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Deficiency of Bim in dendritic cells contributes to overactivation of lymphocytes and autoimmunity

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Apoptosis in dendritic cells (DCs) can potentially regulate DC homeostasis and immune responses. We have previously observed that inhibition of the Fas signaling pathway in DCs results in spontaneous T-cell activation and the development of systemic autoimmunity in transgenic mice. However, the role for different apoptosis pathways in DCs in regulating DC homeostasis and immune tolerance remains to be determined. Bim, a BH3-only protein of the Bcl-2 family, was expressed at low levels in DCs and was significantly up-regulated by signaling from CD40 or toll-like receptors (TLRs). Because Bim$^{-/-}$ mice develop spontaneous systemic autoimmunity, we investigated whether Bim$^{-/-}$ DCs contributed to lymphoproliferation and autoimmunity in these mice. Bim$^{-/-}$ DCs showed decreased spontaneous cell death, and induced more robust T-cell activation in vitro and in vivo. Moreover, Bim$^{-/-}$ DCs induced autoantibody production after adoptive transfer. Our data suggest that Bim is important for regulating spontaneous cell death in DCs, and Bim-deficient DCs may contribute to the development of autoimmune diseases in Bim$^{-/-}$ mice. (Blood. 2007;109:4360-4367)

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Introduction

Dendritic cells (DCs) represent the most efficient antigen-presenting cells in capturing, processing, and presenting antigens for lymphocyte activation.[1-5] Several studies have shown that DCs undergo rapid turnover in vivo.[6-9] DCs may also undergo accelerated clearance from the lymphoid organs after interacting with antigen-specific T cells.[6] It is possible that the life span of DCs can influence their duration for stimulating lymphocytes, thereby affecting the outcome of lymphocyte activation and immune responses. In support of this possibility, ablation of DCs with diphtheria toxin in transgenic mice has been shown to impair the priming of antigen-specific cytotoxic T cells,[10] while inhibition of apoptosis in DCs enhances the antigen-specific immune responses.[8]

Apoptosis plays essential roles in multiple cellular processes, including development, tissue homeostasis, immune tolerance, and immune surveillance.[11-13] The critical role for apoptosis in maintaining peripheral tolerance is demonstrated by systemic autoimmune diseases that result from mutations in the proapoptotic Fas receptor or Fas ligand genes, in both humans and mice.[12-14] DCs may contribute to the maintenance of immune tolerance.[5,15,16] We have observed that targeted inhibition of apoptosis in DCs with p35, a caspase inhibitor that preferentially targets caspase-8 in the Fas-signaling pathway,[17] can induce spontaneous T-cell activation and the development of systemic autoimmunity in transgenic mice.[18] However, whether other apoptosis pathways in DCs help to regulate self-tolerance remains to be tested.

The Bcl-2 family proteins are critical regulators of mitochondrial apoptosis pathway.[19,20] They share one or more Bcl-2 homology (BH) domains and can be divided into 3 subfamilies,[19,20] including the antiapoptotic subfamily proteins, such as Bcl-2, Bcl-xL, and Mcl-1; the proapoptotic Bax- and Bak-like proteins; and the proapoptotic BH3-only subfamily, such as Bim and Bid. In particular, BH3-only proteins emerge as the upstream sensors for different apoptosis signaling in specific cell types.[21] BH3-only proteins either inhibit the antiapoptotic molecules as "derepressors" or directly activate proapoptotic Bax or Bak to induce apoptosis.[21,22] Bcl-2 family proteins may also play important roles in the regulation of apoptosis in DCs.[8,9] It has been shown that overexpression of Bcl-2 in DCs can prolong DC survival and enhance the immunogenicity of DCs in transgenic mice.[8] This suggests that the mitochondrion-dependent apoptosis regulated by Bcl-2 family proteins may play an important role in regulating DC survival and functions.

Bim is a proapoptotic BH3-only protein in the Bcl-2 family that has been shown to play a critical role in regulating homeostasis of lymphocytes.[21,23] Although Bim can be rapidly induced in DCs by different stimuli,[9] whether apoptosis and homeostasis of DCs can be regulated by Bim has not been determined. Deficiency in Bim causes significant expansion of lymphocytes and autoimmunity in mice.[23] In Bim$^{-/-}$ mice, defective negative selection for autoreactive T cells and B cells has been detected that likely contributes to the development of autoimmune diseases in these mice.[24,25] However, whether DCs contribute to the onset of autoimmunity in Bim$^{-/-}$ mice has not been defined.

In the current study, we have investigated the role of Bim in regulating DC apoptosis and function. DCs deficient in Bim underwent less spontaneous apoptosis and were more efficient in inducing T-cell activation both in vitro and in vivo. Moreover, Bim$^{-/-}$ DCs displayed a propensity for inducing autoantibody production, suggesting that Bim-deficient DCs contribute to the overactivation of lymphocytes and the development of autoimmunity.

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Materials and methods

Mice

Wild-type, *lpr,* OT1- and OT2-transgenic, and Bim$^{-/-}$ mice on the C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME).

Purification of DC subsets and T cells

Bone marrow–derived DCs (BMDCs) were generated by culturing bone marrow monocytes for 7 days with 10 ng/mL GM-CSF and 10 ng/mL IL-4 (Biosources, Camarillo, CA). CD11c$^+$ DCs were then purified using CD11c magnetic-activated cell sorting (MACS) beads (Miltenyi Biotec, Auburn, CA). Typically, between 93% and 96% of the purified cells are CD11c$^+$ (data not shown). In some experiments, DCs were incubated with 100 ng/mL LPS and 100 nM CpG during the last 16 to 24 hours of culture. To purify splenic DCs, mouse spleens were treated with liberase (0.4 mg/spleen; Roche, Indianapolis, IN) at room temperature for 20 minutes. Single cell suspension was prepared and the red blood cells were lysed with the ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2). The cells were blocked with 1 μg/mL CD16/CD32 and 10 μg/mL rat IgG, and then incubated with 1 μg/mL biotinylated antibodies to CD3, Thy1.2, CD19, IgM, DX5, and TER119 (BD Biosciences, San Jose, CA), followed by incubation with streptavidin-conjugated Biomag beads (Qiagen, Valencia, CA). After removal of the cells bound to the Biomag beads with a Cell Separation Magnet (BD Biosciences), live cells were purified by Ficoll-gradient separation and incubated with anti–PDCA-1 MACS beads (Miltenyi Biotec). The cells bound to anti–PDCA-1 beads were purified with a MACS column and were typically more than 95% positive for CD11c and PDCA-1 staining. The unbound cells were incubated with anti-CD11c MACS beads (Miltenyi Biotec) to enrich myeloid DCs (mDCs). Typically, approximately 95% of cells were CD11c$^+$ and more than 90% were CD11c$^+$CD11b$^+$ mDCs. DCs were either used for lysis and Western blot or cultured in RPMI complete medium containing 10 μg/mL anti-CD40 (BD Biosciences) plus 1 μg/mL protein A (Sigma, St Louis, MO), 5 μg/mL LPS (Sigma), 50 μg/mL lipoteichoic acid (LTA; Sigma), 0.5 μM phosphorothioate-stabilized CpG oligonucleotide (TCCATGACGTTCCTGATGCT), or 100 ng/mL polyinosinic-polycytidylic acid (poly I:C; Sigma). To purify T cells, splenocytes were incubated with biotinylated anti-Thy1.2 (BD Biosciences), and Thy1.2$^+$ cells were purified with streptavidin-conjugated MACS beads (Miltenyi Biotec). Activated T cells were generated by stimulating splenocytes with 5 μg/mL concanavalin A (ConA; Amersham, Arlington Heights, IL) for 2 days, followed by culturing in 100 U/mL IL-2 for 2 days.

Flow cytometry

Single cell suspensions from mouse spleens were first blocked with 1 μg/mL anti-CD16/CD32, 10 μg/mL rat IgG, and 10 μg/mL hamster IgG. The cells were then stained with various antibodies conjugated to FITC, PE, or PE-Cy5 and analyzed on an EPICX flow cytometer (Beckman Coulter, Hialeah, FL). The data were analyzed by using the FlowJo software (TreeStar, Ashland, OR). The conjugated antibodies to CD11c, CD11b, CD40, B7.1, B7.2, I-A^b, ICAM-1, CD4, and CD8 were obtained from BD Biosciences. PE-conjugated anti–PDCA-1 was from Miltenyi Biotec. To quantitate T regulatory (Treg) cells, cells from mouse spleens or lymph nodes were stained with cychrome anti-CD4 (BD Biosciences). The cells were then fixed and permeabilized for intracellular staining with PE-conjugated anti-FoxP3 according to the manufacturer's protocol (eBioscience, San Diego, CA).

Antibodies and Western blot

Cells were lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1x protease inhibitor cocktail from Roche) on ice for 30 minutes. Cell lysates were quantitated by the Bio-Rad Protein Assay (Hercules, CA) and used for Western blot by probing with various antibodies, followed by horseradish peroxidase–conjugated secondary antibodies (Southern Biotechnology, Birmingham, AL). The blots were then developed using Supersignal Dura substrate (Pierce, Rockford, IL). Antibodies for Western blot analyses were as follows: monoclonal mouse anti–Bcl-xL (BD Biosciences); anti–α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal goat antiactin (Santa Cruz Biotechnology); polyclonal rabbit antibodies to Bim, Bik/Blk, Bmf, Bid (Biovision, Palo Alto, CA), Bad (BD Biosciences), phospho–Ser112-Bad, phospho–Ser136-Bad, and phospho–Ser155-Bad (Cell Signaling, Beverly, MA); Bcl-2 (Upstate Biotechnologies); and Mcl-1 (Santa Cruz Biotechnology).

Apoptosis assays for DCs

DCs (0.5×10^5/well) were incubated with different treatments in 96-well flat-bottom tissue culture plates for 0 hour (as control) or indicated time. The cells were then harvested and stained with propidium iodide and FITC–annexin V (Sigma) plus propidium iodide to quantitate live cells by flow cytometry. Percentages of apoptosis were calculated as described.[26]

T-cell proliferation in vitro

DCs derived from bone marrow of wild-type or Bim$^{-/-}$ mice were either untreated or pulsed with 20 μg/mL $OVA_{323\text{-}339}$ or $OVA_{SIINFEKL}$ peptide at 37°C for 2 hours. OT1 or OT2 T cells (10^5/well) were incubated with indicated numbers of DCs in 96-well plates for 3 days. The plates were pulsed with 1 μCi (0.037 MBq)/well [^{3}H]-thymidine during the last 8 hours of culture. The cells were harvested and incorporated [^{3}H]-thymidine was quantitated in a scintillation counter.

T-cell proliferation in vivo

CD8$^+$ T cells from OT1 mice were purified, labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR), and injected into C57BL/6 mice retro-orbitally as described.[18] DCs (with or without 16 hours of LPS activation) were pulsed with $OVA_{SIINFEKL}$ peptide and injected at the footpad 24 hours later. Three days later, draining popliteal lymph node cells were harvested and stained with cychrome anti-CD8 (BD Biosciences) and PE-H-2K^b/SIINFEKL tetramer (Protein Core Laboratory, Baylor College of Medicine, Houston, TX) and analyzed by flow cytometry. CFSE$^+$ T cells were gated to analyze CFSE dilution due to cell division. CD8$^+$ OT1 T cells positive for the staining with H-2K^b-SIINFEKL tetramer were also analyzed. To analyze stimulation of CD4$^+$ T cells, CD4$^+$ T cells from OT2 mice were purified, labeled with CFSE, and injected into C57BL/6 mice retro-orbitally as described.[18] DCs (with or without 16 hours of LPS activation) were pulsed with $OVA_{323\text{-}339}$ peptide (10^6/mouse) and injected intradermally at footpad 24 hours later. Three days later, popliteal lymph nodes were collected and CD4$^+$CFSE$^+$ T cells were analyzed by flow cytometry.

CFSE-labeled DCs in the draining lymph nodes

BMDCs from wild-type or Bim$^{-/-}$ mice were labeled with 5 μM CFSE (Molecular Probes) at room temperature for 10 minutes. After washing with PBS 3 times, the labeled DCs were injected into the footpad of C57BL/6 mice (1×10^6/mouse). The draining (popliteal) lymph nodes were harvested at days 1, 2, and 5 after injection and treated with 0.4 mg/mL liberase at room temperature for 10 minutes (Roche). Total numbers of lymph node cells were counted, and the cells were then stained with PE-conjugated anti-CD11c (BD Biosciences) and the percentage of CD11c$^+$CFSE$^+$ DCs was analyzed by flow cytometry. The total number of CD11c$^+$CFSE$^+$ DCs in the draining lymph node of each mouse was calculated. In parallel experiments, recipient mice were injected with OT1 T cells retro-orbitally (2×10^6/mouse). One day later, WT or Bim$^{-/-}$ DCs were pulsed with $OVA_{SIINFEKL}$ peptide, followed by labeling with CFSE and injection into the footpad of recipient mice (1×10^6/mouse). Alternatively, recipient mice were injected with OT2 T cells retro-orbitally (2×10^6/mouse). One day later, WT or Bim$^{-/-}$ DCs were pulsed with $OVA_{323\text{-}339}$ peptide and labeled with CFSE, followed by injection into the footpad of recipient mice (1×10^6/mouse). CFSE$^+$CD11c$^+$ DCs in the draining lymph

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node were quantitated at days 1, 2, and 5 after injection of DCs as described in this paragraph.

Adoptive transfer of DCs and ELISA

DCs (5×10^6/mouse) were injected into 8-week-old C57BL/6 mice (7-8 mice/group) intraperitoneally essentially as described.[27] The mice were then injected with LPS (30 μg/mouse) intraperitoneally one day later. Sera were collected from the recipient mice at 1 and 2 weeks after DC transfer. Antinuclear antibody (ANA) was detected by enzyme-linked immunosorbent assay (ELISA) as described.[18] To detect anti-ssDNA and anti-dsDNA, 96-well polyvinyl plates (Becton Dickinson, Lincoln Park, NJ) were coated with 10 μg/mL ssDNA or dsDNA (Sigma) in 0.1 M sodium carbonate buffer, pH 8.8, at 4°C overnight. After blocking with PBS containing 10% FCS and 0.05% Tween at 37°C for 2 hours, serially diluted sera were added and incubated at 37°C for 2 hours, followed by incubation with 1:1000 HRP anti–mouse IgG plus HRP anti–mouse IgM (Southern Biotechnology) at 37°C for 1 hour. The plates were developed and measured as described.[18] For cytokine ELISA, IL-12p70 in the culture supernatants was measured using OptiEIA cytokine ELISA sets according to the manufacturer's instructions (BD Biosciences).

Intracellular IL-12 staining

$CD11c^+$ bone marrow–derived DCs (10^6/mL) were cultured in the absence or presence of TLR stimuli for 24 hours. Brefeldin A (1 μg/mL) and monensin (2 μM) were added during the last 6 hours to inhibit IL-12 secretion. Cells were stained with FITC anti-CD11c, followed by fixation and permeabilization with Cytofix/Cytoperm solution (BD Biosciences) and staining with PE-conjugated anti–IL-12p40/p70 (BD Biosciences) or PE-conjugated rat IgG1 as isotype control. The cells were than analyzed by flow cytometry.

Semiquantitative RT-PCR

Total RNA was extracted from DCs using Trizol following the manufacturer's instructions (Invitrogen, Frederick, MD). cDNA was synthesized using SuperScript First-Strand synthesis System for reverse-transcription–polymerase chain reaction (RT-PCR; Invitrogen). Bim and actin were then amplified by PCR as described[28] using the following primers in the following sequence: Bim forward, 5′-CTGAGTGTGACAGAGAAGGTGG-3′ and reverse, 5′-GTGGTCTTCAGCCTCGCGGT-3′; actin forward, 5′-ATCCGTAAAGACCTCTATGC-3′ and reverse, 5′-AACGCAGCTCAGTAACAGTC-3′.

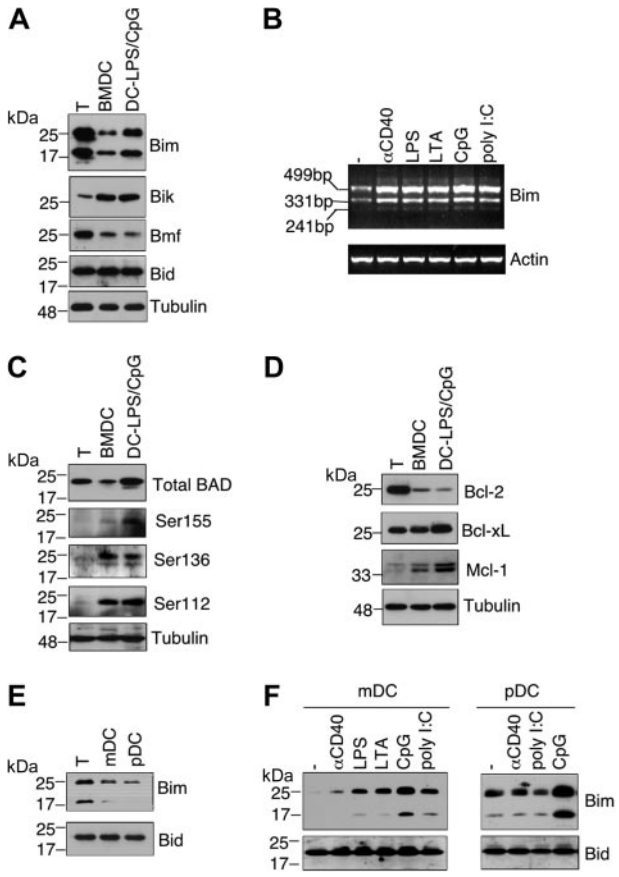

Figure 1. Expression of BH3-only proteins in DC subsets. (A) T cells were generated by activation of mouse spleen cells with ConA and IL-2. Western blot analyses were performed to detect BH3-only proteins in T cells, or bone marrow–derived DCs with or without treatments with LPS and CpG. (B) RNA was prepared from bone marrow–derived DCs with or without stimulation of CD40 or TLRs for 20 hours and used for semiquantitative RT-PCR for Bim or actin. The 499-bp, 331-bp, and 241-bp bands correspond to BimEL, BimL, and BimS, respectively.[28] (C) Cell lysates prepared as in panel A were used for Western blot analyses of BAD and phosphorylated BAD. (D) Cell lysates prepared as in panel A were used for Western blot analyses of antiapoptotic molecules. (E) Freshly purified T cells, mDCs, and pDCs from mouse spleens were lysed for Western blot analysis of Bim and Bid. (F) mDCs and pDCs purified from mouse spleens were treated with anti-CD40 or different TLR stimuli for 24 hours. Cells were lysed for Western blot analyses of Bim and Bid.

Results

Expression of BH3-only proteins in DCs

To determine the role of BH3-only proteins in regulating DC survival, we first examined the expression of various BH3-only proteins in bone marrow–derived DCs (BMDCs). We have previously observed that DCs expressed limited amounts of certain BH3-only members compared with T cells.[18] Consistent with the previous finding, we found that BMDCs expressed lower levels of Bim than T cells (Figure 1A). Stimulation with LPS and CpG induced significant up-regulation of Bim in BMDCs (Figure 1A). Semiquantitative RT-PCR analysis indicated that Bim mRNA was up-regulated in DCs after stimulation of CD40 or TLRs (Figure 1B), suggesting that the up-regulation of Bim involves increased transcription. In comparison, the level of Bid was comparable between BMDCs and T cells and not induced by stimulation with LPS and CpG (Figure 1A). BMDCs expressed higher levels of Bik and lower levels of Bmf than T cells, however, neither Bik nor Bmf was induced by stimulation with LPS and CpG (Figure 1A).

We also observed that total protein of BAD in DCs was up-regulated by TLR stimulation (Figure 1C), however, higher levels of constitutive phosphorylation of BAD at Ser^{112} and Ser^{136} were detected in DCs than in T cells (Figure 1C). Moreover, up-regulation of phosphorylation at Ser^{155} was detected in DCs after TLR stimulation (Figure 1C). Because phosphorylation suppresses the proapoptotic activity of BAD by inhibiting its binding to Bcl-xL,[29] BAD is potentially less active in DCs than in T cells.

Because Bim potentially functions as a derepressor by binding to and inhibiting antiapoptotic Bcl-2 family proteins,[21,22] we examined whether TLR signaling also induced expression of these antiapoptotic molecules. We observed that Mcl-1 and Bcl-xL, but not Bcl-2, were induced by TLR signaling (Figure 1D). This suggests that TLR signaling induces both proapoptotic Bim and antiapoptotic molecules in DCs.

Consistent with the observations in BMDCs (Figure 1A), splenic $CD11c^+CD11b^+$ myeloid DCs (mDCs) and $CD11c^{low}PDCA\text{-}1^+$ plasmacytoid DCs (pDCs) also expressed lower levels of Bim than T cells, while Bid was similarly expressed in mDCs, pDCs, and T cells (Figure 1E). We also found that Bim was induced by CD40 cross-linking or treatments with different TLR stimuli in splenic mDCs (Figure 1F). Bim was also induced in pDCs with CpG (Figure 1F). It has

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been reported that pDCs express TLR9, a receptor for CpG, but lack most other TLRs.[2,30] This may explain why only CpG induced up-regulation of Bim in pDCs (Figure 1F). By contrast, Bid was constitutively expressed in mDCs and pDCs and remained unchanged after treatments with CD40 cross-linking or TLR stimulation (Figure 1F). These data suggest that Bim is up-regulated in BMDCs, as well as in splenic mDCs and pDCs after stimulation via TLRs.

Bim in the regulation of spontaneous DC apoptosis

We next examined whether Bim regulated apoptosis in DCs. We purified mDCs and pDCs from wild-type (WT) and Bim$^{-/-}$ mouse spleens, and measured the effects of Bim deficiency on their spontaneous cell death. We observed that WT mDCs underwent rapid cell death in vitro, with only 30% cell survival after 24-hour culture (Figure 2A). Although spontaneous turnover of pDCs is significantly slower than mDCs in vivo,[18,31] pDCs underwent more cell loss (approximately 90%) after 24-hour in vitro culture, with only 10% surviving cells (Figure 2A). This suggests that removing DCs from the splenic microenvironments represents a stress that leads to rapid DC death, with pDCs being more sensitive to this stress. Cell-cell contact and cytokines in the spleen might provide critical survival signaling to DCs. Of interest, Bim deficiency significantly improved the survival of mDCs (to approximately 50%) and pDCs (to approximately 30%) during the same period (Figure 2A), while Fas-deficient *lpr* DCs remained as sensitive to spontaneous cell death as WT DCs (Figure 2B). These data suggest that Bim plays an important role in sensing the loss of the splenic microenvironment to trigger the apoptosis machinery in both mDCs and pDCs, while the Fas signaling pathway is not involved in regulating such spontaneous cell death in DCs.

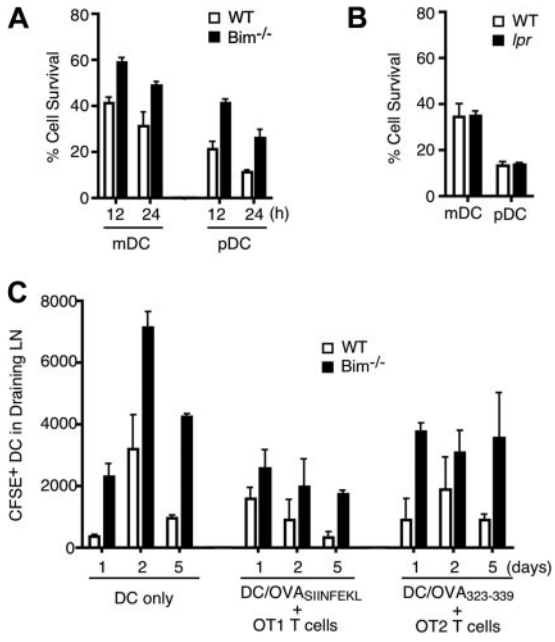

Figure 2. Increased survival of Bim$^{-/-}$ DCs. (A) mDCs and pDCs enriched from wild-type (WT) or Bim$^{-/-}$ mouse spleens were cultured in vitro for 12 or 24 hours, followed by analysis of cell loss by flow cytometry. Data shown (mean ± SD) are averages of 3 sets of mice of each genotype. (B) mDCs and pDCs enriched from 3 to 5 wild-type (WT) or *lpr* mouse spleens were cultured in vitro for 20 hours, followed by analysis of cell loss. Data shown (mean ± SD) are averages of 3 sets of mice of each genotype. (C) Mice were either untreated or injected with OT1 or OT2 cells. Twenty-four hours later, WT or Bim$^{-/-}$ DCs with or without pulsing of corresponding OVA peptides were labeled with CFSE and injected into the footpad of these mice. CFSE$^+$ DCs in the draining lymph nodes (LNs) were quantitated at days 1, 2, and 5 after DC injection. Data shown are the representative of 2 separate experiments and are presented as mean ± SD of 3 mice per injected group at each time point. Statistical significance between mice injected with wild-type and Bim$^{-/-}$ DCs was analyzed by Student *t* test, and the *P* values are as follows: .024 (day 1), .043 (day 2), .001 (day 5); in groups injected with DC/OVA$_{SINFEKL}$: .149 (day 1), .220 (day 2), .013 (day 5); and in groups injected with DC/OVA$_{323\text{-}339}$, .029 (day 1), .205 (day 2), .048 (day 5).

We also tested the effects of Bim deficiency on the survival of DCs after adoptive transfer. We injected recipient mice with CFSE-labeled DCs from wild-type or Bim$^{-/-}$ mice, followed by analyzing CFSE$^+$ DCs in the draining lymph nodes. We consistently detected more Bim$^{-/-}$ DCs in the draining lymph nodes at days 1, 2, and 5 after adoptive transfer (Figure 2C). This is consistent with the possibility that Bim deficiency enhances the survival of DCs in vivo.

We also pulsed bone marrow–derived DCs from wild-type or Bim$^{-/-}$ mice with OVA$_{SIINFEKL}$ peptide, followed by CFSE labeling and injection into the footpad of recipient mice that had previously received OVA-specific OT1 T cells. In mice that received both OVA$_{SIINFEKL}$-pulsed CFSE$^+$ DCs and OT1 T cells, however, lower numbers of CFSE$^+$ DCs were detected in the draining lymph nodes than in mice that received unpulsed DCs alone (Figure 2C). This is consistent with previous observations that interactions with antigen-specific T cells led to the disappearance of antigen-loaded DCs in the lymph nodes.[6] It has been shown that DCs are susceptible to killing by T cells through Fas and perforin.[18,32,33] Decreases in DCs in the presence of antigen-specific T cells are probably due to T-cell–mediated killing of antigen-specific DCs during T-cell–DC interactions.[18] Of interest, more OVA$_{SIINFEKL}$-pulsed Bim$^{-/-}$ DCs than wild-type DCs could be detected in the draining lymph nodes of mice that received OT1 T cells at day 5 after transfer of DCs (Figure 2C), although the differences in numbers of WT and Bim$^{-/-}$ DCs recovered were less significant at days 1 and 2 (Figure 2C). Similar effects of Bim deficiency were observed for the interactions of OVA$_{323\text{-}339}$-pulsed DCs with CD4$^+$ OT2 T cells in the draining lymph nodes (Figure 2C). These data are consistent with the possibility that Bim deficiency enhances the survival of DCs during interactions with antigen-specific T cells in vivo.

We also observed that Bim in BMDCs was increased after 24-hour culture in the absence of GM-CSF (Figure 3A). Spontaneous apoptosis in DCs was reduced in the presence of GM-CSF that was correlated with decreased Bim expression (Figure 3A). We also compared BMDCs from wild-type and Bim$^{-/-}$ mice (Figure 3B). Consistent with a role for Bim in spontaneous apoptosis in DCs, spontaneous apoptosis in the absence of GM-CSF was decreased in Bim$^{-/-}$ BMDCs after 24-hour in vitro culture (Figure 3B). Treatments with LPS, LTA, CpG, or poly I:C further suppressed spontaneous apoptosis in Bim$^{-/-}$ DCs (Figure 3B). These data support the possibility that Bim plays an important role in regulating spontaneous apoptosis of DCs.

DC accumulation and the expression of costimulatory molecules on DCs in Bim$^{-/-}$ mice

We next investigated whether improved survival of Bim$^{-/-}$ DCs resulted in DC accumulation. CD11c$^+$ DCs in the spleens of WT and Bim$^{-/-}$ mice were quantitated by flow cytometry. Despite a slight decrease in the percentage of CD11c$^+$ cells due to significant expansion of lymphocytes, the absolute numbers of CD11c$^+$ DCs were increased in Bim$^{-/-}$ mice (Figure 4A left panel). Both CD11c$^+$CD11b$^+$ mDCs and CD11c$^+$PDCA-1$^+$ pDCs were increased in Bim$^{-/-}$ mice (Figure 4A right panel). To examine whether the loss of Bim led to elevated activation status of Bim$^{-/-}$ DCs, we measured surface expression of costimulatory molecules, including CD40, I-A^b, B7.1, B7.2, and ICAM-1, on Bim$^{-/-}$ DCs by flow cytometry (Figure 4B). However, no increases in the levels of these molecules were detected (Figure 4B), suggesting that the loss of Bim did not promote the activation of DCs.

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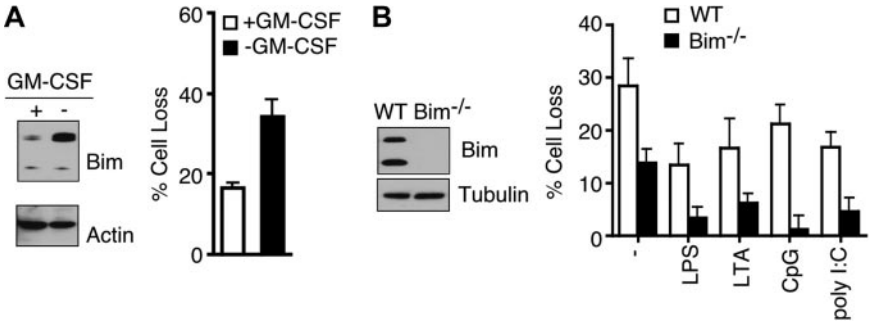

Figure 3. Roles of Bim in spontaneous apoptosis in DCs with or without TLR stimulation. (A) CD11c$^+$ BMDCs were cultured in the presence or absence of GM-CSF for 24 hours. Cells were either lysed for Western blot analyses (left) or used to measure cell loss (right). (B) CD11c$^+$ BMDCs derived from WT or Bim$^{-/-}$ mice were used for Western blot analyses (left). Spontaneous apoptosis of WT or Bim$^{-/-}$ CD11c$^+$ BMDCs with or without treatments with different TLR stimuli were quantitated 24 hours later (right). Data shown (mean ± SEM) are averages of 3 independent experiments.

Increased immunogenicity of Bim$^{-/-}$ DCs

We next examined whether prolonged survival induced by Bim deficiency enabled DCs to induce better priming of antigen-specific T cells. We first measured proliferation of OVA-specific CD4$^+$ OT2-transgenic T cells induced by wild-type or Bim$^{-/-}$ DCs pulsed with $OVA_{323\text{-}339}$ peptide. Bim$^{-/-}$ DCs induced better proliferation of OT2 T cells by [^{3}H]-thymidine incorporation in vitro (Figure 5A). Similarly, CD8$^+$ OVA-specific OT1-transgenic T cells also showed better proliferation when stimulated with $OVA_{SIINFEKL}$-pulsed Bim$^{-/-}$ DCs than with pulsed wild-type DCs (Figure 5B). This indicates that Bim$^{-/-}$ DCs induce better T-cell stimulation in vitro.

We also examined whether Bim$^{-/-}$ DCs enhanced cytokine production. Of interest, more IL-12 could be detected by ELISA in the supernatants of Bim$^{-/-}$ DCs than in those of wild-type DCs after treatments with CpG and other TLR stimuli (Figure 5C). However, intracellular staining of IL-12 showed that the percentages of IL-12–producing cells, as well as mean fluorescence intensities of IL-12 staining, were similar between wild-type and Bim$^{-/-}$ DCs (Figure 5D). This suggests that IL-12 production on a per cell basis was not changed in Bim$^{-/-}$ DCs. Rather, enhanced IL-12 production could be due to better survival of Bim$^{-/-}$ DCs.

We also measured T-cell proliferation induced by Bim$^{-/-}$ DCs in vivo. We transferred CFSE-labeled OT2 T cells into recipient mice. Wild-type or Bim$^{-/-}$ DCs with or without LPS pretreatment were pulsed with $OVA_{323\text{-}339}$ peptide and then injected at the footpad of these recipient mice. As measured by CFSE dilution, CD4$^+$ OT2 T cells in the draining (popliteal) lymph nodes proliferated better to antigen-pulsed Bim$^{-/-}$ DCs than to wild-type DCs (Figure 6A upper panel). LPS-treated DCs induced better T-cell proliferation, while Bim deficiency further improved T-cell proliferation (Figure 6A upper panel). Similar results were observed with activation of CFSE-labeled CD8$^+$ OT1 T cells in the draining lymph nodes (Figure 6A lower panels). In addition, more OVA-specific CD8$^+$ T cells were detected in the draining lymph nodes by staining with H-2K^b/$OVA_{SIINFEKL}$ tetramer after immunization with Bim$^{-/-}$ DCs than wild-type DCs (Figure 6B). Similar results were observed with LPS-treated DCs (Figure 6B). These data suggest that Bim$^{-/-}$ DCs with or without LPS pretreatments induce better proliferation of antigen-specific T cells than wild-type DCs in vivo.

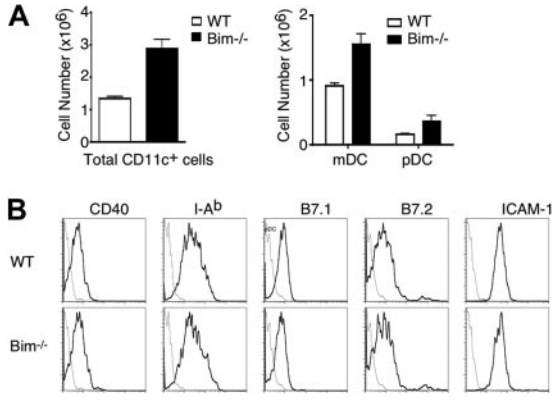

Figure 4. DC accumulation and unaltered DC activation in Bim$^{-/-}$ mice. (A) Total splenocytes of 5-month-old Bim$^{-/-}$ mice and wild-type controls were counted. Splenocytes were then stained for FITC anti-CD11c, PE anti–PDCA-1, or PE anti-CD11b, and percentage of CD11c$^+$ cells was analyzed by flow cytometry. Total numbers of CD11c$^+$ cells (left panel) as well as CD11c$^+$CD11b$^+$ mDCs and CD11c$^+$PDCA-1$^+$ pDCs (right panel) were quantitated. Data represent mean ± SD of 5 mice of each genotype. Statistical significance between wild-type and Bim$^{-/-}$ mice was analyzed by Student *t* test, with $P < .001$ for total CD11c$^+$ DCs, $P < .07$ for pDCs, and $P < .004$ for mDCs. (B) Unaltered expression of costimulatory molecules on Bim$^{-/-}$ DCs. Splenocytes were enriched for DCs by depletion of T and B cells. Cells were then stained with PE anti-CD11c and FITC-conjugated antibodies to CD40, I-A^b, B7.1, B7.2, or ICAM-1 (solid line) or isotype controls (dotted line) and analyzed by flow cytometry. Data shown are representative of 5 mice of each genotype.

Propensity for Bim$^{-/-}$ DCs in inducing the production of autoantibodies

Bim$^{-/-}$ mice develop systemic autoimmunity featuring lymphocyte expansion and autoimmune tissue destruction.[23] Thymic negative selection is important for the deletion of high-affinity self-reactive T cells.[34,35] In Bim$^{-/-}$ mice, defective negative selection in the thymus has been detected.[24] This suggests that autoreactive T cells may escape thymic selection and induce autoimmune responses in Bim$^{-/-}$ mice. The thymus is also critical for the development of the CD4$^+$FoxP3$^+$ T regulatory (Treg) cells that can actively inhibit immune responses of responder T cells in the periphery.[36,37] Abundant evidence suggests that Treg cells play an important role in suppressing autoreactive T cells in the periphery as a dominant tolerance mechanism.[37-40] We therefore examined whether the Treg cells might be decreased in Bim$^{-/-}$ mice. Compared with control mice, Bim$^{-/-}$ mice actually contained slightly increased CD4$^+$FoxP3$^+$ Treg cells in both the spleen and the lymph nodes (Figure 7A). This implies that the development of autoimmunity in Bim$^{-/-}$ mice is not due to a loss of Treg cells.

Because Bim$^{-/-}$ DCs showed increased potency in activating T cells (Figures 5-6), we tested whether Bim$^{-/-}$ DCs were capable of inducing autoimmune responses. We examined whether Bim$^{-/-}$ DCs induced autoantibodies in recipient mice by adoptive transfer similar to established protocols.[27] Wild-type or Bim$^{-/-}$ DCs were transferred into syngenic C57BL/6 recipient mice followed by injection of LPS one day later. Sera in the recipient mice were collected 1 and 2 weeks later to measure the levels of autoantibodies by ELISA. Wild-type DCs did not induce significant levels of anti-dsDNA, anti-ssDNA, or ANAs in the recipients (Figure 7B). By contrast, Bim$^{-/-}$ DCs induced elevated levels of anti-dsDNA, anti-ssDNA, and ANA one week after adoptive transfer of DCs (Figure 7B). Two weeks after DC transfer, the levels of ANA, anti-dsDNA, and anti-ssDNA decreased but were still higher in mice that received the transfer of Bim$^{-/-}$ DCs (Figure 7B). IgG

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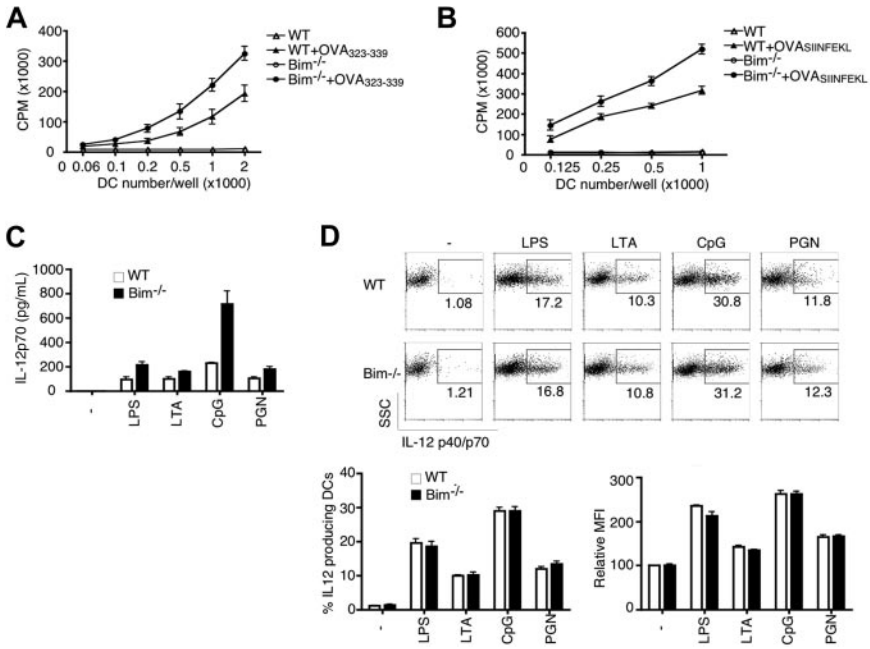

Figure 5. Bim$^{-/-}$ DCs in T-cell stimulation. (A) CD4$^+$ OT2 cells (10^5/well) were cultured with indicated numbers of wild-type (WT) or Bim$^{-/-}$ CD11c$^+$ DCs with or without pulsing of $OVA_{323-229}$ peptide. Three days later, the cells were labeled with [^{3}H]-thymidine during the last 8 hours of culture to measure [^{3}H]-thymidine incorporation. Data (mean ± SEM) are averages of 3 independent experiments. (B) CD8$^+$ OT1 cells (10^5/well) were cultured with indicated numbers of WT or Bim$^{-/-}$ CD11c$^+$ DCs with or without pulsing of $OVA_{SIINFEKL}$ peptide. [^{3}H]-thymidine incorporation was measured 3 days later. Data (mean ± SEM) are averages of 3 independent experiments. (C) CD11c$^+$ BMDCs from WT or Bim$^{-/-}$ mice were treated with various TLR stimuli as indicated. IL-12p70 in the supernatants was quantitated by ELISA. Data (mean ± SD) are representative of 3 experiments. (D) CD11c$^+$ BMDCs from WT or Bim$^{-/-}$ mice were treated with various TLR stimuli and were used for intracellular staining for IL-12 by flow cytometry. The percentage of IL-12–producing cells and the mean fluorescence staining (MFI) (means ± SD) for IL-12 staining were plotted.

deposition in the glomeruli of kidneys was detectable in approximately 50% of the recipients transferred with Bim$^{-/-}$ DCs, but not in mice that received wild-type DCs (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). These results suggest that Bim$^{-/-}$ DCs have the potential to induce the production of autoantibodies. However, we did not detect increased capacity for Bim$^{-/-}$ DCs in directly promoting B-cell proliferation (Figure S2). This suggests that autoantibody production induced by Bim$^{-/-}$ DCs is potentially mediated through overactivation of T cells, rather than through a direct effect on B cells.

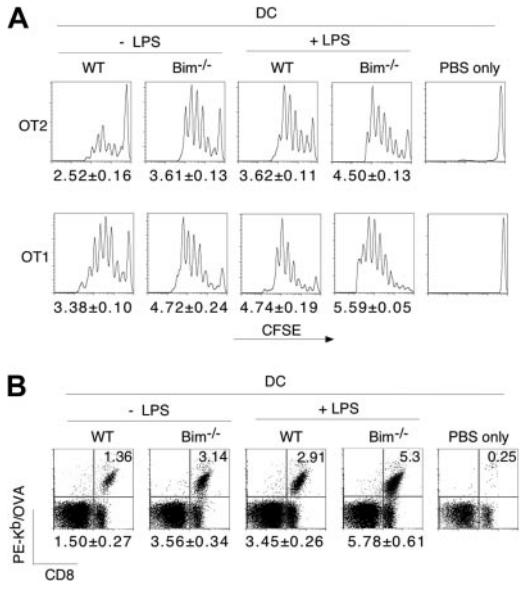

Figure 6. Increased immunogenicity of Bim$^{-/-}$ DCs in vivo. (A) CFSE-labeled OT1 or OT2 T cells were injected into recipient mice. DCs from WT or Bim$^{-/-}$ mice with or without LPS stimulation were pulsed with corresponding OVA peptides, or PBS alone, and then injected into recipient mice. Three days later, CFSE$^+$ cells in the draining nodes were analyzed for CFSE dilution of dividing cells. The average numbers of cell division (means ± SEM) from 5 independent mice were also calculated. Statistical significance was analyzed by Student *t* test, and the *P* values are as follows: for mice injected with OT2 cells, .001 (WT vs Bim$^{-/-}$) and .001 (WT + LPS vs Bim$^{-/-}$ + LPS); and for mice injected with OT1 cells, .001 (WT vs Bim$^{-/-}$) and .001 (WT + LPS vs Bim$^{-/-}$ + LPS). (B) CD8$^+$ OVA-specific T cells in the draining lymph nodes in panel A were also detected by staining with anti-CD8 and H-2k^b/OVA tetramer. The percentages of CD8$^+$K^b/OVA$^+$ cells are averages of 5 independent mice (means ± SEM). Statistical significance was analyzed by Student *t* test, and the *P* values are as follows: .001 (WT vs Bim$^{-/-}$), .001 (WT + LPS vs Bim$^{-/-}$ + LPS).

Discussion

In this study, we investigated the roles of Bim in regulating apoptosis and functions of DCs. Bim was expressed at low levels in DCs. Activation of DCs through CD40 or TLRs up-regulated Bim (Figure 1). DCs deficient in Bim showed improved survival (Figures 2-3), induced more robust T-cell activation in vitro and in vivo (Figures 5-6), and displayed the propensity for triggering autoantibody production (Figure 7). Our results suggest that Bim plays an important role in regulating DC apoptosis, and that defective apoptosis of Bim$^{-/-}$ DCs potentially contributes to the development of autoimmunity in Bim$^{-/-}$ mice. Previously, we have shown that inhibition of apoptosis in DCs in transgenic mice expressing a caspase inhibitor, p35, can lead to spontaneous T-cell activation and the onset of systemic autoimmunity.[18] p35 preferentially inhibits caspase-8–mediated death-receptor–dependent apoptosis pathway.[17] Our current study indicates that defective DC apoptosis due to the loss of Bim, which mediates mitochondrion-dependent apoptosis,[21] may also contribute to the development of autoimmunity.

Different BH3-only proteins may function in different cell types. Bim has been found to regulate apoptosis in T cells and B cells.[23-25] Our data suggest that Bim is also involved in regulating apoptosis in DCs. Bim was expressed at low levels in resting DCs, but was significantly up-regulated by TLR stimuli (Figure 1), while deficiency in Bim improved DC survival after TLR stimulation (Figure 3B). These data suggest that Bim-mediated DC apoptosis may be involved in controlling the duration of DCs in inducing immune responses. Although increased expression of costimulatory molecules on DCs has been shown to enhance immunogenicity of DCs,[1] we did not observe elevated expression of CD40, MHC class II, B7.1, B7.2, and ICAM-1 on Bim$^{-/-}$ DCs (Figure 4B).

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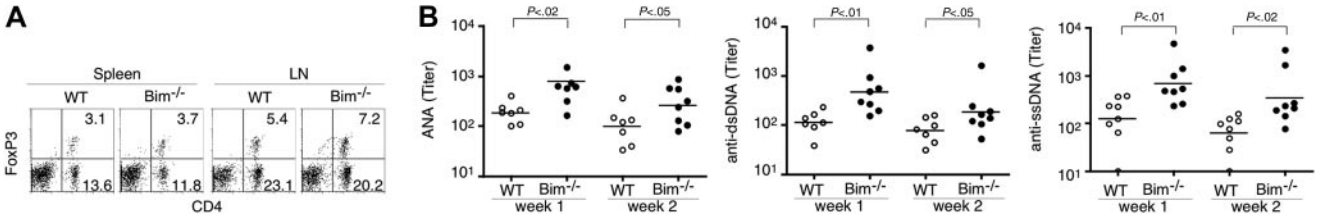

Figure 7. Roles of Bim$^{-/-}$ DCs in inducing autoantibody production. (A) Splenic and lymph node (LN) cells from 2-month-old Bim$^{-/-}$ or wild-type (WT) control mice were incubated with cychrome anti-CD4, followed by intracellular staining with PE anti-FoxP3 and analysis by flow cytometry. Percentages of CD4$^+$FoxP3$^+$ Treg cells (mean ± SD) in 4 WT and Bim$^{-/-}$ mice were as follows: in the spleens, 2.9 ± 0.30 (WT) and 3.6 ± 0.44 (Bim$^{-/-}$), $P = .32$; in the LNs, 5.6 ± 0.61 (WT) and 6.9 ± 0.35 (Bim$^{-/-}$), $P = .25$. (B) CD11c$^+$ WT or Bim$^{-/-}$ BMDCs were injected into syngenic C57BL/6 recipient mice intraperitoneally, followed by injection of LPS intraperitoneally (30 μg/mouse) 24 hours later. Sera were collected from recipient mice 1 and 2 weeks later for quantitation of ANA, anti-ssDNA, and anti-dsDNA by ELISA. The titers of the antibodies shown are representative of 3 independent experiments. Bars indicate geometric means. Statistical significances were analyzed by Student *t* test using GraphPad Prism version 4 for Macintosh (GraphPad Software, San Diego, CA). A *P* value less than .05 was considered statistically significant.

Therefore, the enhanced immunogenicity of Bim$^{-/-}$ DCs is likely due to prolonged survival rather than enhanced activation status.

Of interest, despite up-regulation of Bim expression, TLR stimulation increased DC survival in culture. This could be explained by concomitant up-regulation of antiapoptotic Bcl-X_L and Mcl-1 expression in DCs by TLR stimulation (Figure 1). The increased expression of the prosurvival proteins (Bcl-xL and Mcl-1) likely antagonized the effects of elevated Bim expression, resulting in improved survival of TLR-stimulated DCs. Our data therefore support the notion that the balance between proapoptotic (such as Bim) and antiapoptotic (such as Bcl-xL) Bcl-2 family proteins and their temporal expression patterns determine DC survival and life span.

We observed that DC numbers recovered from draining lymph nodes following OVA-specific T-cell stimulation were reduced compared with those from mice immunized by unpulsed DCs (Figure 2C). We have previously shown that antigen-specific T cells could kill DCs through Fas and perforin pathways.[18] Therefore, the accelerated disappearance of antigen-loaded DCs in the lymph nodes following encounter with antigen-specific T cells could be attributed to T-cell–mediated killing of DCs.

An existing viewpoint suggests that immature DCs induce tolerance, while mature DCs induce immunity. However, immature DCs do not necessarily induce T-cell inactivation.[41] Immature DCs are also capable of inducing the activation of immunity.[42-44] We found that immature DCs could also induce potent antigen-specific activation (Figures 5-6), although at levels lower than those triggered by mature DCs. We compared unstimulated versus LPS-treated DCs for OT1/OT2 T-cell proliferation in vivo (Figure 6). While LPS-stimulated DCs were better in inducing T-cell proliferation, DCs without LPS stimulation also induced significant T-cell proliferation. Enhanced survival of Bim$^{-/-}$ DCs likely contributed to the increased immunogenicity of Bim$^{-/-}$ DCs with or without LPS treatment.

Multiple mechanisms are involved in the establishment and maintenance of self-tolerance.[45] Negative selection in the thymus is important for the deletion of high-affinity self-reactive T cells during T-cell development.[34,35] The thymus is also involved in the development of CD4$^+$FoxP3$^+$ natural Treg cells.[36,37] Treg cells are important for suppressing autoreactive T cells in the periphery as a dominant tolerance mechanism.[37-40] In Bim$^{-/-}$ mice, defective negative selection for autoreactive T cells and B cells has been observed,[24,25] suggesting that the escape of autoreactive lymphocytes may contribute to the development of autoimmunity in Bim$^{-/-}$ mice. However, Treg cells were not decreased in Bim$^{-/-}$ mice (Figure 7A), implying that the development of autoimmunity in Bim$^{-/-}$ mice is not due to the loss of Treg cells.

Although negative selection helps to delete T cells with high autoreactive potential, T cells that develop and populate the peripheral lymphoid organs are positively selected by self-ligands in the thymus and therefore carry certain degrees of autoreactivity.[46] Different mechanisms, such as apoptosis in expanded lymphocytes during and after immune responses, clonal anergy, and Treg cells may help to maintain lymphocyte homeostasis and peripheral tolerance.[12,13,47] Injection of overactivated DCs has been associated with the development of systemic and tissue-specific autoimmune diseases,[48,49] suggesting that a surplus of DCs can potentially disrupt peripheral tolerance. We detected increased numbers of DCs in Bim$^{-/-}$ mice (Figure 4A). Moreover, DCs from Bim$^{-/-}$ mice induced stronger T-cell activation than wild-type DCs in vitro and in vivo (Figures 5-6). Bim$^{-/-}$ DCs also displayed the propensity for inducing autoantibody production after adoptive transfer into recipient mice (Figure 7B). This suggests that defective apoptosis in Bim-deficient DCs may also contribute to the onset of autoimmune diseases in Bim$^{-/-}$ mice.

It is possible that the onset of autoimmune diseases involves the breakdown of several different tolerance mechanisms. DCs may influence the outcome of immune responses by regulating the duration of DCs in stimulating lymphocytes. Apoptosis may play an important role in limiting the life span of DCs to help preserve self-tolerance. Our data suggest that Bim plays a key role in regulating the life span of DCs. In combination with other defects, such as defective negative selection in the thymus, defective Bim-dependent apoptosis in DCs may contribute to abnormal DC survival, chronic lymphocyte activation, and the development of autoimmunity.

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Authorship

Contribution: M.C. and J.W. designed the research, performed research, analyzed the data, and wrote the paper; L.H. performed research and analyzed the data.

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