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The NF-κB Regulator Bcl-3 Governs Dendritic Cell Antigen Presentation Functions in Adaptive Immunity

Ilaria Tassi,* Estefania Claudio,∗ Hongshan Wang,∗ Wanhu Tang,* Hye-lin Ha,* Sun Saret,* Madhu Ramaswamy, † Richard Siegel, † and Ulrich Siebenlist*

Bcl-3 is an atypical member of the IκB family and modulates gene expression via interaction with p50/NF-κB1 or p52/NF-κB2 homodimers. We report in the present study that Bcl-3 is required in dendritic cells (DCs) to assure effective priming of CD4 and CD8 T cells. Lack of Bcl-3 in bone marrow–derived DCs blunted their ability to expand and promote effector functions of T cells upon Ag/adjuvant challenge in vitro and after adoptive transfers in vivo. Importantly, the critical role of Bcl-3 for priming of T cells was exposed upon Ag/adjuvant challenge of mice specifically ablated of Bcl-3 in DCs. Furthermore, Bcl-3 in endogenous DCs was necessary for contact hypersensitivity responses. Bcl-3 modestly aided maturation of DCs, but most consequentially, Bcl-3 promoted their survival, partially inhibiting expression of several antiapoptotic genes. Loss of Bcl-3 accelerated apoptosis of bone marrow–derived DCs during Ag presentation to T cells, and DC survival was markedly impaired in the context of inflammatory conditions in mice specifically lacking Bcl-3 in these cells. Conversely, selective overexpression of Bcl-3 in DCs extended their lifespan in vitro and in vivo, correlating with increased capacity to prime T cells. These results expose a previously unidentified function for Bcl-3 in DC survival and the generation of adaptive immunity. The Journal of Immunology, 2014, 193: 4303–4311.

Dendritic cells (DCs) are the most potent APCs and are crucial for the initiation of adaptive immune responses. The immunogenicity of DCs is determined by their ability to capture, process, and present Ags, their production of cytokines and other soluble mediators, and their expression of costimulatory molecules, but also by their longevity. Enhanced survival or expansion of DCs can result in autoimmune (1–3). Upon activation, the lifetime of DCs may need to be strictly regulated to maintain a balanced and functional immune response (4–6). However, how DCs manage to carefully control their own survival, particularly during priming of T cells, is largely unknown.

NF-κB is a master regulator of inflammation, and several NF-κB subunits have been described to control DC functions (7–11). The dimeric NF-κB transcription factors are composed of five variously combined polypeptides that comprise the Rel/NF-κB family (RelA [p65], RelB, c-Rel, p50 [NF-κB1], and p52 [NF-κB2]). Both p50 and p52 lack transactivation domains, and the abundant p50 homodimers have been implicated in inhibition of NF-κB–dependent gene transcription (12, 13). NF-κB activity is regulated by the IkB family proteins, which include the classical members IkBα, IkBβ, and IkBe; the p105/NF-κB1 and p100/NF-κB2 precursors, and the atypical members IkBζ, IkBNS, and Bcl-3. The atypical members modulate transcriptional activities of NF-κB complexes in the nucleus.

Bcl-3 exclusively binds homodimers of p50 or p52 and may convert these homodimers into transactivating complexes owing to transactivation domains present within Bcl-3, yet Bcl-3 may also enhance their inhibitory function. The exact outcome may depend on the particular target gene and cellular context, which also involves not well-understood posttranslation modifications of Bcl-3 (12, 14). The specific cellular functions and mechanisms of action of Bcl-3 in biologic contexts remain poorly understood. Nevertheless, much evidence points to profound roles of Bcl-3 in vivo. Its gene is a partner in recurring chromosomal translocations, especially in B cell leukemias (15, 16), and its expression is elevated in various solid tumors (17). Bcl-3 is critical for both innate and adaptive immune responses to pathogens and contributes to immune system development (18–22).

In this study we have explored the role of Bcl-3 in DCs. We discovered that Bcl-3 was required for efficient priming of CD4 and cross-priming of CD8 T cells in vitro and in vivo. Mice specifically ablated for Bcl-3 in CD11c+ cells failed to generate a proper Ag-specific CD4 T cell response and failed to develop a normal CD8–dependent contact hypersensitivity reaction. Mechanistically, Bcl-3 contributed to expression of costimulatory factors on DCs and modestly reduced expression of some T cell inhibitory factors, but most notably it promoted survival of DCs to allow for optimal priming of T cells. Conversely, transgenic (Tg) mice overexpressing Bcl-3 in CD11c+ cells prolonged the lifespan of DCs in vitro and in vivo and enhanced T cell priming. Our findings reveal an unexpected and critical role for Bcl-3 in DCs to assure adequate survival and efficient priming of T cells.

Materials and Methods

Mice
All mice used were on C57BL/6 backgrounds. OT-I, OT-II, and LPR mice were from Taconic, and IL-10−/− Igax-cre (CD11c-cre) mice were from...
The Jackson Laboratory, Bcl-3<sup>-/-</sup> (18) and Bcl-3<sup>Tg</sup> mice have been described (23), and Bcl-3<sup>Tg<sub>Cre</sub></sup> mice are described in Supplemental Fig. 2. All 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.

Flow cytometry

Samples were stained at 4°C with Fc Block present (2.4G2; BD Biosciences) in flow cytometry buffer (PBS/2% FBS). Abs used included: allophycocyanin-conjugated anti-CD4 (RM4-5), allophycocyanin-anti-CD11c (HL3), allophycocyanin-anti-NK1.1 (PK136), allophycocyanin-anti-CD8 (53-6.7), FITC–anti-CD45R0 (145-2C11), PE–anti-CD86 (B7-2), FITC–anti-CD54 (3E2), PE–anti-CD40 (3/23), PerCP–anti-Vε2 TCR (B20.1), and PE-Cy7-anti-MHC II (HL-3) (all from BD Biosciences); PE-Cy7–anti-IFN-γ (XMG1.2), PE-Cy5–anti-MHC class II (MHC-II; MS/14.15.2), PE–anti-MHC-II (M5/11.14.5.2), allophycocyanin-anti-CD207 (eBioRMUL.2), PE–anti-Fas ligand (Fasl; MFL3), eFluor 450–anti-CD11b (M1/70), and PE–anti-PD-L1 (MH5), PE–anti-CD103 (2E7) (all from eBioscience); and allophycocyanin–anti-CD8 (53-6.7), allophycocyanin–anti-CD49b (DX5), allophycocyanin–anti-CD25 (PC61), allophycocyanin–Cy7–MHC-II (MS/14.15.2), FITC–anti-CD80 (16-10A1), and FITC–anti-MHC class I (34-1-2s) (all from BioLegend). For propidium iodide/annexin V analysis, the Annexin V eFluor 450 apoptosis detection kit was used (eBioscience). Caspase-3 activation was measured with a NuView 488 caspase-3 assay kit (Biotium, Hayward, CA). Dead cells were excluded with an aqua Live/Dead fixable kit (Invitrogen). Stained cells were analyzed on a FACSCanto and data were analyzed with FlowJo software (BD Bio sciences).

In vitro priming of T cells

Bone marrow–derived DCs (BMDCs) were generated with GM-CSF for 7–9 d (24). DC yield was monitored with flow cytometry after anti-CD11b and anti-CD11c staining. BMDCs were stimulated with ultrapure LPS (Escherichia coli 0111:B4; List Biological Laboratories). Surface markers were stained and analyzed by flow cytometry 24 h after LPS (100 ng/ml) overnight. Cytokines were assessed with flow cytometry 24 h after LPS (100 ng/ml). Cytokines present in cell supernatants of BMDCs (10<sup>5</sup>/well) after 16 h with LPS were assessed with flow cytometry 24 h after LPS (100 ng/ml). Cytokines were assessed with RT-qPCR (SABiosciences/Qiagen).

BMDCs were stimulated for various times with LPS (100 ng/ml), and nuclear and cytoplasmic fractions were prepared by a nuclear extraction–protein extraction reagent kit (Pierce). Proteins from cell lysates were separated by standard SDS-PAGE and analyzed by immunoblotting with Abs specific for p65 (CT; Millipore), p50 (sc-114,; Santa Cruz Biotechnology), Lamin B (sc-6216) (all from Santa Cruz Biotechnology).

DC apoptosis

BMDCs (3 × 10<sup>5</sup>/well) were incubated for 3 h with 100 μg/ml OVA, stimulated with LPS (100 ng/ml) overnight, washed, and cocultured with 1.5 × 10<sup>6</sup> OT-II cells per well (24-well plate) for 72 h. Apoptosis of BMDCs was analyzed with flow cytometry after staining for CD11c, annexin V/7-aminocanthoinycin D (7-AAD) or caspase-3, and gating on CD11c<sup>+</sup>. WT and Bcl-3<sup>-/-</sup> mice were injected i.v. with 30 μg LPS or PBS. After 48 h, splenocytes were isolated, stained for CD11c, and MHC-II was assessed with flow cytometry. Absolute numbers of CD11c<sup>+</sup> MHC-II<sup>+</sup> in the spleen were determined using CountBright absolute counting beads (Invitrogen). In situ apoptosis of splenic DCs was determined with TUNEL assay (Histoserv) and quantitated by counting in 0.63-mm<sup>2</sup> areas.

Western analysis

BMDCs were stimulated for various times with LPS (100 ng/ml), and nuclear and cytoplasmic fractions were prepared by a nuclear extraction–protein extraction reagent kit (Pierce). Proteins from cell lysates were separated by standard SDS-PAGE and analyzed by immunoblotting with Abs specific for p65 (CT; Millipore), p50 (sc-114,), actin (sc-7477), and Lamin B (sc-6216) (all from Santa Cruz Biotechnology).

Statistical analysis

Data were recorded as the means ± SEM. Differences between groups were analyzed by unpaired, two-tailed Student t tests. A p value ≤ 0.05 was considered significant (Prism; GraphPad Software). For multiple comparisons, data were analyzed by the one-way ANOVA followed by a Bonferroni multiple comparisons test. The numbers of independent data points (n) for each experiment are stated in the figure legend.

Results

Bcl-3 promotes DC-mediated priming of CD4 T cells

BMDCs from WT and Bcl-3–deficient (Bcl-3<sup>-/-</sup>) animals were loaded with OVA, stimulated with LPS, and cocultured with CFSE-labeled OT-II CD4 T cells for 3 d (lack of Bcl-3 did not affect generation of BMDCs; Supplemental Fig. 1A). Bcl-3<sup>-/-</sup> BMDCs were much more effective in inducing proliferation of T cells than WT BMDCs (Fig. 1A). This was not due to a defect in either uptake or processing of Ag (Supplemental Fig. 1B, 1C), and T cell proliferation was not rescued upon loading with OVA peptide (Fig. 1B). The defect in priming was not specific to TLR4, because CD4 proliferation was also impaired upon stimulation with CpG or polyinosinic-polycytidylic acid (Fig. 1C). We also observed diminished induction of CD25, IL-2, and IFN-γ when T cells were primed by Bcl-3<sup>-/-</sup> BMDCs (Fig. 1D).

To investigate priming in vivo, CD45.1 mice were injected with CFSE-labeled CD45.2 OT-II cells, then with LPS-stimulated and OVA-pulsed WT or Bcl-3<sup>-/-</sup> BMDCs, and proliferation of splenic CD45.2 CD4 OT-II cells was monitored 3 d later. Compared to
WT, Bcl3−/− BMDCs induced significantly less OT-II proliferation in vivo (Fig. 1E). Of note, Bcl3−/− BMDCs migrated to the spleen as efficiently as did WT BMDCs (Fig. 1F). Therefore, expression of Bcl-3 in BMDCs is required for efficient priming of CD4 T cells in vitro and in vivo.

Bcl-3 promotes DC-mediated cross-priming of CD8 T cells

To investigate cross-priming of CD8 T cells, we cultured LPS-stimulated and OVA-pulsed WT or Bcl3−/− BMDCs together with CFSE-labeled OT-I CD8 T cells. Both proliferation of T cells (Fig. 2A) and levels of IL-2 (Fig. 2B) were significantly reduced when BMDCs lacked Bcl-3. To assess cross-priming in vivo, WT and Bcl3−/− mice were injected with CFSE-labeled CD8 OT-I cells, OVA was administered 1 d later, and proliferation of OT-I CD8 T cells was monitored in spleen and draining lymph node 3 d later (Fig. 2C). Cross-priming in Bcl3−/− compared with WT mice resulted in reduced proliferation of T cells in both spleen and lymph node. Therefore, efficient cross-priming of CD8 T cells requires Bcl-3 expression in DCs in vitro and in vivo (see also below).

Ablation of Bcl-3 in CD11c+ cells impairs priming of T cells in mice

To investigate the role of Bcl-3 in endogenous Ag-presenting DCs we made use of mice in which DCs lacked Bcl-3. We generated
conditional Bcl-3 knockout mice (Bcl3flox/flox) and deleted Bcl-3 in DCs with CD11c-driven Cre (CD11c-Cre;Bcl3flox/flox; hereafter referred to as Bcl-3-D-DC) (Supplemental Fig. 2A, 2B). We confirmed that BMDCs generated from Bcl-3-D-DC mice were defective in priming CD4 T cells in vitro (Supplemental Fig. 2C) and that loss of Bcl-3 in CD11c+ cells did not alter the total numbers of DCs, CD4, CD8, or B cells in spleens (Supplemental Fig. 2D).

CFSE-labeled OT-II CD4 cells were injected into Bcl3flox/flox (WT) and Bcl-3-Δ-DC mice, followed by challenge with OVA and LPS, and proliferation of OT-II cells in draining lymph nodes was measured 3 d later. OT-II proliferation was significantly reduced in Bcl-3-Δ-DC compared with WT mice (Fig. 3A). Bcl-3 was thus critical within endogenous CD11c+ cells to properly prime CD4 T cells. To assess migration of DCs from skin to lymph node, we painted shaved bellies of WT and Bcl-3-Δ-DC mice with FITC, injected LPS s.c., and enumerated FITC-labeled CD11c+ cells in draining lymph nodes 18 h later (Fig. 3B). Loss of Bcl-3 did not appear to affect migration.

To further explore the role of Bcl-3 in DCs in cross-priming in vivo, we employed a CD8-dependent contact hypersensitivity model. Bcl-3-Δ-DC and WT mice were sensitized ventrally to the hapten oxazolone, rechallenged on ears 5 d later, and ear thickness was measured as a readout of inflammation at time of challenge and during the course of the next 4 consecutive days. Bcl-3-Δ-DC mice had notably reduced ear thickening compared with WT mice (Fig. 3C). To exclude the possibility that the observed reduction in contact hypersensitivity was not due to the lack of one or more DC subtypes in the skin, we analyzed the skin DC subsets in ears of WT and Bcl3flox/flox mice (25, 26). We did not find any significant

FIGURE 2. Bcl-3 is required for efficient DC-mediated cross-priming in vitro and in vivo. (A) WT and Bcl3Δ−/− BMDCs were stimulated with LPS (100 ng/ml) overnight, pulsed with OVA (100 μg/ml) for 3 h, and cocultured with CFSE-labeled OT-I T cells for 72 h. T cells were analyzed by flow cytometry after staining and gating for CD8. Representative FACS plots and proliferation and division indices (FloJo) are shown; mean ± SEM; n = 5/group. (B) WT and Bcl3Δ−/− BMDCs were treated as in (A) and IL-2 production in supernatants was analyzed with CBA; mean ± SEM; n = 5/group. (C) CFSE-labeled CD45.1 OT-I T cells (5 × 10⁶) were injected i.v. into CD45.2 WT and Bcl3Δ−/− mice. Twenty-four hours later animals were injected intradermally with OVA (1 μg). Cells were isolated from spleens and draining lymph nodes 72h later, stained and gated for CD45.1 and CD8, and analyzed by flow cytometry. Data are shown as in (A), with n = 10 mice/group based on two experiments. *p < 0.05, **p < 0.01.
numbers of CD11c⁺MHC-II⁺FITC⁺ DCs were obtained using CountBright absolute counting beads. Data are shown as means ± SEM; n = 8 mice/group based on two experiments. (B) Shaved abdomens of WT and Bcl-3⁻Δ-DC mice were painted with FITC solution and injected s.c. with LPS (30 μg). Inguinal lymph nodes were obtained after 18 h, stained for CD11c and MHC-II, and absolute numbers of CD11c⁺MHC-II⁺FITC⁺ DCs were obtained using CountBright absolute counting beads. Data are shown as means ± SEM; n = 4 mice/group. (C) WT and Bcl-3⁻Δ-DC mice were sensitized with oxazolone applied to shaved abdominal skin on 2 consecutive days. Five days later mice were challenged on ears and ear swelling was measured blindly at indicated time points after challenge, presented as mean of increase ± SEM in thickness over basal level of solvent only–treated ears; n = 5 mice/group. An additional experiment yielded similar data. *p < 0.05.

FIGURE 3. Selective ablation of Bcl-3 in CD11c⁺ cells impairs CD4 and CD8 responses in mice. (A) CFSE-labeled OT-II T cells (5 × 10⁶) were injected i.v. into WT and Bcl-3⁻Δ-DC mice. Twenty-four hours later animals were injected s.c. with OVA (1 μg) and LPS (30 μg). Cells were isolated from draining lymph nodes 72 h later, stained and gated for Vp2 TCR and CD4, and analyzed by flow cytometry. Representative FACS plots and proliferation and division indices are shown; mean ± SEM; n = 8 mice/group based on two experiments. (B) Shaved abdomens of WT and Bcl-3⁻Δ-DC mice were painted with FITC solution and injected s.c. with LPS (30 μg). Inguinal lymph nodes were obtained after 18 h, stained for CD11c and MHC-II, and absolute numbers of CD11c⁺MHC-II⁺FITC⁺ DCs were obtained using CountBright absolute counting beads. Data are shown as means ± SEM; n = 4 mice/group. (C) WT and Bcl-3⁻Δ-DC mice were sensitized with oxazolone applied to shaved abdominal skin on 2 consecutive days. Five days later mice were challenged on ears and ear swelling was measured blindly at indicated time points after challenge, presented as mean of increase ± SEM in thickness over basal level of solvent only–treated ears; n = 5 mice/group. An additional experiment yielded similar data. *p < 0.05.

Bcl-3 contributes to BMDC maturation

Efficient priming requires engagement of T cells with Ag-bound MHC-II, with costimulatory ligands on DCs and stimulation by DC-produced cytokines (27). Lack of Bcl-3 in BMDCs partially reduced LPS-induced increases in expression of the costimulatory proteins CD80 and CD86 (Fig. 4A). LPS-induced levels of MHC-II were also somewhat lower, whereas those of PD-L1 were enhanced. However, addition of PD-L1 blocking Abs to cocultures failed to improve priming (not shown). Expression levels of MHC class I, CD40, and ICAM-1 were not noticeably different in the absence of Bcl-3 (Fig. 4A).

WT and Bcl-3⁻/⁻ BMDCs were stimulated with LPS overnight to measure protein levels of IL-6, IL-12p70, TNF-α, and IL-10. Levels of IL-10 were modestly increased in Bcl3⁻/⁻ BMDCs, noted previously (28), whereas production of IL-6, TNF-α, and IL-12p70 were normal (Fig. 4B). To assess the relevance of increased IL-10 production, BMDCs from WT, Bcl3⁻/⁻, and Bcl3⁻A⁻⁻ IL-10⁻/⁻ mice (double knockout) were used in standard cocultures; double knockout BMDCs failed to improve T cell proliferation (Fig. 4C). Furthermore, IL-10-blocking Abs in cocultures failed to improve priming by Bcl3⁻/⁻ BMDCs (Supplemental Fig. 3A).

To determine whether soluble factors differentially secreted between LPS-stimulated WT and Bcl3⁻/⁻ BMDCs caused defective T cell priming, we seeded OVA-loaded and LPS-stimulated WT or Bcl3⁻/⁻ BMDCs together with OT-II T cells in the bottom chambers, and LPS-stimulated WT or Bcl3⁻/⁻ BMDCs in the upper chambers of a transwell plate, allowing for all combinations of BMDCs. Only the presence or absence of Bcl-3 in BMDCs in the lower chamber mattered for priming of T cells (Fig. 4D). This suggests that soluble factors were not primarily responsible for defective priming.

Bcl-3 promotes survival of DCs in vitro and in vivo

LPS-activated WT and Bcl3⁻/⁻ BMDCs were transcriptionally profiled with PCR arrays. Expression of several proapoptotic genes was increased in LPS-stimulated Bcl3⁻/⁻, compared with WT BMDCs (especially caspase-4 and -12, NF-κB1, and RIPK1, but also including FasL and Bax) (Fig. 5A). We then investigated whether Bcl-3 might promote survival in DCs, especially since the life expectancy of DCs may be critical for efficient priming of T cells (1, 2). We therefore assessed the survival of BMDCs in standard 3-d coculture experiments with annexin V and 7-AAD staining (Fig. 5B, 5C) or caspase-3 activation (Fig. 5D). Bcl3⁻/⁻ BMDCs survived significantly less well than did WT BMDCs, evident by day 2, and they exhibited increased caspase-3 activation, indicating a role for Bcl-3 in preventing premature apoptosis. Of note, the absence of Bcl-3 in BMDCs did not affect the cytoplasmic and nuclear levels of p50 and p65 during a time course of stimulation with LPS (Supplemental Fig. 3B). This was expected, as Bcl-3 is not known to affect the transcriptional activation/nuclear translocation of NF-κB; instead, it affects the transcriptional activity of these complexes.

To test survival of DCs in vivo, we injected WT, Bcl3⁻/⁻, and Bcl3⁻Δ-DC mice i.v. with LPS and monitored numbers of CD11c⁺MHC-II⁺ splenic DCs at days 0 and 2. DCs undergo rapid activation-induced cell death under these conditions (29, 30). We observed an even more pronounced reduction in splenic DCs lacking Bcl-3 (Fig. 5E, 5F). Splenic sections from LPS-treated Bcl3⁻Δ-DC and WT mice were stained for TUNEL⁺ (apoptotic) cells; their numbers were significantly higher in Bcl3⁻Δ-DC than in WT mice (Fig. 5G). These data implicate Bcl-3 in
survival of DCs in vitro and in vivo, suggesting a mechanism by which Bcl-3 promoted T cell priming.

Surface staining for FasL confirmed increased, albeit still low, expression levels in Bcl3<sup>−/−</sup> BMDCs (Supplemental Fig. 3C). Because FasL may impair priming due to Fas-mediated apoptosis of T cells and/or BMDCs (1, 2, 31), we added the Fas/Fc-soluble inhibitor to cocultures. However, limited Fas engagement on naive T cells during activation may promote proliferation, whereas only extensive engagement may favor apoptosis (32, 33). Consistent with this, Fas/Fc significantly reduced proliferation of T cells primed by WT BMDCs and further reduced poor proliferation when primed by Bcl3<sup>−/−</sup> BMDCs; however, the difference between WT and Bcl3<sup>−/−</sup> BMDCs was no longer as apparent in the presence of Fas/Fc (Supplemental Fig. 3D). To assess apoptosis of cocultured T cells, induced via Fas or otherwise, we measured caspase-3 activation and stained for live/dead cells (Supplemental Fig. 3E, 3F). We observed a limited increase in apoptosis of OT-II cells when primed by Bcl3<sup>−/−</sup> compared with WT BMDCs. We furthermore tested Fas-deficient CD4 OT-II T cells (from OT-II/LPR crosses). These cells proliferated less well than did Fas-sufficient OT-II cells, consistent with the noted positive role of Fas. However, the difference in proliferation of these cells when primed by WT compared with Bcl3<sup>−/−</sup> BMDCs was no longer as marked (Supplemental Fig. 3G). Therefore, increased expression

FIGURE 4. Bcl-3 contributes to BMDC maturation but is dispensable for inflammatory cytokine production. (A) WT and Bcl3<sup>−/−</sup> BMDCs were left unstimulated or stimulated with LPS (100 ng/ml). Twenty-four hours later BMDCs were stained for markers indicated and analyzed by flow cytometry, gated on CD11c<sup>+</sup>CD11b<sup>+</sup> cells. Representative mean fluorescence intensity (MFI) plots are shown in the top row; summaries for differentially expressed markers are shown in the bottom row. Isotype control staining is represented by shaded area. Data are presented as means ± SEM; n = 10/group. (B) WT and Bcl3<sup>−/−</sup> BMDCs (10<sup>5</sup>) were stimulated with LPS for 18 h in 96-well plates, and indicated cytokines present in supernatants were measured with CBA. Data are shown as means ± SEM; n = 3–4/group. (C) WT, Bcl3<sup>−/−</sup>, and Bcl3<sup>−/−</sup>/IL-10<sup>−/−</sup> BMDCs were loaded with OVA (100 μg/ml), stimulated with LPS (100 ng/ml) overnight, and cocultured with CFSE-labeled OT-II T cells for 72 h. Cells were stained and gated for CD4, analyzed with flow cytometry, and data are shown as means ± SEM; n = 2/group. (D) WT and Bcl3<sup>−/−</sup> BMDCs were treated and seeded together with CFSE-labeled OT-II T cells as in (C) in bottom chambers of a transwell plate. LPS-stimulated WT and Bcl3<sup>−/−</sup> BMDCs were seeded in upper chambers, allowing for every combination of BMDCs in the two chambers as indicated. After 72 h, cells in bottom chamber were analyzed as in (C). Data are shown as means ± SEM; n = 4/group based on two experiments. *p < 0.05, **p < 0.01.
of FasL on BMDCs appears to have moderately increased T cell apoptosis, but was unlikely to be primarily responsible for impaired priming by Bcl3−/− BMDCs.

Overexpression of Bcl-3 in DCs promotes survival and T cell priming

BMDCs were generated from Bcl3 Tg mice that express the transgene in DCs only (CD11c-Cre–mediated removal of loxP-flanked stop cassette) (23). When WT and Bcl3−/− BMDCs were used in standard coculture experiments, the Tg BMDCs caused a significant increase in T cell proliferation above that of WT BMDCs (Fig. 6A). Importantly, Tg BMDCs also exhibited significantly improved survival in these cocultures (Fig. 6B). To assess survival of DCs in vivo, we injected WT and Tg mice i.v. with LPS and monitored CD11chighMHC-II+ splenic DCs at days 0 and 2. As discussed, LPS caused a drastic reduction of DCs in WT mice after 2 d, whereas significantly more DCs remained at that time in mice expressing the Bcl3 Tg in DCs (Fig. 6C). Thus, survival of DCs was extended by increased Bcl-3 levels and correlated with improved T cell priming.

Discussion

The present study explores the function of the NF-κB regulator Bcl-3 in Ag-presenting DCs. We discovered that Bcl-3 plays a surprisingly critical role in DC-mediated priming of CD4 and cross-priming of CD8 T cells. We demonstrate this with BMDCs and, importantly, with Ag-challenged mice specifically ablated of Bcl-3 in DCs. Thus, Bcl-3 was essential within the DC population normally present in animals to initiate an appropriate adaptive
Bcl-3 contributed in several ways: it promoted expression of some coactivators associated with DC maturation, and it delimited expression of some potentially negative mediators of T cell activation. However, these contributions were relatively minor and the most consequential function of Bcl-3 was to prevent the premature demise of DCs. Loss of Bcl-3 shortened the lifetime of activated DCs both in vitro and in vivo. In contrast, overexpression of Bcl-3 increased their lifetime in vitro and in vivo, correlating with increased priming. Our findings suggest that activated Bcl-3–deficient DCs failed to survive long enough to assure efficient continuous priming of T cells, thereby compromising the development of an adequate adaptive immune response in vivo.

DC maturation signals invariably activate NF-κB, and prior studies have suggested roles for various Rel subunits in this process, including expression of cytokines (8–10, 34). Bcl-3 interacts with homodimers of p50/NF-κB1 and p52/NF-κB2 (14). NF-κB2 and NF-κB1 have been suggested to act as negative regulators of DC functions, based on studies with BMDCs and/or mice lacking these proteins. Ablation of NF-κB2 increased expression of some costimulatory molecules, postulated to be due to loss of the RelB inhibitor p100/NF-κB2 (35). Ablation of NF-κB1 increased expression of some inflammatory cytokines, especially TNF-α (7), but also of some maturation markers, and it enhanced IFN-γ production by cocultured T cells (36). It was furthermore suggested that loss of inhibitory p50 homodimers might be primarily responsible for these changes. However, these interpretations are confounded by the fact that NF-κB1 and NF-κB2 encode the IκB-like precursors p105 and p100 as well as the processed p50 and p52 subunits, which form NF-κB heterodimers in addition to homodimers. The different forms have distinct activities and it is unclear which are critical for DC-mediated priming. Prior studies have suggested that Bcl-3 enforces inhibitory activities of p50 homodimers (37), and based on the above-cited studies, Bcl-3 would then be expected to inhibit DC functions. However, contrary to this notion, we found that Bcl-3 was indispensable for efficient Ag-specific priming of both CD4 and CD8 T cells, as demonstrated both in vitro and in mice conditionally ablated for Bcl-3 in Ag-presenting DCs, including a CD8 T cell–dependent contact hypersensitivity model. Rather than enforcing inhibitory functions of p50 homodimers, Bcl-3 may reverse these functions, at least for some genes, which is conceivable given that Bcl-3 contains transactivation domains. Additionally, Bcl-3 may execute important functions via p52 homodimers.

To understand how Bcl-3 may aid DCs in effective priming of T cells, we investigated Ag uptake, processing, maturation, and cytokine expression. LPS-induced expression of MHC-II and the costimulators CD80 and CD86 was modestly reduced in Bcl-3–deficient BMDCs, and expression of the potential inhibitors PD-L1, FasL, and IL-10 was somewhat increased. However, blocking or eliminating these inhibitors failed to overcome defective T cell priming by Bcl-3–deficient BMDCs, and in the case of FasL, appeared to result in an only moderate reversal. This argues against significant roles for these inhibitors, although in vivo contributions cannot be ruled out. In contrast, loss of Bcl-3 markedly increased apoptosis in DCs, which we demonstrated in BMDCs in vitro as well as in DCs in vivo. Prior reports have suggested that the lifespan of DCs significantly affects the magnitude of immune responses to Ags and is critical for the balance between tolerance and inflammation (1, 4–6). Extending the lifetime of DCs, such as via overexpression of antiapoptotic or elimination of apoptotic proteins, enhanced Ag-specific T cell responses and/or led to autoimmunity and inflammation; conversely, elimination of the antiapoptotic regulator Bcl-XL shortened DC survival in vivo and blunted an Ag-specific T cell response (38). Therefore, the observed premature apoptosis of Bcl-3–deficient DCs may be the primary reason for impaired priming of T cells. In line with this interpretation, overexpression of Bcl-3 increased survival of DCs in vitro and in vivo and enhanced priming of T cells. The Bcl-3–mediated survival of activated and Ag-loaded DCs may increase their chances to encounter cognate T cells and, additionally, may aid activation of these cells via prolonged interaction.

How may Bcl-3 assure adequate survival of DCs? Transcriptional profiling indicated elevated expression of several proapoptotic genes in Bcl-3–deficient BMDCs, including Bax, caspase-12 and -4, RipK1, and NF-κB1. Loss of NF-κB1 has been suggested to prolong the lifespan of DCs (36), and it is conceivable that Bcl-3 may act in part by limiting expression of this protein, not just by modulating its functions. RPK1 is involved in apoptosis and necrosis, and pretreatment of BMDCs with the inhibitor of necrosis Nec-1 did not rescue T cell priming (data not shown) (39). Bcl-3 appears to modulate the expression of multiple proteins and it may be the combination of these changes that impacts the physiology of DCs.

The ability of DCs to prime T cells has also recently been linked to a major shift in metabolic programming from oxidative phosphorylation to aerobic glycolysis (40). A similar switch occurs in highly proliferative and stressed tumor cells and activated lymphocytes. It may be in the context of metabolic shifts and stress conditions that Bcl-3 is required to carefully control the adequate survival of activated DCs, thereby assuring an effective adaptive immune response.
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Disclosures

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References


Supplemental Figure 1. (A) WT and Bcl-3−/− BMDCs were cultured with GM-CSF for 7-8 days, stained for CD11c and CD11b and analyzed by flow cytometry. FACS data are representative of 10 experiments. Similar numbers of WT and Bcl-3−/− BMDCs were obtained in these cell culture experiments. (B) WT and Bcl-3−/− BMDCs were incubated with increasing amounts of OVA-FITC (Invitrogen) for 30 min, stained and gated for CD11c and CD11b and uptake analyzed by flow cytometry. Mean ±SEM; n=3 mice/group. (C) WT and Bcl-3−/− BMDCs were pulsed with DQ-OVA (Invitrogen) for 15 min, extensively washed and incubated for 30 min at either 4°C or 37°C. Ag processing unquenched DQ-OVA fluorescence, which was recorded as mean fluorescent intensity (MFI). Similar data were obtained in 2 additional experiments.
**Supplemental Figure 2.** (A) Schematic diagram of the targeting vector used to generate Bcl-3^{flx/flx} conditional knockout mice. The Neo cassette is flanked by FRT sites, and exon 1 is flanked by LoxP sites. A tandem affinity purification (TAP) tag was introduced at the translation start site of Bcl-3 (the TAP tag was derived from the pNTAP vector (Agilent Technologies). Bcl-3^{flxNe/WT} mice were generated by Ozgene (Australia) via homologous recombination of the targeting vector....
vector in C57BL/6J-derived ES cells. The Neo cassette was removed by crossing the $Bcl-3^{WT/\text{flo}x\text{Neo}}$ mice with mice carrying an FLPe recombinase transgene. The FLPe transgene was subsequently removed from $Bcl-3^{\text{flo}x/WT}$ mice in crosses. $Bcl-3^{\text{flo}x/WT}$ mice were generated by germline Cre-mediated deletion (Ella-cre, Ozgene) of the loxP-flanked sequences and this Cre transgene was also subsequently removed in crosses. $Bcl-3^{\text{flo}x/\text{flo}x}$, $Bcl-3^{\text{flo}x/\text{flo}x}$, and $Bcl-3^{-/-}$ mice were generated by appropriate intercrosses of the latter lines, and Bcl-3-Δ-DC mice (Bcl-3 knockout in CD11c+ cells) were generated by crosses of $Bcl-3^{\text{flo}x/\text{flo}x}$ and $Bcl-3^{-/\text{flo}x}$ with mice carrying the CD11c-driven Cre recombinase transgene. (B) Detection of the Bcl-3 loxP-flanked (floxed; flx) allele and the Cre-mediated loxP-deleted allele (KO) in CD11c+ sorted BMDCs generated from mice with genotypes as indicated (with or without CD11c-Cre transgene). (C) WT and Bcl-3-Δ-DC BMDCs were loaded with different doses of OVA, stimulated with LPS (100ng/ml) o.n., and cultured with CFSE-labeled OT-III T cells for 72h. After staining and gating for CD4, T cells were analyzed by flow cytometry (division index). Mean ± SEM; n=3 mice/group. (D) Splenocytes from WT and Bcl-3-Δ-DC mice were stained for CD4, CD8α, B220, MHC-II and CD11c and CD4, CD8, B and DC cell populations were assessed with countbright beads. Total numbers shown as mean ± SEM; n=3 mice/group. (E) Flow cytometric analysis of dermal and epidermal cell suspensions from ears of WT and $Bcl-3^{-/}$ mice. After gating out dead cells, live cells were gated on CD11c+MHC-II+ and analyzed for expression of Langerin (Lang), CD11b and CD103, as indicated. Lang+ cells in the epidermis are Langerhans cells. The cell numbers for each population were assessed with countbright beads. Total numbers shown as mean ± SEM; n=5 mice/group.
Supplemental Figure 3. (A) WT and Bcl3⁻/⁻ BMDCs were co-cultured with CFSE-labeled OT-II cells and T cells analyzed as in Figure 4C (standard conditions and analysis), except that indicated co-cultures also contained anti-IL-10 blocking antibodies (10 µg/ml) (JES5-2A5, BioXcell). (B) WT and Bcl3⁻/⁻ BMDCs were stimulated for the indicated times with LPS (100 ng/ml). Cytoplasmic (left panels) and nuclear (right panels) fractions were analyzed by immunoblot with anti-p65.
and anti-p50 antibodies. As a control for protein loading, the membranes were immunoblotted with an anti-actin antibody for the cytoplasmic fraction, and with anti-laminB antibody for the nuclear fraction. (C) WT and Bcl-3−/− BMDCs were left unstimulated or stimulated with LPS (100ng/ml) for 24h, then stained for CD11c, CD11b and FasL and analyzed by flow cytometry for FasL (MFI) after gating on CD11c and CD11b. Isotype control staining is shown by shaded histograms.

Representative FACS data in left panels and summary in right panel, shown as mean ± SEM; n=4 mice/group. (D) WT and Bcl3−/− BMDCs were OVA loaded, LPS stimulated and co-cultured with CFSE-labeled OT-II cells and T cells analyzed as in (A) (standard conditions and analysis; Figure 4C), except for the presence of FAS-Fc or control CTR-Fc in co-cultures. T cell proliferation data shown as mean ± SEM; n=3 mice/group. (E) WT and Bcl3−/− BMDCs were OVA loaded, LPS stimulated, then co-cultured with OT-II cells and T cells analyzed after staining and gating for CD4 (standard analysis). Here T cells were analyzed for live/dead (live/dead fixable kit) and caspase-3 activity. FACS plots shown are representative of 4 experiments. (F) WT and Bcl3−/− BMDCs were treated and co-cultured with CFSE labeled OT-II and CD4+ T cells gated on as in (C), but now analyzed for live/dead and CFSE. FACS plots shown are representative of 4 experiments. (G) WT and Bcl3−/− BMDCs were treated, co-cultured with CFSE-labeled OT-II or OT-II/LPR T cells and T cells analyzed as in (A). Data shown as mean ± SEM; n=4/group.