



HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2016 November 03.

Published in final edited form as:

Oncogene. 2016 December 1; 35(48): 6203–6211. doi:10.1038/onc.2016.152.

The B-cell tumor promoter Bcl-3 suppresses inflammation-associated colon tumorigenesis in epithelial cells

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Abstract

Bcl-3 is an atypical member of the I κ B family. It associates with p50/NF- κ B1 and p52/NF- κ B2 homodimers in nuclei where it modulates transcription in a context-dependent manner. A subset of B cell tumors exhibits recurrent translocations of *Bcl-3*, resulting in overexpression. Elevated expression without translocations is also observed in various B cell lymphomas and even some solid tumors. Here we investigated the role of Bcl-3 in AOM/DSS-induced colon tumors, a mouse model for colitis-associated colorectal cancers in humans. Contrary to expectations, Bcl-3 suppressed colorectal tumor formation: Bcl-3-deficient mice were relatively protected from DSS-induced epithelial damage and developed more polyps after AOM/DSS treatment, though polyp size was unaffected. DSS-challenged mutant mice exhibited increased recruitment of myeloid-derived suppressor cells (MDSCs), consistent with protection of the epithelium. Loss of Bcl-3 in intestinal epithelial cells was sufficient to increase tumorigenesis. The added tumor burden in mutant mice was dependent on TNF α , a tumorigenic, NF- κ B-mediated signaling pathway that was dampened by Bcl-3. These findings reveal a tumor-suppressive role for Bcl-3 in this inflammation-associated cancer model. Bcl-3 thus functions as a tumor promoter or suppressor, depending on the cellular and environmental context.

Keywords

Bcl-3; NF- κ B; colorectal cancer

INTRODUCTION

NF- κ B transcription factors are homo- or heterodimers composed of members of the Rel-related protein family. These factors are activated by a vast array of stimuli, in particular stress- and pathogen-related signals as well as cytokines, and they regulate expression of

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CONFLICTS OF INTEREST

Authors declare no conflict of interest.

Supplementary Information accompanies this paper on the *Oncogene* website (<http://www.nature.com/onc>).

The largely inflammatory cell-derived cytokine TNF α has been reported to play a critical role in inflammation-associated cancers^{13,14}. TNF α targets not only hematopoietic immune cells, but also intestinal epithelial cells; its actions are mediated at least in part via the IKK/NF- κ B signaling pathway, a pathway essential for colitis-associated tumorigenesis, although some individual NF- κ B factors may indeed have opposite functions^{15,16}. In addition, NF- κ B regulates the expression of the cytokine IL-6, which plays an important role in AOM/DSS-induced tumorigenesis by promoting proliferation and survival of intestinal epithelial cells, primarily via activation of Stat3¹⁷.

Inflammation-associated tumorigenesis involves the interplay between immune and epithelial/stromal cells. In addition to the potential tumor-promoting functions of Bcl-3 in epithelial cells discussed above, prior work has shown that Bcl-3 can also modulate inflammatory responses via functions in both stromal and immune cells^{4,18,19}. The role of Bcl-3 may therefore be complex and contrary to the notion that Bcl-3 acts as a tumor promoter, we demonstrate here that Bcl-3 instead suppressed colorectal tumor formation. Mice deficient in Bcl-3 were relatively protected from DSS-induced epithelial damage and developed more polyps after AOM/DSS treatment, while tumor growth was not impacted. DSS treatment led to increased numbers of myeloid suppressor cells (MDSCs) in the absence of Bcl-3, which may have afforded protection to the epithelial layer. The increased tumor burden also appeared to be dependent on TNF α signaling, a pathway dampened by Bcl-3. Finally loss of Bcl-3 in epithelial cells was sufficient for epithelial protection and for the increase in tumor polyps.

RESULTS

Bcl-3 suppresses AOM/DSS-induced colorectal tumorigenesis

To study the role of Bcl-3 in inflammation-associated tumorigenesis, we utilized the AOM/DSS-induced colorectal mouse tumor model. Both wild type (WT) and Bcl-3 knockout (KO) mice were injected once with AOM and 5 days later subjected to three cycles of 2.5% DSS treatment (5 days with DSS, then 16 days without); mice were sacrificed 10 days after completion of the last cycle. Body weights were monitored weekly, and there was no statistically significant difference in weight loss between WT and KO mice (Figure 1a). AOM/DSS treatment induced tumorigenic polyps primarily in the distal part of the colon, which most closely resembles what is seen in patients with ulcerative colitis^{11,12}. KO mice developed significantly more polyps than WT mice (Figure 1b and c). Polyp sizes were comparable (Figure 1d), suggesting that Bcl-3 primarily influenced tumor initiation rather than tumor growth. This conclusion was supported by histochemical analysis of polyp sections, which revealed a similar percentage of PCNA staining cells in WT and KO mice (Figure 1e).

Loss of Bcl-3 affected the immune response to AOM/DSS treatment. Splens of KO mice were more enlarged than those of WT mice and they contained a higher proportion of activated T cells, especially of CD4⁺ effector/memory T cells (Supplementary Figure S1a and b). We additionally observed a notably higher proportion of myeloid-derived suppressor cells (MDSCs; Gr-1⁺ CD11b⁺) in mesenteric lymph nodes of treated KO compared to WT mice (Figure 1f).

Loss of Bcl-3 ameliorates epithelial cell death in DSS-induced colitis

We investigated whether Bcl-3 played a role in responses to either AOM or DSS treatments alone. Injection with AOM alone failed to induce significant weight loss in KO or WT mice (Figure 2a), and also did not alter the appearance of the mucosal epithelium in H&E stained sections (Supplementary Figure S2a). AOM treatment did cause some apoptotic cell death in crypts by 12 h, but there was no significant difference between WT and KO animals (Supplementary Figure S2b). We injected mice with AOM once a week for 6 consecutive weeks. This regimen yielded only few polyps 5 months later, much less than what was observed upon AOM/DSS treatment, with no difference in numbers between KO and WT mice (Figure 2b).

Next we treated mice with 3% DSS for 5 days and monitored their body weight daily for 2 weeks. In agreement with a previous study²⁰, WT mice lost more body weight and recovered more slowly than KO mice (Figure 2c). After 3 days of DSS, but not mock treatment we already observed more apoptotic epithelial cells in WT than KO mice (Figure 2d; quantitated in summary graph), although crypts were still largely intact at this time with no significant difference in mucus production between the two genotypes (Figure 2e). However, by day 6 (one day after 5 days of DSS) colons of WT mice exhibited severe epithelial damage, while the epithelium of KO mice was still largely intact, as assessed with H&E stained sections (Figure 2f). We also noted overall fewer proliferating cells in colons of WT relative to KO mice at this time of DSS treatment (but not after mock-treatment), as analyzed with PCNA staining; this was true even when examining WT sections with relatively better preserved architecture (Figure 2g, left panels; quantitation of total PCNA+ cells shown in top right graph). Rather than reflecting an inherently lower rate of proliferation in WT colonic epithelial cells, the lower numbers of such proliferating cells in WT mice may be due primarily to the increased loss of crypts in these mice (quantitated in bottom right graph in Figure 2g). Thus loss of Bcl-3 may not inherently promote proliferation in colonic epithelial cells in mice challenged with DSS. We furthermore analyzed primary WT and KO mouse colon epithelial cells isolated from unchallenged mice (no DSS) and found no significant differences in their growth in culture or their rate of cell death *ex vivo* (Supplementary Figures S2c and d). These data suggest that Bcl-3 did not intrinsically alter these parameters under homeostatic conditions, consistent with *in vivo* data.

Intestinal epithelial cell-specific loss of Bcl-3 promotes tumor formation

To determine in which cell type Bcl-3 acted to suppress inflammation-associated tumorigenesis in this model, we first performed reciprocal bone marrow transfer experiments and then treated recipient mice with AOM/DSS to induce colorectal tumors. We consistently observed more polyps in KO recipients compared to WT recipients, regardless of the type of bone marrow transferred (Figure 3a). This result indicated that radiation resistant cells (primarily stromal cells) were critical for the protective effect of Bcl-3.

To determine whether Bcl-3 might promote DSS-induced damage and suppress tumorigenesis via functions in colonic epithelial cells, we generated mice conditionally ablated of Bcl-3 in intestinal epithelial cells (*Bcl-3^{flx/flx}; Villin-Cre*) (see Supplementary Figure S3a). Similar to mice globally lacking Bcl-3, those lacking Bcl-3 only in intestinal

epithelial cells were largely protected from DSS-induced colitis when compared to Bcl-3-sufficient control mice, as judged by ameliorated weight loss and a better-preserved colonic epithelium (Figure 3b and Supplementary Figure S3b). Furthermore, DSS-treated mice lacking Bcl-3 in intestinal epithelial cells recruited more MDSCs into mesenteric lymph nodes and colon lamina propria than mice sufficient in Bcl-3 (Figure 3c). Finally, mice ablated of Bcl-3 in intestinal epithelial cells developed significantly more polyps compared with control mice after treatment with AOM and DSS, though possibly slightly fewer than mice with germline deletion (Figure 3d). Therefore, Bcl-3 functioned primarily in intestinal epithelial cells to suppress inflammation-associated tumorigenesis.

Bcl-3 suppresses TNF α responses

Anti-TNF α therapy has shown some efficacy in treatment of IBD patients²¹. Although it remains unsettled whether TNF α is critical for development of colitis in mouse models, possibly owing to differences in models and experimental details in various studies, this cytokine has been more clearly linked to tumor development in the AOM/DSS model^{13,22,23}. TNF α mediates its functions in large part via activation of NF- κ B, whose activity is also strongly associated with tumor formation. Given that Bcl-3 can dampen nuclear NF- κ B activity, we hypothesized that Bcl-3 might suppress TNF α -mediated pro-tumorigenic activities. To this end we first examined TNF α mRNA levels after 3 days of DSS treatment and found no significant difference between WT and KO mice (Figure 4a). We then investigated whether Bcl-3 might modulate responses to TNF α . We generated primary MEFs from WT and KO animals, then treated these cells with 10 ng/mL TNF α for 1 h and 4 h and assessed mRNA levels for IL1 β , IL-6, Bcl-2, I κ B α and TNF α with RT-PCR assays. MEF cells lacking Bcl-3 (KO) exhibited higher expression levels for all these TNF α target genes than WT MEFs, except for TNF α itself (Figure 4b). We additionally assessed mRNA levels of several chemokines that might play a role in recruiting MDSCs, and found higher TNF α -induced levels for CXCL2, but not CXCL1 or CCL2, in KO compared to WT MEFs (Figure 4b). We also isolated intestinal epithelial cells to examine their responses to TNF α *in vitro*. Responses were modest in these cells, but loss of Bcl-3 did result in higher TNF α -induced expression of IL1 β , IL-6, Bcl-2 and I κ B α , but not TNF α , consistent with the results obtained with MEFs (Figure 4c), although we could not discern a difference in what were very low expression levels of CXCL2. Nevertheless, we did detect significantly elevated expression of CXCL2 in colons of DSS-treated mice specifically lacking Bcl-3 in intestinal epithelial cells when compared with WT mice (Figure 4d, left graph), which supports the notion that this chemokine may have a role in increased recruitment of MDSCs. We found no difference in TNF α expression in the colons of these latter mice (Figure 4d, right graph), consistent with above findings. Together these data do suggest that Bcl-3 dampens several, though not all TNF α -induced responses.

To gain insight into how Bcl-3 might inhibit responses to TNF α , we first investigated for potential effects on the activation of the classical IKK/NF- κ B pathway in both MEFs and colon epithelial cells. The loss of Bcl-3 did not affect TNF α -induced degradation of I κ B α in either cell type (Supplementary Figure S4a and b). Furthermore, there was no apparent difference between WT and KO MEFs in TNF α -induced nuclear translocation of RelA (Supplementary Figure S4c). However, ChIP analyses of the IL-1 β and IL-6 promoters

revealed higher levels of TNF α -induced association of RelA in KO as compared to WT MEFs (Figure 4e), consistent with the mRNA expression data above; this suggests Bcl-3 may delimit access/binding of RelA to these particular promoters (Figure 4e).

We furthermore investigated for potential effects of Bcl-3 on the alternative/non-canonical NF- κ B signaling pathway. Upon treatment of primary mouse colon epithelial cells with Light *in vitro* we did not observe a difference in induced processing of p100/NF- κ B2 to p52 between WT and KO cells (Supplementary Figure S4d). In addition, we detected similar levels of Light-induced nuclear translocation RelB/p52 complexes, as judged by co-immunoprecipitation (Supplementary Figure S4d, bottom panel). Finally, Bcl-3 did not affect p100 to p52 processing in MEFs upon treatment with TWEAK (Supplementary Figure S4e).

The suppressive effect of Bcl-3 on tumor formation is TNF α -dependent

Since Bcl-3 negatively regulated TNF α responses *in vitro*, we asked whether the suppressive effect of Bcl-3 on tumor formation might be dependent on TNF α *in vivo*. To address this question we generated mice lacking both Bcl-3 and TNF α (double knockout; dKO) and initially subjected these mice, WT mice and mice lacking either Bcl-3 or TNF α to DSS for 5 days; we measured body weights for 9 days, at which point Bcl-3 KO mice had already lost less weight than WT mice. Absence of Bcl-3 in mice lacking TNF α (dKO) failed to reduce the weight loss experienced by TNF α KO mice, which was similar to the weight loss seen in WT mice (Figure 5a). Furthermore, dKO mice experienced epithelial damage to a degree similar to that in TNF α KO mice (and similar to that of WT mice), as revealed with IHC analysis (Figure 5b). These results indicated that loss of Bcl-3 on a TNF α knockout background no longer afforded these mice added protection against DSS-induced damage, while loss of Bcl-3 on an otherwise WT background did help to protect mice. We then challenged mice with AOM/DSS. Mice lacking TNF α had a tendency to develop slightly fewer polyps relative to WT mice, but this trend did not reach statistical significance with the numbers of animals analyzed. Importantly, loss of Bcl-3 on the TNF α KO background failed to increase the number of polyps, even though loss of Bcl-3 on a TNF α -sufficient background significantly increased polyp numbers relative to WT mice (Figure 5c). Consistent with this, dKO mice did not exhibit increased recruitment of MDSCs in mesenteric lymph nodes or lamina propria compared to TNF α KO mice, and there was no difference in expression of mRNA for CXCL2 (Figure 5d and Supplementary Figure S5). Thus the increased tumor burden seen in mice deficient in Bcl-3 no longer occurred in the absence of TNF α .

DISCUSSION

Here we investigated the role of Bcl-3 in an inflammation-associated colorectal carcinogenesis model. Bcl-3 has been ascribed oncogenic activities, based in large part on recurrent translocations of this gene in certain B cell leukemias, translocations that lead to overexpression of this protein, absent any mutations⁴. Although no translocations have been noted in solid tumors, some of these cancers exhibit elevated levels of Bcl-3⁶. However, there is as yet no definitive evidence for a causative role of Bcl-3 in the genesis of these

solid tumors. Nevertheless, some findings have linked Bcl-3 to cell survival and proliferation, which would be consistent with oncogenic activity⁶. It was therefore unexpected that Bcl-3 suppressed, rather than promoted colorectal tumor formation in the AOM/DSS model. We chose to investigate the role of Bcl-3 in this inflammation-associated carcinogenesis model, because prior work suggested that Bcl-3 functions in both immune cells and stromal/epithelial cells and thus may control the interplay between these cell populations at barrier sites^{18–20,24,25}.

Our study demonstrates that Bcl-3 suppressed tumor initiation, limiting the number of polyps, rather than controlling tumor progression, as judged by the size of polyps and their proliferative status, which were similar in Bcl-3 sufficient and deficient mice. This suggests that Bcl-3 acted during the early phase in the AOM/DSS model to suppress tumorigenesis. Germline loss of Bcl-3 helped protect mice from excessive DSS-induced colitis, as also noted previously²⁰. We now demonstrate here that loss of Bcl-3 specifically in colonic epithelial cells was sufficient to protect the epithelial cells from excessive DSS-induced cell death. The preservation of the epithelial layer in Bcl-3-deficient mice most likely underlies the increase in numbers of tumors, as presumably more of the AOM-mutated cells survived the DSS-induced stress, enlarging the pool of potential tumor-initiating stem cells.

The protection that loss of Bcl-3 afforded DSS-stressed epithelial cells may have been mediated, at least in part, by the significant increase in myeloid-derived suppressor cells (MDSCs) observed in mesenteric lymph nodes and the lamina propria of mutant mice. These cells usually accumulate at sites of strong inflammatory reactions/cell damage and are thought to help resolve inflammation and support tissue repair. As such, MDSCs are likely to provide an environment beneficial to survival of DSS-stressed and AOM-mutated epithelial cells, thereby promoting tumor development^{26,27}. Increased accumulation of MDSCs correlated with increased expression of CXCL2 in the colon of DSS-challenged mice lacking Bcl-3 in intestinal epithelial cell; CXCL2 is a chemokine known to be involved in the recruitment of MDSCs²⁸. However, the exact mechanisms by which Bcl-3 controlled recruitment of MDSCs remains to be determined.

Bcl-3 acted in colonic epithelial cells to suppress AOM/DSS-induced tumorigenesis. Loss of this regulator specifically in these cells was sufficient to increase polyp numbers to nearly the same degree as germline deficiency did. It is possible that other radioresistant mutant cells may have made additional, albeit limited contributions in mice with germline deficiency in Bcl-3. Nevertheless, the fact that loss of Bcl-3 in colonic epithelial cells was sufficient raised the possibility that Bcl-3 may function in these cells at least in part by modulating responses in the TNF α /NF- κ B signaling pathway. TNF α signaling into colonic epithelial cells and the resulting activation of NF- κ B has been reported as critical for AOM/DSS-induced tumor development^{13,22}. While loss of Bcl-3 increased tumor burden relative to WT mice, loss of Bcl-3 in TNF α -deficient (dKO) mice failed to increase tumor burden relative to TNF α KO. Thus the tumorigenic effect resulting from loss of Bcl-3 appeared to be dependent on TNF α signaling. We furthermore found that Bcl-3-deficient fibroblasts and epithelial cells were relatively hyper-responsive to TNF α , exhibiting more pronounced induction of some NF- κ B-dependent targets genes. Since Bcl-3 has previously been shown to inhibit certain NF- κ B-regulated genes in epithelial/stromal cells¹⁸, Bcl-3 may blunt NF-

κ B activity on genes relevant in the present context. Indeed the roles of Bcl-3 revealed in our study are exactly opposite those of IKK β or IKK γ /Nemo, primary mediators of classical NF- κ B activation pathways. Signaling via IKK β /Nemo in epithelial cells protected mice from DSS-induced colitis, while it promoted AOM/DSS-induced colorectal tumorigenesis^{15,29}. Bcl-3 may thus suppress tumorigenic functions of classically activated NF- κ B within epithelial cells, while also promoting DSS-induced colitis via these cells. Bcl-3 did not however modulate the classical IKK-dependent signaling pathway per se, as it did not impair TNF α -induced I κ B α degradation or nuclear translocation of RelA, but instead delimited binding/access of RelA complexes to select regulatory gene regions, as shown here. Bcl-3 also did not modulate the non-canonical/alternative signaling pathway per se. It remains to be shown how exactly Bcl-3 functions to modulate actions of various NF- κ B complexes in specific gene regulatory regions, including whether its functions are mediated locally via binding to p50/NF- κ B1 and/or p52/NF- κ B2 homodimers. Of note, different NF- κ B factors appear to have distinct and even opposing roles in AOM/DSS-induced inflammation and tumorigenesis, based on studies with mice deficient in individual factors¹⁶; it is however unclear how these findings relate to our studies, given that an individual NF- κ B factor can be part of physically and functionally distinct NF- κ B dimers, and given that the study cited involved germline-deficient mice, in which functions of all cells are altered.

It is intriguing that IL-6 was among the TNF α -induced and NF- κ B-dependent target genes whose expression was increased in Bcl-3-deficient epithelial cells and fibroblasts. This particular pro-inflammatory cytokine is considered crucial for colorectal tumor development via its ability to activate Stat3 in epithelial cells, which in turn is thought to promote proliferation and especially survival of these cells. Also, Stat3-deficiency in epithelial cells was reported to exacerbate DSS-induced colitis. Thus a TNF α /NF- κ B/IL-6/Stat3 axis may help assure survival of DSS-stressed, AOM-mutated epithelial cells, preserving the epithelial barrier, but at the cost of promoting tumorigenesis^{17,30}. Bcl-3 may normally dampen this tumorigenic pathway, and thus loss of Bcl-3 would enhance it.

Bcl-3 modulates NF- κ B-regulated responses, and as such may help maintain homeostasis by properly balancing interactions between epithelial/stromal and immune cells. As shown here, loss of Bcl-3 ameliorated DSS-induced colitis damage, but at the same time promoted tumor development after AOM/DSS challenge. While colitic inflammation is required to induce tumors in this model, excessive inflammation may in fact suppress tumor initiation. Bcl-3 may act primarily in epithelial cells to dampen the tumor promoting effects of the TNF α /NF- κ B pathway and to delimit the accumulation of MDSCs. Although Bcl-3 is an established tumor promoter in B cells, it appears to function as a tumor suppressor in epithelial cells in the AOM/DSS model. Bcl-3 is thus a prime example of a regulator whose contributions to tumor development are entirely dependent on context. The Bcl-3 pathway may provide new avenues to therapeutically control colitis-associated cancer development.

MATERIALS AND METHODS

Mice

Bcl-3 germline-deficient and conditionally-deficient mice have been described^{25,31,32}. TNF α deficient (005540) and Villin-Cre (018963) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). 8 to 12 weeks old littermates were used for all experiments. Mice were housed in National Institute of Allergy and Infectious Diseases facilities, and all experiments were done with approval of the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee and in accordance with all relevant institutional guidelines.

Immunohistochemistry

Colon tissue samples were fixed with 10 ml of 10% formalin buffer and embedded in paraffin. Sections (5 μ m) were cut and stained with hematoxylin and eosin (H&E). PAS and Alcian blue staining were performed to detect mucus production. The ApopTag kit (Millipore, Billerica, MA, USA) was used to detect apoptotic cells, following the manufacturer's instructions. Histology of DSS treated colon was scored by a combination of inflammatory cell infiltration (score 0–3) and tissue damage (score 0–3) as previously described³³. (Presence of occasional inflammatory cells in lamina propria scored as 0, increased numbers of inflammatory cells in lamina propria scored as 1, confluence of inflammatory cells extending into submucosa scored as 2, and transmural extension of the infiltrate scored as 3). For tissue damage, no mucosal damage was scored as 0, lymphoepithelial lesions scored as 1, surface mucosal erosion or focal ulceration scored as 2, and extensive mucosal damage and extension into deeper structures of the bowel wall scored as 3.

Flow cytometry

Spleen and lymph node samples were forced through 100 μ m filters to prepare single-cell suspensions. Dead cells were removed by gradient centrifugation with lymphocyte M (Cedarlane, Burlington, NC, USA) and live cells were stained with surface antibodies for flow cytometric analysis. Data were collected in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Antibodies used: Gr-1 (RB6-8C5, BD Biosciences, San Jose, CA, USA), CD11b (M1/70, eBioscience, San Diego, CA, USA), CD62L (MEL-14, eBioscience, San Diego, CA, USA), CD44 (IM-7, eBioscience, San Diego, CA, USA) and EpCAM (G8.8, eBioscience, San Diego, CA, USA).

AOM/DSS-induced colorectal tumor model and acute DSS treatment

8 to 12 week old mice were injected intraperitoneally with AOM (NCI, Frederick, Maryland, USA) at 12.5 mg/kg body weight. Five days after injection, 2.5% DSS (MW 36 – 50 kDa, MP Biomedicals, Santa Ana, CA, USA) was given in the drinking water for five days, followed by 16 days of regular water. This cycle was repeated twice more and mice were sacrificed ten days after the last complete cycle. The colon was washed with PBS and cut open longitudinally. Tumor polyps were measured under a dissecting microscope and their

volume was calculated as $1/2 \times \text{length} \times (\text{width})^2$. For acute DSS treatment 3% DSS was given in drinking water.

Intestinal epithelial cell isolation

To isolate intestinal epithelial cells, thoroughly washed intestine pieces were treated with 5 mM EDTA and 1 mM DTT in $1 \times$ HBSS for 30 min at RT, then passed through 100 μm filters and centrifuged at 1600 rpm.

Real-time PCR

RNA was isolated using the RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized with oligo(dT) using Superscript III (Life Technologies, Grand Island, NY, USA). Gene expression was quantified with the TaqMan real time PCR primers (Life Technologies). The results are then normalized against β -actin.

Chromatin immunoprecipitation

MEF cells were left untreated or treated with 10 ng/mL TNF α for 30 min (Peprotech, Rocky Hill, NJ, USA). ChIP analysis was performed using Magna ChIP A kit (Millipore, Billerica, MA, USA). ChIP grade antibodies indicated in Figures were from Santa Cruz Biotechnology (Dallas, TX, USA). Real-time PCR was performed with SYBR Green master mix (Life Technologies, Frederick, MD, USA) and previously reported promoter primers (*I1b* 5'-CCCCTAAGAATTCCTCAAGC-3', 5'-GAGCTGTGAAATTTCCCTTGG-3' and *I16* 5'-CCCACCCTCCAACAAAGATT-3', 5'-GCTCCAGAGCAGAATGAGCTA-3')³⁴.

Statistical analysis

All animals were randomized and analyzed in a blinded fashion for *in vivo* experiments. Each mouse in a given experiment was reported on, no mice were excluded. Group sizes were estimated based on the results of preliminary experiments. No statistical method was used to predetermine group size. There is an estimate of variation within each group, and the variance between groups was similar. Statistical significance was determined with the two-tailed unpaired Student's t-test when comparing two groups that passed normality test, except when the Mann-Whitney U test was used, as indicated. One-way ANOVA test was used when comparing multiple groups. Normality test, Student's t-test, Mann-Whitney U test and one-way ANOVA test were calculated in GraphPad Prism. All data are expressed as the mean \pm SD. *p* values were considered to be statistically significant when less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We greatly appreciate the constructive inputs provided by all members of the Siebenlist laboratory. This research was supported by the Intramural Research Program of NIAID, NIH.

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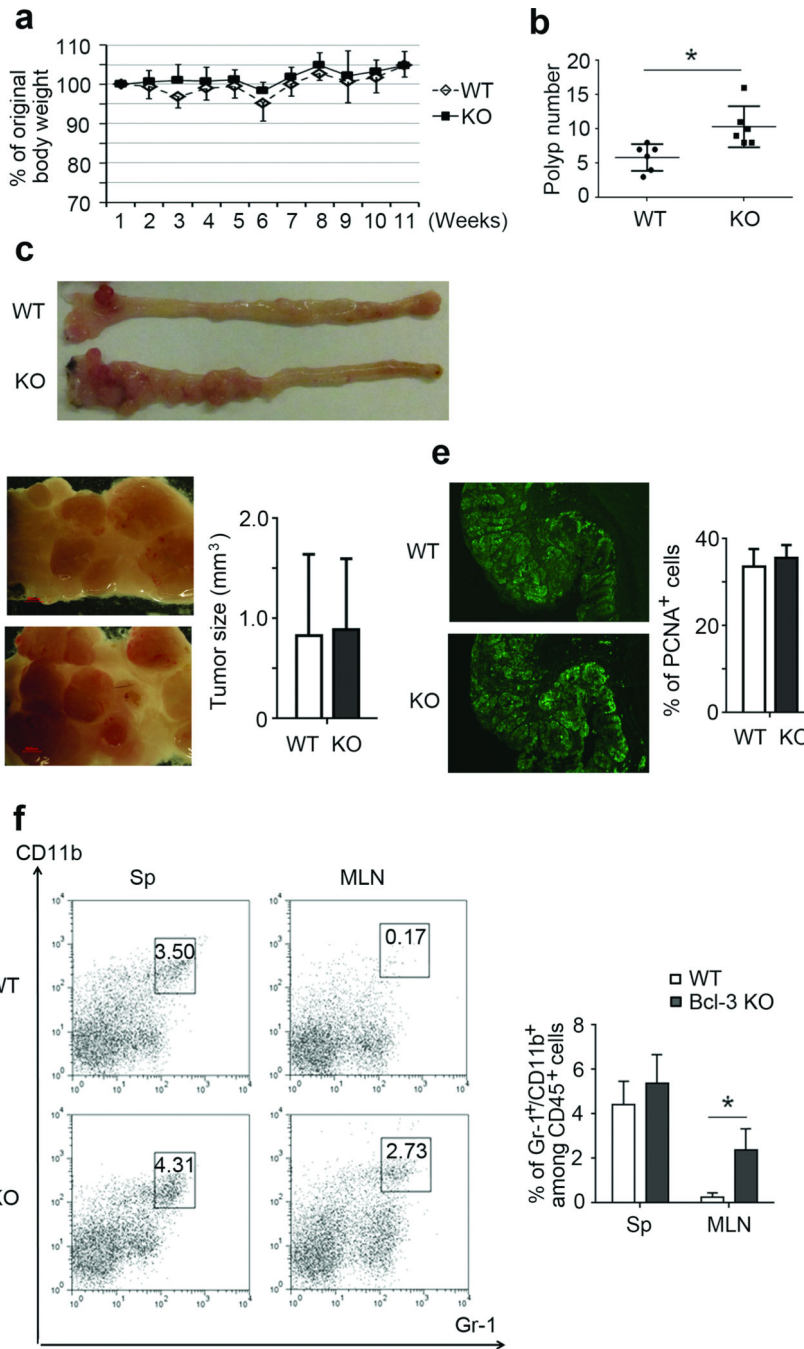


Figure 1. Loss of Bcl-3 promotes AOM/DSS-induced colorectal tumorigenesis. Weekly weights (a) and polyp numbers (b) of WT and (Bcl-3) KO mice after AOM/DSS treatment (n=6 mice per group; two additional experiments yielded similar results). (c), Representative examples of colons from WT and KO mice in (a,b) after AOM/DSS treatment. (d) Higher magnification of WT and KO tumors from a representative example as in (c) and average size of the tumors from WT and KO mice in (a,b). (e) PCNA staining of WT and KO tumor sections, representative of sections from n=5 mice per group from two independent

experiments as in (a,b). (f) Representative example and summary of flow cytometric analysis of cells for markers as indicated, with cells isolated from spleen and mesenteric lymph node (MLN) from WT and KO mice after AOM/DSS treatment (n=6 mice per group with examples from two independent experiments as in (a,b)). Data represented as means \pm SD. * $p < 0.05$.

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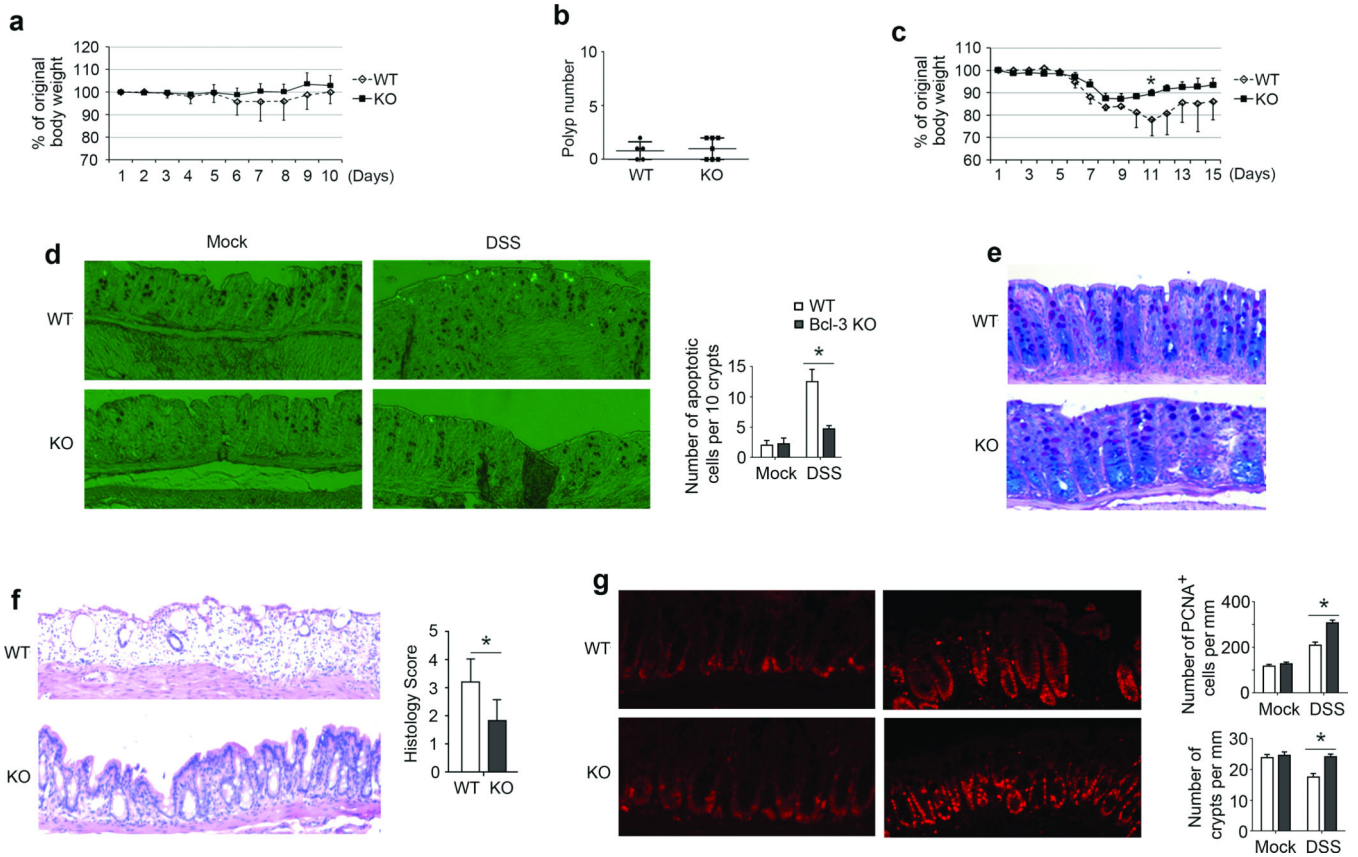


Figure 2. Loss of Bcl-3 protects against DSS-induced colitis. **(a)** Daily weights of WT and KO mice after a single AOM injection (n= 5 (WT) and 7 (KO) mice; an additional experiment yielded similar results). **(b)** Polyp numbers of WT and KO mice five months after six consecutive weekly AOM injections. **(c)** Daily weights of WT and KO mice treated with 3% DSS for 5 days (n= 5 (WT) and 6 (KO); two additional experiments yielded similar results). **(d, e)** Representative examples of tissue sections showing apoptotic cell death and summary graph **(d)** and PAS & Alcian blue staining **(e)** after 3 days of mock and/or DSS treatment of WT and KO mice. **(f, g)** H&E staining (with summary of histology scores) on day 6 of DSS treatment **(f)** and PCNA staining **(g)** on day 6 of mock (left panels) and DSS treatment (right panels) of KO and WT mice and quantitation for numbers of PCNA⁺ cells (top right graph) and numbers of crypts (bottom right graph). For statistical analyses in (d, f, g) we analyzed 5mm portions each of 3 sections per mouse from n=5 mice per group, taken from two independent experiments. Data represented as means ± SD. * *p* < 0.05.

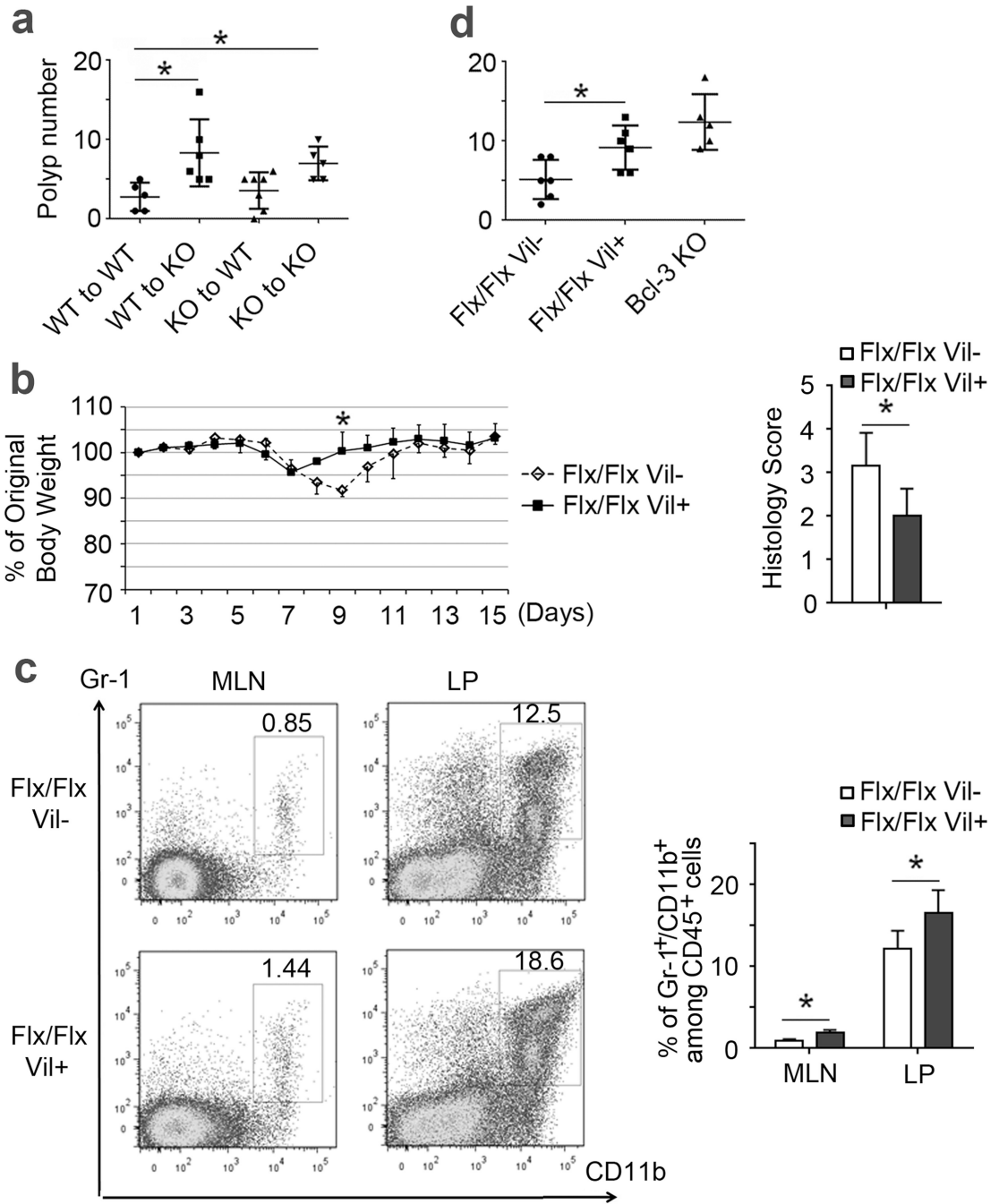


Figure 3. Intestinal epithelial-cell specific deficiency in Bcl-3 promotes tumor formation. (a) Polyp numbers of bone marrow chimeras with genotypes as indicated after AOM/DSS treatment (an additional experiment yielded similar results). (b) Daily weights and histology scores of WT (Flx/Flx) and intestinal epithelial cell-specific Bcl-3 KO mice (Flx/Flx, Vil+), treated with 3% DSS for five days (n= 6 (WT) and 5 (KO) mice; an additional experiment yielded similar results). Man-Whitney U tests were performed to determine significance in (a,b). (c) Representative example and summary of flow cytometric analysis of cells for markers as

indicated, with cells from MLN and lamina propria (LP) of WT and intestinal epithelial cell-specific Bcl-3 KO mice on day six after DSS treatment (n=7 mice per group from two experiments). **(d)** Polyp numbers in individual mice with genotypes as indicated after AOM/DSS treatment (an additional experiment yielded similar results). Data represented as means \pm SD. * $p < 0.05$.

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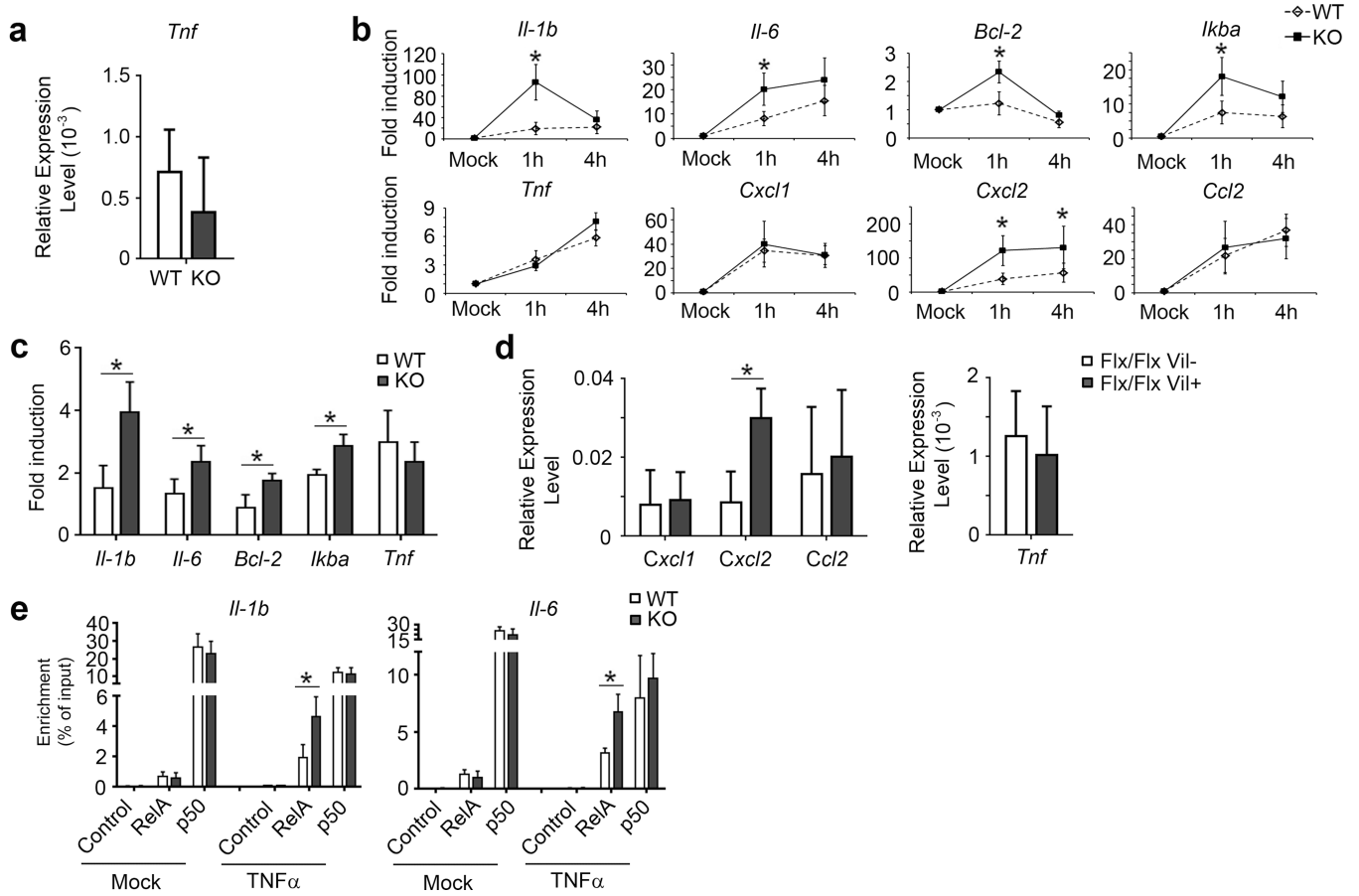


Figure 4. Bcl-3 deficiency leads to increased responses to TNF α . (a) RT-PCR analysis for TNF α expression in colons from WT and Bcl-3 KO mice after treatment with 3% DSS treatment for 3 days (n=5 mice per group; two additional experiments yielded similar results). (b) RT-PCR analysis of WT and KO mouse embryo fibroblasts (MEFs) for expression of genes as shown after TNF α treatment (10 ng/mL) for indicated times (n=6 mice per group from two experiments; an additional experiments yielded similar results). (c) RT-PCR analysis of intestinal epithelial cells from WT and KO mice for expression of genes as shown after TNF α treatment (10 ng/mL) for 4 h (n=9 mice per group from three experiments). (d) RT-PCR analysis for expression of genes as indicated in colons from mice treated with 3% DSS for 3 days (n=10 mice per group from three experiments). (e) ChIP analysis of indicated NF- κ B subunits binding to *IL1B* and *IL6* promoter in WT and Bcl3 KO MEF cells (n = 9 per group from three experiments). Data represented as means \pm SD. * $p < 0.05$.

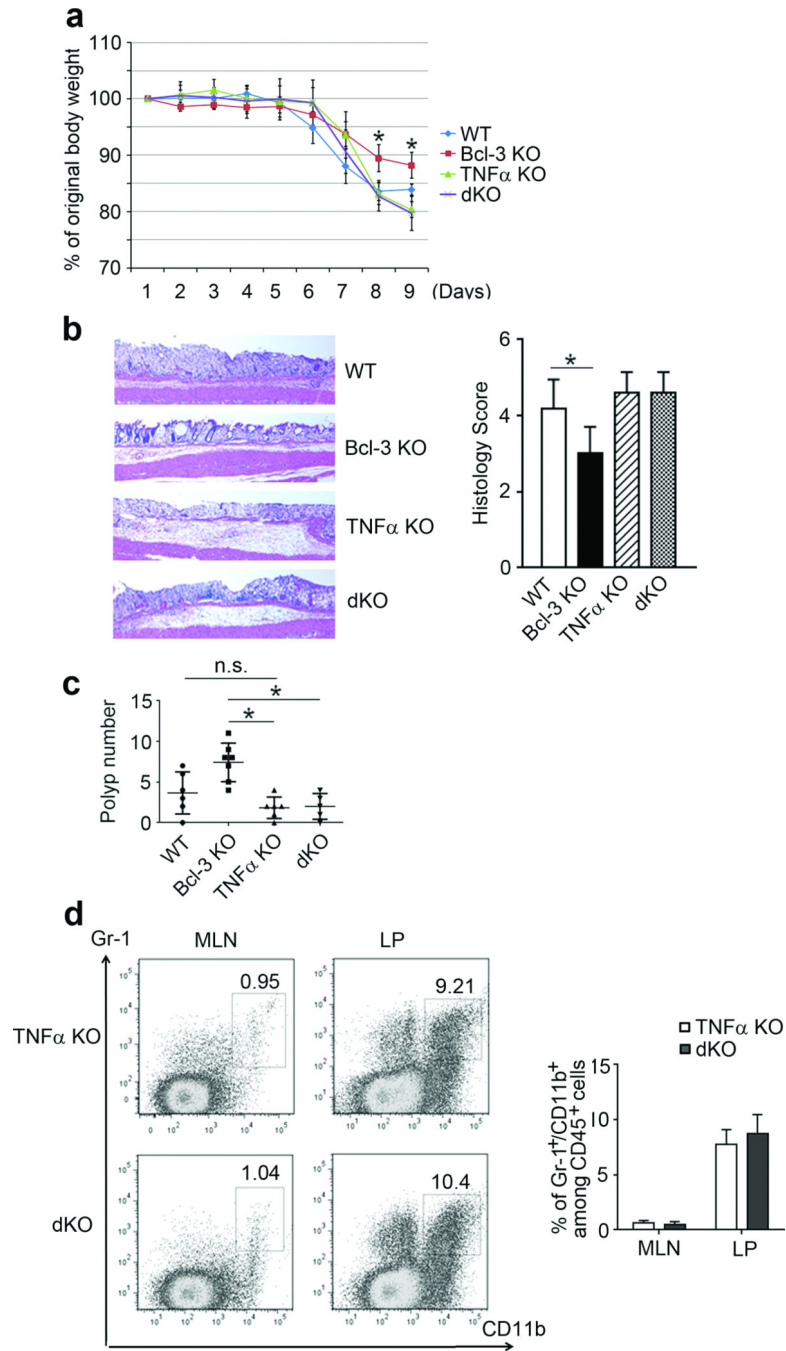


Figure 5.

The tumor-promoting effect associated with loss of Bcl-3 depends on TNF α signaling. **(a)** Daily weights of mice treated with 3% DSS for five consecutive days ($n=5$ (WT), 6 (Bcl-3 KO), 5 (TNF α KO) and 7 (dKO) (an additional experiment yielded similar results). **(b)** Representative H&E stained sections and histology scores on day 9 of mice from (a). **(c)** Polyp numbers in mice with indicated genotypes after AOM/DSS treatment (an additional experiment yielded similar results). **(d)** Representative example and summary of flow cytometric analysis of cells for markers as indicated, with cells from MLN and LP of TNF α

KO and Bcl-3/TNF α dKO mice on day six after treatment as in (a) (n=4 mice per group; an additional experiment yielded similar results). Data represented as means \pm SD. * $p < 0.05$.

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