of cellular infiltrates within the mucosa of Bcl-3TOE mice. We found increased numbers of infiltrating CD4⁺ T cells, CD11c⁺ dendritic cells, F4/80⁺ macrophages and MPO⁺ neutrophils compared with littermate controls (Fig. 2g). Quantification of this analysis revealed a significantly increased cell infiltration of all tested immune cells in the colon of Bcl-3-overexpressing mice, confirming the increased score of colitis as measured by mini-endoscopy (Supplementary Fig. 1). These data demonstrate that increased Bcl-3 expression in T cells leads to severe intestinal inflammation in mice.
T cells in Bcl-3TOE mice. We found a dramatic decrease in the percentage and total cell numbers of CD44\textsuperscript{high} CD62L\textsuperscript{low} effector/memory CD4\textsuperscript{+} T cells in lymph nodes (LNs), mesenteric LN (mLN) and spleens of Bcl-3TOE mice compared with controls, whereas accordingly the percentage of naïve T cells was significantly increased (Fig. 3d,e). This effect we already detected in young mice at the age of 4 weeks before the onset of colitis (Supplementary Fig. 3c,d). As proinflammatory cytokines IFN-γ, IL-17A and granulocyte-macrophage colony-stimulating factor have been implicated in playing important roles in the development and pathogenesis of colitis, we investigated their production by T cells in the intestinal lymphocyte (LPL) compartments of Bcl-3TOE mice as assessed by flow cytometry (Fig. 3f,g and Supplementary Fig. 5) and immunohistochemistry (Fig. 3h). Thus, γδ T cells seem to represent a major population involved in the intestinal inflammatory response of Bcl-3TOE mice.

IL-10 is known to have an ameliorating effect on colitis, we also investigated the production of this cytokine by CD4\textsuperscript{+} T cells. Here, a significant reduction of IL-10 production by Bcl-3TOE CD4\textsuperscript{+} T cells was found, probably contributing to the observed colitis phenotype in Bcl-3TOE mice. However, the effector CD4\textsuperscript{+} T cells themselves do not seem to be responsible for the induction of colitis, as their numbers as well as their production of the inflammatory cytokines IFN-γ and IL-17A are significantly reduced.

Previously, it was demonstrated that the numbers of activated γδ T cells are increased in the colitic area of CD and UC patients\textsuperscript{5,6}. Interestingly, we detected an increased number of γδ T cells, in particular CD8\textsuperscript{+} γδ T cells in the intestinal epithelial cell (intestinal epithelial lymphocyte, IEL) and LP lymphocyte (LPL) compartments of Bcl-3TOE mice as assessed by flow cytometry (Fig. 3f,g and Supplementary Fig. 5) and immunohistochemistry (Fig. 3h). Thus, γδ T cells seem to represent a major population involved in the intestinal inflammatory response of Bcl-3TOE mice.
Figure 3 | Bcl-3-driven gut inflammation is dominated by γδ T cells. (a,b) Naive CD4⁺ CD25⁻ T cells (5 x 10⁵) from Bcl-3 TOE mice (n = 4) or controls (n = 3) were injected i.p. into RAG1⁻/⁻ recipients (n = 5 per group). Recipients were examined for signs of colitis by mini-endoscopy shown as (a) endoscopic score and (b) body weight loss once a week for 5 weeks (mean ± s.e.m.). **P < 0.01 and ***P < 0.001 using unpaired Student’s t-test. (c) In vitro proliferation assay of CD4⁺ CD25⁻ T cells from Bcl-3 TOE mice (n = 3) and littermate controls (n = 3). CD4⁺ T cells were labelled with CFSE and cultured with anti-CD3/CD28 for 4 days. CFSE dilution was analysed by FACS. Upper histogram: CFSE dilution of control CD4⁺ compared with CD4⁺ expression. Numbers in quadrants represent percentage. (d) FACS analysis of LN, mLNs and splenic (spl) cells from 8 weeks old Bcl-3 TOE mice (n = 5) and littermate controls (n = 5) pre-gated on CD4⁺ and analysed for CD44 and CD62L expression. Numbers in quadrants represent percentage. (e) Upper panel: mean percentage and lower panel: total cell numbers of CD4⁺CD62L⁺ and CD4⁺CD62L⁺. (f) FACS analysis of intraepithelial lymphocytes (IEL) and LPL from colon of indicated mice (n = 3). Cells were gated on live cells and analysed for TCRβ and TCRγδ expression. Numbers represent percentage. (g) FACS analysis of IEL of Bcl-3 TOE mice (n = 3) compared with littermate controls (n = 3). Cells were analysed for CD8β and TCRγδ surface expression (percentage displayed). (h) Representative immunohistochemistry of colonic cryosections from indicated mice stained for TCRγδ (red). Nuclei were counterstained with Hoechst 33342 (blue). Scale bars, 50 μm (right) and 200 μm (left), n = 5. Data shown are representative for at least three independent experiments with similar results. Bcl-3γδ littermate mice without Cre were used as controls.
Bcl-3 overexpression impairs suppressive capacity of Tregs. Tregs are essential for the maintenance of immunological tolerance and immune homeostasis by suppressing the activation and expansion of potentially self-reactive T cells. We found a significant reduction in the percentage as well as total numbers of Foxp3\(^+\) Tregs in LNs and spleens of Bcl-3\(^{TOE}\) mice compared with controls (Fig. 4a,b). This reduction was already present in Bcl-3\(^{TOE}\) mice at the age of 4 weeks compared with littermate controls (Supplementary Fig. 3e,f). In mLNs of Bcl-3\(^{TOE}\) mice, only the percentage of Foxp3\(^+\) cells was reduced, whereas the total cell number was similar to the numbers in littermate controls (Fig. 4b,c). Hence, Bcl-3 appears to regulate the development and maintenance of mature Tregs.

Glucocorticoid-induced tumour necrosis factor receptor (GITR) was described as a critical regulator of the interface between Tregs and immune effector cells\(^{36}\). FACS analysis of Treg cells from Bcl-3\(^{TOE}\) mice revealed decreased number of GITR\(^+\) Treg cells and, among them, reduced expression levels of GITR as measured by mean fluorescence intensities (Fig. 4e). Similarly, we found less cells expressing CTLA-4 and lower expression levels of this co-inhibitory molecule (Fig. 4e), which was shown to be a potent negative regulator of T-cell immune responses.

To further evaluate the suppressive capacity of Bcl-3\(^{TOE}\) Tregs, we investigated the expression of the anti-inflammatory cytokine IL-10, a key cytokine mediating the inhibitory activity of Tregs, by intracellular staining. Upon stimulation, Bcl-3\(^{TOE}\) Tregs expressed decreased levels of IL-10 compared with Tregs from littermate controls (Fig. 5a). Similarly, quantitative RT–PCR confirmed a significant reduction in messenger RNA levels of genes relevant for Treg cell development or function, such as Ctla-4, Foxp3, Il10 and Il2r (Fig. 5b). Thus, our results suggest that Bcl-3 suppresses Treg expansion through downregulation of genes critically required for their function.

To test the function of Bcl-3\(^{TOE}\) expressing T cells in vivo, we examined their ability to suppress gut inflammation. Strikingly, the transfer of Bcl-3\(^{TOE}\) Tregs failed to prevent the development of colitis mediated by naïve T cells, in contrast to Tregs from littermate controls (Fig. 5c). To further confirm that the
Bcl-3 regulates Treg development and function. To determine whether Bcl-3 influences Treg cell development and function by extrinsic factors or via an intrinsic mechanism, we crossed Bcl-3\textsuperscript{TOE} mice with the Foxp3-IRES Cre mouse strain\textsuperscript{37} to obtain Bcl-3\textsuperscript{OE} mice without Cre were used as littermate controls. Of note, in Bcl-3\textsuperscript{TOE} mice 14% of Foxp3$^+$ T cells expressed wild-type (wt) levels of Bcl-3 in the same experiments show that endogenous Bcl-3 binds directly to p50 so far. We therefore performed immunoprecipitation experiments that escaped CD4-Cre-mediated recombination (Fig. 6c). In Bcl-3\textsuperscript{TregOE} mice, this effect was much more pronounced, with about 50% of Foxp3$^+$ GFP$^-$ T cells. Interestingly, the analysis of CTLA-4 expression in GFP$^+$ and GFP$^-$ Tregs of Bcl-3\textsuperscript{TOE} and Bcl-3\textsuperscript{TregOE} mice revealed a significant reduction of CTLA-4 expression and geometric mean fluorescence intensity in the GFP$^+$ Treg compartment when compared with GFP$^-$ Tregs (Fig. 6d). Taken together, these data indicate that Bcl-3 negatively regulates the development and function of Tregs intrinsically.

Bcl-3 interacts with NF-κB member p50 in Treg cells. As changes in NF-κB member expression levels might be the cause of impaired Treg function, we analysed whether Bcl-3 overexpression leads to altered expression levels or localizations of some NF-κB family members. As the expression levels and the nuclear localizations of p50, p52 and p65 were similar to those of controls (Fig. 7a), we hypothesized that increased Bcl-3 expression directly modulates the activity of NF-κB B member p50 in Tregs. As changes in NF-κB member expression levels might be the cause of impaired Treg function, we analysed whether Bcl-3 overexpression leads to altered expression levels or localizations of some NF-κB family members. As the expression levels and the nuclear localizations of p50, p52 and p65 were similar to those of controls (Fig. 7a), we hypothesized that increased Bcl-3 expression directly modulates the activity of NF-κB B member p50 in Tregs.
directed against endogenous Bcl-3 and transfected p50. Indeed, both proteins localize in close proximity in the nucleus of Treg cells (Supplementary Fig. 6a).

As Bcl-3 overexpression led to reduced expression of Foxp3, as well as IL-10, CTLA-4 and IL-2R (Fig. 4g), we performed electrophoretic mobility shift assays (EMSA) with DNA probes from intergenic regions within these genes. These probes highlight active H3K4 trimethylation in Tregs\(^\text{39,43,44}\) and contain a recognizable κB enhancer-binding site\(^\text{42}\). Incubating these probes with nuclear lysates from cells overexpressing p50 revealed binding of p50 specifically to κB sites, as complexes were nearly absent with DNA probes with point mutations in κB-binding sites (Supplementary Fig. 6b). We now wondered whether Bcl-3 could alter p50 binding to sequences from these regions as it has been previously suggested from different contexts\(^\text{39,43,44}\). Therefore, we performed pull-down assays using Treg lysates and Bcl-3-expressing lysates with baits is appropriate from the IL-2R\(_a\), Foxp3 and IL-10 gene segments, as well as chromatin immunoprecipitations (ChIP) for p50 in Tregs from Bcl-3\(_{TOE}\) mice.

Pull-down assays revealed that binding of endogenous p50 to DNA was inhibited in the presence of Bcl-3 protein to an extent similar to reactions using a NF-κB-binding site as an NF-κB-targeted gene (Supplementary Fig. 6c). Most importantly, this effect of Bcl-3 inhibiting p50 binding to DNA occurs in Tregs themselves on Treg-relevant genes as seen by CHIP experiments with p50-specific antibodies. We could observe inhibited binding of p50 to the endogenous Foxp3, as well as CTLA-4 promoter in Tregs of Bcl-3\(_{TOE}\) mice, compared with Treg cells of littermate controls (Fig. 7d). Although we observe in all assays only a ~50% reduction in binding of p50 to DNA by elevated levels of Bcl-3, this inhibition was consistently observed through all experiments and reflects also the extent to which NF-κB targeted genes were reduced in expression (Fig. 5b). Together, these experiments demonstrate that Bcl-3 can inhibit the binding of p50 to promoters and sequences containing a NF-κB site and are important for Treg development and function.

**Bcl-3 inhibits p50-mediated NF-κB gene activity.** To test whether Bcl-3 overexpression had a direct effect on p50-mediated gene regulation in Tregs, we designed a reporter vector containing the Foxp3 promoter with a κB-binding site that has been previously shown to bind p50 (ref. 45). Indeed, these
reporter assays revealed diminished activity of the Foxp3 promoter in Tregs from Bcl-3TOE compared with Tregs isolated from littermate controls (Fig. 8a). To test whether this inhibitory effect of Bcl-3 overexpression on gene activity was due to its direct interaction and regulation of p50, we used a Bcl-3 mutant that is unable to interact with p50 (ref. 46) (Fig. 8b). Strikingly, this mutant was not able to repress gene activity compared with wt Bcl-3 construct. In the presence of this construct, we observed enhanced promoter activity (Fig. 8c), possibly functioning as a dominant-negative protein. As NF-kB family members are described to act as heterodimers for activation and as homodimers for repression of genes19–22, we tested whether Bcl-3 affects gene expression through its interaction with the p50/p65 heterodimer. Indeed, we found that endogenous p65 from Tregs is inhibited in its binding to designed IL-2Ra, Foxp3 CN2 (from top to bottom) with protein extracts from Tregs to which Bcl-3 or empty vector overexpressing lysates were added. One out of two experiments is demonstrated. (d) CHIP assay using p50 and isotope control antibodies antibodies were performed in vitro differentiated Tregs from Bcl3TOE mice and littermate controls. Primer were designed as indicated for two sites within the CTLA-4 and Foxp3 Promoter region. One of two representative biological examples is shown.

Discussion

IBD is characterized by the infiltration of T cells that cause colon damage and attract innate inflammatory immune cells. We found drastically elevated expression levels of Bcl-3 in CD4+ T cells isolated from patients with CD and UC, underlining a role for Bcl-3 in the pathogenesis of IBD. In mice, we could define a clear
role for Bcl-3 in intestinal inflammation, as mice overexpressing Bcl-3 in T cells develop spontaneous colitis. This disease was accompanied by infiltration of various pro-inflammatory cells into the colon, including γδ T cells, which might account for the increased levels of IL-17A detected in the colon of these mice.

Previous studies using Bcl-3 deficient animals suggested an intrinsic function of Bcl-3 in constraining the plasticity of pathogenic Th1 cells27. Interestingly, we found that high expression of Bcl-3 in CD4+ cells leads to a reduced number of pathogenic effector T cells. Indeed, the spontaneous colitis seen in our model is not driven by conventional γδ T cells, as these cells fail to proliferate and differentiate into pathogenic T cells and in accordance fail to induce colitis in a passive T-cell transfer model.

We found a suppressive dysfunction of Tregs from Bcl-3TOE mice, as these cells secrete decreased levels of IL-10, shown to be crucial for suppressing γδ T-cell expansion and for the prevention of spontaneous colitis in vivo3. Whether or not the reduced IL-10 production by Bcl-3-overexpressing Tregs is the sole reason for the colitis is not clear, as we also noticed decreased expression levels of Foxp3, CTLA-4, GITR and IL-2R in these Treg cells. In accordance to the above data, the transfer of wt Tregs into Bcl-3TOE mice blocked the development of colitis and inhibited γδ T-cell expansion in the colonic compartment of these mice. These findings underline the suppressive dysfunction of Tregs overexpressing Bcl-3.

Surprisingly, mice overexpressing Bcl-3 specifically in Treg cells (Bcl-3Foxp3OE mice) did not develop any signs of colitis probably due to the very high percentage of GFP−Foxp3+ Treg cells, which was less pronounced using CD4-Cre. These GFP−Foxp3+ cells could have escaped Cre-mediated recombination and fill up the Treg compartment through a proliferative and/or survival advantage over the cells that overexpress Bcl-3. However, also the different time points of Cre activity and the cells targeted could be a potential explanation of this inconsistency. Using the CD4-Cre mouse, Bcl-3 is overexpressed in CD4-expressing T cells during T-cell development, which results in elevated Bcl-3 levels already before Treg development. In addition, in these mice all conventional T cells overexpress Bcl-3 possibly indirectly also influencing Treg cell development/proliferation and/or viability of these cells. In contrast, enforced expression of Bcl-3 using Foxp3-Cre mice occurs later, starting only after Treg cells already developed and only in Foxp3+ Treg leaving all conventional T cells unaffected, and therefore functionally normal. This difference in time of overexpression and the targeted cells may affect how Treg cells are capable to deal with Bcl-3 overexpression.

It is well established that Bcl-3 plays an important role in NF-kB regulation, either promoting or inhibiting target gene expression depending on the cell type and stimulus received47. Bcl-3 selectively interacts with p50 and p52 subunits of NF-kB via its ankyrin domains, thereby regulating NF-kB-dependent gene transcription48–50. In addition to its numerous posttranslational modifications influencing its mode of action, Bcl-3 was also shown to act as an adaptor molecule, possibly building a platform for other coactivators or repressors to p50/p50 homodimers, thereby possibly influencing its mode of action51. Previously, it has been suggested that NF-kB activity is important for Treg development and differentiation, as mice lacking p65, p50 and c-Rel show diminished Treg cell development and defective Foxp3 gene expression15–17. However, p50 and c-Rel-deficient mice do not develop spontaneous autoimmunity even though these mice display decreased numbers of Tregs and defective peripheral Treg differentiation. Therefore, the exact mechanistic role of NF-kB signalling in controlling immune homeostasis remains unknown.

Aberrant expression of Bcl-3 results in a wide range of defects within the immune system but so far, no clear correlation between Bcl-3 expression and subsequent NF-kB activation has been reported for Treg function. In contrast, for T-helper subsets a clear role for Bcl-3 in regulating NF-kB activity has been demonstrated. Here, it was shown that Bcl-3 controls T-helper cell plasticity by preventing the binding of c-Rel and p65 to NF-kB binding sites in the RORγt promoter27. In the absence of Bcl-3, c-Rel and p65 induce Rorc expression leading to Th17 differentiation32. In Th1 cells, Bcl-3 mediates the stabilization of inhibitory p50 homodimers on NF-kB-binding sites, thereby restricting RORγt expression27. Of note, our data using Bcl-3 transgenic mice clearly demonstrate that Bcl-3 overexpression has a global effect on T-helper cell differentiation and impairs Treg function. We found that enforced Bcl-3 expression has a cell type-specific suppressive function in Treg cells leading to repression of NF-kB target gene expression, whereas inflammation and/or NF-kB-driven Bcl-3 expression, as seen
the gut of those mice, results in elevated NF-κB target gene expression. This is in line with previously suggested context-dependent functions of Bcl-3 (ref. 47). We show that in Tregs Bcl-3 directly interacts with p50, thereby preventing its binding to DNA and thus directly inhibiting NF-κB target gene expression, thereby fine-tuning the function of Tregs. How this is achieved is not clear, as Bcl-3 cannot bind to DNA, but it does exert its functions in the nucleus. We show that Bcl-3 is also localized in the nucleus of Treg cells. One possible mechanism is the described interaction of Bcl-3 with histone deacetylases leading to transcriptional termination43,44. We also observe transcriptional inhibition in Treg cells by enforced Bcl-3 expression on p50-regulated genes. In addition, we also show weaker binding of p50 to DNA in the presence of high levels of Bcl-3. However, the exact molecular mechanism for this still remains unclear.

We further propose that the direct interaction of Bcl-3-p50 also prevents the binding of p50/p65 heterodimers to DNA but not through a direct mechanism. p65 was recently illustrated to be essential for Treg stability and controlling the functionality of Tregs, as p65 inactivation specifically in Tregs induces multi-focal autoimmune disease in mice17. Therefore, inhibition of p50/p65 activation by Bcl-3/p50 complexes is likely to be responsible for the suppressive dysfunction of Bcl-3-overexpressing Tregs, leading to chronic inflammatory colitis in Bcl-3OE mice. Here we define a cell-type-specific molecular mechanism by which Bcl-3 regulates NF-κB-dependent gene expression in Treg cells. Our analyses demonstrate the importance to fine-tune Bcl-3 expression for the development and function of Treg cells, as elevated levels of Bcl-3 expression leads to dysfunctional Tregs resulting from diminished NF-κB activity. Specifically targeting the activity of Bcl-3 in IBD may represent an effective strategy for the inhibition of gut inflammation.

**Methods**

**Patients biopsy collection.** For the analysis of Bcl-3 expression in humans, colonic tissue samples were obtained from patients with IBD (CD and UC) and control patients without IBD, who underwent colonic resection or routine colonoscopy (Table 1). Colonic specimens from biopsies and surgical resections from patients with IBD were studied and compared with control samples. The collection of human samples was approved by the Ethics Committee of the University Hospital, Friedrich-Alexander-Universität Erlangen-Nürnberg. Human samples derived from the University Medical Center of the Johannes Gutenberg were analysed from therapeutically indicated biopsies following informed consent and was approved by the Ethical Committee of the Landesärztekammer Rheinland-Pfalz.

**Immunohistochemistry in IBD patients and control subjects.** Immunofluorescence of cryo- or paraffin sections from gut specimens of control patients and patients with IBD was performed using the TSA Fluorescein system (PerkinElmer) and a fluorescence microscope (Olympus IX81)35. In brief, sections were fixed in 4% paraformaldehyde in PBS, before staining paraffin was removed, followed by incubation with avidin–biotin blocking reagent (Vector, SP-2001) and Roti-Immunoblock (ROTH, T144.1, 1:10 dilution in TBST + 2% BSA) for 1 hour in a humidified chamber with primary antibody (anti-human CD4, OKT4, BioLegend, 1:1,000 dilution in Perm buffer; anti-mouse CD3, eBioscience or BioLegend (see Supplementary Table 10). Cells were fixed with 2% formaldehyde to retain EGFP. For intracellular staining of Foxp3 with EGFP, slides were incubated at room temperature for 30 min with biotinylated secondary antibody (111-065-144, Jackson ImmunoResearch, 1:1,000 dilution in TBS). Histological sections that were not optimal for a proper analysis were discarded from the analysis.

**Isolation of human LP T cells.** LP mononuclear cells were isolated from freshly obtained specimens from control and UC patients using Laminap Propria Dissociation Kit (Miltenyi Biotech, catalogue number: 130-097-410). In brief, tissue was incubated in Hank's balanced salt solution with EDTA and dithiothreitol (DTT). After mechanical dissection by vortexing and passing through a cell strainer, cell suspension of epithelial cells and IEL was removed. After incubation with collagenase, DNase and dispase for 30 min at 37 °C, the suspension was subjected to further purification of T cells. LP T cells were prepared from the

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**Table 1 | Patient's characteristic.**

| Group | Gender | Age | Localization |
|-------|--------|-----|--------------|
| Control | ♂ | 64 | Sigmoid colon |
| Control | ♀ | 30 | Sigmoid colon |
| Control | ♂ | 26 | Descending colon |
| Control | ♀ | 33 | Sigmoid colon |
| Control | ♂ | 28 | Transverse colon |
| Control | ♀ | 41 | Term. ileum |
| Control | ♂ | 60 | Descending colon |
| CD | ♂ | 31 | Anastomose |
| CD | ♂ | 35 | Term. ileum |
| CD | ♂ | 28 | Sigmoid colon |
| CD | ♂ | 29 | Caecum |
| CD | ♂ | 30 | Term. ileum |
| UC | ♂ | 36 | Sigmoid colon |
| UC | ♂ | 39 | Sigmoid colon |
| UC | ♂ | 51 | Sigmoid colon |
| UC | ♂ | 56 | Sigmoid colon |
| UC | ♂ | 23 | Sigmoid colon |
| UC | ♂ | 46 | Rectum |
| UC | ♂ | 23 | Sigmoid colon |
| UC | ♂ | 33 | Descending colon |
| UC | ♂ | 39 | Sigmoid colon |
| UC | ♂ | 51 | Sigmoid colon |

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**Mice.** Bcl-3OE mice were generated as described previously30. Bcl-3OE mice were crossed to CD4-Cre mice37 to generate Bcl-3 OE mice and to Foxp3-ires-Cre mice36 to generate Bcl-3OE mice. Age- and gender-matched genetically modified animals carrying loxP sites without Cre transgene (Bcl-3OE mice) were used as control mice on C57BL/6 background. Animals requiring veterinary attention were provided with appropriate care and excluded from experiments. All experiments were performed with 4- to 18-week-old mice (unless otherwise specified). RAG1-/-, Bcl-3OE and Bcl-3OE mice were bred on the animal facility at the University of Mainz. All animal experiments were in accordance with the guidelines of the Translational Animal Research Center, University of Mainz, or to the guidelines of the Helmholtz Zentrum Münchener.

**Real-time PCR analysis.** Total RNA was isolated using RNAeasy Kit (catalogue number: 74104, Qiagen) according to the manufacturer’s instruction. Quantitative real-time PCR was performed using Quantitec Primer Assay (Qiagen). Catalogue number: IL-6: QT00099875; IFN-γ: QT00103882; TNFα: QT00104006; IL-10: QT00103278; TGFβ: QT00106169.

**Flow cytometry.** Single-cell suspensions were prepared from different organs. Red blood cells in cell suspensions from the spleen were lysed with tris-annummonium chloride pH 7.2. Cells were incubated with combinations of antibodies to cell surface determinants. CD4, CD8α, CD25, CD44, CD46, CTLA-4, Foxp3, GTR, Helios, IL-10, TCRβ and TCRγ/δ antibodies were purchased either from BD, eBioscience or Biolegend (see Supplementary Table 10). All samples were acquired on a FACS Canto II BD and results were analysed with FlowJo software. Absolute numbers of thymocyte, LN and splenocyte subpopulations were calculated based on their percentage and total number.

**Staining of Foxp3 together with retention of GFP.** Single-cell suspensions were prepared from mLN. Cells were stained and fixed as described previously38. In brief, cells were surface stained with antibodies purchased from eBioscience, BD and BioLegend (see Supplementary Table 10). Cells were stained with 2% formaldehyde to retain GFP. For intracellular staining of Foxp3 with EGFP retention, 1 × Perm buffer (eBioscience) was used. Samples were acquired on a FACS Canto II BD and results were analysed with FlowJo software.
Cell purification. Cells from spleen and LNs were purified using CD4^+ MicroBeads (Miltenyi Biotech, catalogue number: 130-049-201), the CD4^+ CD62L^+ T-cell streapset (Miltenyi Biotech, catalogue number: 130-093-227) or the CD4^+ CD25^+ Treg cell isolation kit (Miltenyi Biotech, catalogue number: 130-091-041) according to the manufacturer’s instruction. Purity as determined by flow cytometry was over 95%.

Isolation of IEL and LPL. IELs and LPLs were isolated as described previously. In brief, large intestine IEL and LPL were isolated by using a combination of mechanical dissociation and enzymatic digestion. The isolated cells were used directly for FACS analysis.

Survival assay. CD4^+ T cells were isolated by using MicroBeads (Miltenyi Biotech, catalogue number: 130-049-201) from spleens and LNs of 5-week-old Bcl-3^TOE mice and control littermates. Triplicates of 1 × 10^6 CD4^+ T cells were cultured for 4 days in T-cell media at 37°C. Each day cell counts were taken, stained for Annexin V (ImmunoTools, catalogue number: 314900616) and 7-aminoactinomycin D (BD Pharmingen, catalogue number: 559925) according to the manufacturer’s instruction and analysed by FACS.

Western blot analysis of NF-kB members using Bcl-3^TOE Tregs. In vitro-generated Tregs were restimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 2 h and nuclear extracts were prepared. The antibodies used were as follows: anti-β-catenin: Santa Cruz (sc-18, 1:1,000 dilution) and Absent (WA-APO3737, 1:1,000 dilution), anti-p50: Santa-Cruz, (E-10, 1:1,000 dilution) and (PMA) and ionomycin for 2 h and nuclear extracts were prepared. The antibodies In vitro was stained for anti-β-catenin, Noxartis, catalogue number: 55397 then anti-CD3 in the section: Luciferase reporter Assays. Cells were re-stimulated with PMA and ionomycin for 2 h and stained for cover glass slides with poly-l-lysine (SIGMA, P4832). Bcl-3 was stained with anti-Bcl-3 antibodies (Santa Cruz, sc-185 C-14) and p50 was visualized by staining for Flag tag with anti-Flag antibody (E. Kremer, 67).

T-cell culture and in vitro differentiation. Naive CD4^+ CD62L^+ T cells were isolated by using MicroBeads (Miltenyi Biotech, catalogue number: 130-104-453) or Dyna and Detacha Beads (Invitrogen, catalogue number: 11445D and catalogue number: 12406D, respectively) from spleens of 6- to 12-week-old C57BL/6 mice or of Bcl-3^TOE and control littermates, and activated with plate-bound anti-CD3 (using first anti-hamster, Novartis, catalogue number: 55397 then anti-IFN-γ (clone: XMG-121, 10 μg ml^-1^). All antibodies were obtained in collaboration with and from Elisabeth Kremmer (Helmholtz Center Munich). For Treg differentiation additionally the following cytokines were added to cultures: TGF-β (both: R&D Systems, catalogue number: sc-185) and TNF (SIGMA, P4832). For expansion of Treg cells, cells were cultured in RPMI and 2,000 units Proteinkin S (MP Biomedicals, catalogue number: 02238131). For experiments, only samples were used that achieved between 35–85% Foxp3 positive cells (Staining Kit, BD Bioscience: catalogue number: 00552300).

Immunoprecipitation in T cells. T10 and Treg cells were generated and expanded as described above and 1 × 10^5 cells were lysed in 4 ml Meister Lysis Buffer (20 mM Tris/HC1 pH 7.5, 0.25% NP40, 150 mM NaCl, 1.5 mM MgCl2, and Protease Inhibitors (Roche, catalogue number: 04693132001) and 1 mM DTT). 60 μl Protein-G beads (Dynabeads Protein G, catalogue number: 10004D) were pre-coupled with 10 μg antibodies (anti-Bcl-3: Santa-Cruz, catalogue number: sc-185; anti-p50: Abcam, catalogue number: ab7971) in PBS and 0.05% Tween and then equilibrated in Meister Lysis Buffer and added to lysed cells and incubated for 4 h. Washing was performed with Lysis Buffer. Proteins were eluted with 80 μl 1 × SDS Lämml loading dye, one-fourth was used for western blot analysis. Blots were incubated with anti-p50 (Santa-Cruz, catalogue number: sc-8414) and anti-Bcl-3 antibodies (Santa-Cruz, catalogue number: sc-185).

Luciferase assay in T cells. Reporter assays were performed as previously described. The Foxp3 promoter (−422 → +20, position at Exon1) was cloned into the pGL4.10 Luciferase reporter plasmid (Promega, catalogue number: E6651). The TK-Renilla reporter plasmid (Promega, catalogue number: E2241) was used as a control. Treg cells, polarized for 42 h as described above and expanded for 1 day were transfected with different Luciferase reporter and control Renilla reporter constructs by using the Mouse T Cell Nucleofector Kit (Lonza, catalogue number: V4XP3032) according to the manufacturer’s instructions. Sixteen hours after electroporation, T cells were re-stimulated for 6 h with PMA (25 ng ml^-1^, Santa Cruz) and Ionomycin (1 μg ml^-1^, Santa Cruz) and then harvested and measured with the Dual Luciferase reporter system (Promega, catalogue number: E9190). Renilla activity was used to normalize transfection efficiency and Luciferase activity.

Electrophoretic mobility shift assays. HEK293T cells (obtained by ATCC, CRL-11269) were transfected by calcium phosphate transfection with p50-expressing plasmids. Nuclear lysates were generated two days after transfection by incubation in 2 ml (per 10 cm plate) Hypotonic Buffer (10 mM Hepes pH 7.6, 10 mM KCl, 0.1 mM EGTA, 1.5 mM MgCl2, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and the complete protease inhibitor mixture (Roche, catalogue number: 11697498001) and then lysed with the addition of 0.01% Triton X-100. After centrifugation, the supernatant was resuspended in 250 μl of the Nuclear Buffer (420 mM NaCl, 20 mM Hepes pH 7.9, 0.2 mM EDTA, 2% Glyceral, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride with complete protease inhibitor mixture). Nuclear proteins were extracted with intermittent vortexing and incubation for 15 min at 4°C. In vitro binding assays were performed with reagents from the light-shift chemiluminescent EMSA kit (Thermo Scientific, catalogue number: 21450) according to the manufacturer’s protocol, with the exception of using IR Dye800-labelled probes (Table 2). Protein–DNA complexes were separated by 6% TBE Gels (Invitrogen) and LiCor Model 2800 used to visualize EMSA bands.

Pull-down with DNA probes in T cells. For one pull-down reaction, 1 × 10^6 T cells polarized and expanded as described above and nuclear extracts were generated as described in the section 'Electrophoretic mobility shift assays', using

Histology and immunohistochemistry in mice. Colonic cryosections were stained with haematoxylin and eosin. For immunohistochemistry, colon samples were isolated from control and colitic mice at indicated time points. Immunofluorescence of cryosections was performed using the TSA Cy3 System (NEL704A001KT, PerkinElmer) and a fluorescence microscope (IX70; Olympus) using primary antibodies against CD4 (catalogue number: 533043, BD Pharmingen, rmε-5, 1.00 dilution), CD11c (catalogue number: 550283, BD, dilution 1:200), MPO (ab15484 Abcam, 1: 20 dilution), F4/80 (MBI bioscience, Lot 14:4801-81, 1: 1000 dilution) and TC3γ/δ (BD Bioscience, clone N3 H57-597 catalogue number: 553169, dilution 1:100). In brief, cryosections were fixed in 4% PFA for 20 min followed by sequential incubation with methanol, avidin/biotin (Vector Laboratories) and protein blocking reagent (catalogue number: T1441 Roti-Immunoblock, Roth) to eliminate unspecific background staining. Slides were then incubated overnight with primary antibody specific for the respective antigen. Subsequently, the slides were incubated for 30 min at room temperature with biotinylated secondary antibodies (Jackson Immunoresearch catalogue number: 127-065-160 and BD Pharmingen catalogue number: 534011). All samples were finally treated with streptavidin-horseradish peroxidase and stained with Tyramide (Cy3) according to the manufacturer’s instructions (catalogue number: NEL704A001KT, PerkinElmer). Before examination, nuclei were counterstained with mounting medium for fluorescence with 4,6-diamidino-2-phenyldione (catalogue number: H-1200, Vector).
Table 2 | DNA sequences used in EMSAs and pulldowns.

| IL-10 promoter:                                      |
|-----------------------------------------------------|
| CTTGGCCAGGAGGCCCCAAGCTCAGGCTTCA                     |
| IL-10 promoter mutant:                               |
| CTTGGCCAAAGGAAGCAGGCAAAGGCGTCTTA                    |
| CTLA-4 H3 intron-2:                                  |
| GTTGACCGGAGCTGACCTCTGAGAAG                        |
| CTLA-4 H3 intron-2 mutant:                           |
| GTTGACAGGAACTGAAATTCTGACAG                        |
| Foxp3 CNS2:                                          |
| ACCCTACTGGCCTTACCGCTACAG                           |
| Foxp3 CNS2 mutant:                                  |
| ACCCTACCTAAGCTTAAAGGCTACAG                         |
| IL-2 RA promoter:                                   |
| GCAAGGTTTGGAGAAGGCCCTTGGGGT                       |
| IL-2 RA promoter mutant:                            |
| GCAAGGTTTAAAGGCAACTTGGGTG                         |

Sequences were chosen by conservation peaks and a potential NF-κB binding site and GC and CC within the motive were mutated.

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Author contributions

S.R. conceived the study and performed experiments. N.H. and A.W. conceptualized and supervised the whole project. N.H., A.W., S.R. and E.G. prepared the manuscript. E.G. designed and supervised Bcl-3 molecular assays, including Bcl-3 and NF-kB expression analysis, immunoprecipitations, localization studies, as well as EMSAs, reporter assays and CHIP experiments. Y.T. was involved in experimental procedures. A.N. performed immunohistochemistry. C.W. performed immunoprecipitations and pulldown experiments. EMSAs and reporter assays in part developed by and/or taken over by C.G. or E.G. R.W., M.F.N., J.M.S. and P.R.G. provided human samples. B.W. and K.G. performed RT–PCR and western blotting with human samples. J.M. performed experiments. F.T.W. was involved in generating Bcl-3KO mice. I.A.M. was involved in correcting the manuscript. All authors discussed results and conclusions and reviewed the manuscript.

Additional information

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