Immune Regulation through Mitochondrion-Dependent Dendritic Cell Death Induced by T Regulatory Cells

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Dendritic cells (DCs) harbor an active mitochondrion-dependent cell death pathway regulated by Bcl-2 family members and undergo rapid turnover in vivo. However, the functions for mitochondrion-dependent cell death of DCs in immune regulation remain to be elucidated. In this article, we show that DC-specific knockout of proapoptotic Bcl-2 family members, Bax and Bak, induced spontaneous T cell activation and autoimmunity in mice. In addition to a defect in spontaneous cell death, Bax<sup>−/−</sup>Bak<sup>−/−</sup> DCs were resistant to killing by CD4<sup>+</sup>Foxp3<sup>+</sup> T regulatory cells (Tregs) compared with wild-type DCs. Tregs inhibited the activation of T effector cells by wild-type, but not Bax<sup>−/−</sup>Bak<sup>−/−</sup>, DCs. Bax<sup>−/−</sup>Bak<sup>−/−</sup> DCs showed increased propensity for inducing autoantibodies. Moreover, the autoimmune potential of Bax<sup>−/−</sup>Bak<sup>−/−</sup> DCs was resistant to suppression by Tregs. Our data suggested that Bax and Bak mediate intrinsic spontaneous cell death in DCs, as well as regulate DC killing triggered by Tregs. Bax- and Bak-dependent cell death mechanisms help to maintain DC homeostasis and contribute to the regulation of T cell activation and the suppression of autoimmunity. *The Journal of Immunology*, 2011, 187: 5684–5692.
not been well characterized. Furthermore, it is not known whether mitochondrion-dependent cell death in DCs plays a role in the regulation of immune tolerance.

Tregs can interact with DCs to inhibit the activation of Ag-specific T cells in vivo (36–42). Tregs may downregulate important costimulatory molecules on DCs (40, 43–48). Interestingly, one report suggested that Tregs may cause the disappearance of DCs in the draining lymph nodes (38). It has not been determined whether Tregs can induce cell death in DCs or whether such interactions help to protect immune tolerance. Using mice with DCs deficient in Bax and Bak, we showed that CD4+Foxp3+ Tregs efficiently induced mitochondrion-dependent cell death in DCs. Different from the killing of DCs by effector T cells (Teffs) through Fas, our current study suggests that Tregs exploit the active mitochondrion-dependent apoptosis pathway in DCs for immune regulation. Such interactions between Tregs and DCs potentially play a fundamental role in the regulation of initiation and expansion of Ag-specific immune responses, as well as in the protection of immune tolerance.

Materials and Methods

Flow cytometry, preparation of DCs, measurement of autoantibodies, cell death assays, and histochemistry

Flow cytometry analyses of different cell types, preparation of bone marrow-derived DCs (BMDCs) and splenic DCs, histochemistry, measurements of spontaneous cell death in DCs, DC turnover in vivo by BrdU labeling, and detection of autoantibodies in mice were performed as described (22, 29).

Proliferation assays

Mice were immunized with OVA (50 μg/mouse) emulsified in CFA at the footpad. Ten days later, total cells (2 × 10^6/well) from the draining popliteal lymph nodes were cultured in 96-well plates with various concentrations of OVA for 72 h. The cells were pulsed with 1 μCi/well [3H]thymidine for the last 12 h and harvested to measure [3H]thymidine incorporation.

CD4+Foxp3+ Tregs were sorted from Foxp3GFP mice and expanded in vitro with anti-CD3– and anti-CD28–coated Dynabeads (Invitrogen; 10^5/mouse). DCs (2 × 10^6 or 5 × 10^5) were injected into the footpad of C57BL/6 mice (six mice/group), with or without Tregs (with Treg/DC at 0.5:1), essentially as described (29, 51). The mice were then injected with 10 μg/ml LPS and 100 μg/ml Cpg at 48 h. Tregs-mediated killing of splenic DCs and activated T or B cells were measured as the killing of BMDCs above. CD4+Foxp3+ Tregs or CD4+Foxp3− T cells sorted from OT2-Foxp3GFP mice were stimulated with anti-CD11c MACS beads (Miltenyi Biotec). Activated CD4+ T cells were generated by stimulating sorted CD4+CD25+ cells anti-CD3– and anti-CD28–coated Dynabeads in the presence of 10 U/ml IL-2 for 3 d. B cells were purified with anti-CD19 MACS beads and stimulated with 1 μg/ml LPS and 100 μg/ml Cpg at 48 h. Tregs-mediated killing of splenic DCs and activated T or B cells were measured as the killing of BMDCs above. CD4+Foxp3+ Tregs or CD4+Foxp3− T cells from OT2-Foxp3GFP mice were stimulated with OVA323–339 peptide-pulsed DCs (TC/DC = 2:1) for 3 d. OT2 Tregs or Teffs were then isolated by removing DCs with anti-CD11c MACS beads (Miltenyi Biotec) and used for killing of DCs, with or without pulsing with OVA323–339 peptide. Percentages of killing of DCs by T cells were measured, as above.

Analyses of interaction between Tregs and DCs

To detect interactions between Tregs and DCs in vivo, DCs and Tregs were labeled at 37°C for 10 min with 10 μM 5-((and -6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTRM) and CFSE, respectively. WT or DCO DCs (1 × 10^6) were injected into the footpad of recipient mice. Tregs (0.5 × 10^6) were also injected into the footpad of some recipient mice. Draining (popliteal) lymph nodes were harvested 24 h later, and frozen sections were analyzed using an LSM 510 confocal microscope (Zeiss).

To determine the conjugate formation between DCs and Tregs in vitro, DCs and Tregs were labeled at 37°C for 10 min with 0.3 μM CMTMR and CFSE, respectively. The cells were mixed at a ratio of 1:1 and centrifuged at 500 × g for 5 min. The cells were incubated at 37°C for 0, 1, 2, or 3 h in the absence or presence of 10 μg/ml anti-LAG3, anti–LFA-1 (BD Bioscience), or rat IgG. The cells were washed with PBS and analyzed by flow cytometry.

Adaptive transfer of DCs

To determine autoantibody production after adoptive transfer, WT or Bax−/− Bak−/− Bak−/− BMDCs (10^5 or 5 × 10^5/mouse) were injected i.p. into 8-wk-old C57BL/6 mice (six mice/group), with or without Tregs (with Treg/DC at 0.5:1), essentially as described (29, 51). The mice were then injected with LPS (30 μg/mouse) i.p. 1 d later. Sera were collected from the recipient mice 1 wk after DC transfer. Anti-nuclear Abs (ANAs), anti-ssDNA, and anti-dsDNA were measured, as above. In parallel experiments, CFSE-labeled WT or Bax−/− Bak−/− BMDCs (10^5 or 5 × 10^5/mouse) were injected into 8-wk-old C57BL/6 mice (six mice/group), with or without Tregs (with Treg/DC at 0.5:1) for the footpad. Twenty-four hours later, draining (popliteal) lymph nodes were collected. Total cell numbers were counted, and percentages of CFSE− DCs were determined by flow cytometry. Total CFSE− DCs in the draining lymph node cells were calculated.
**Western blot**

To determine Treg-mediated signaling in DCs, WT or DKO DCs were incubated with Tregs at a ratio of 2:1 for 6 h at 37°C. The cells were incubated with anti-CD11c MACS beads (Miltenyi Biotec) to isolate DCs. DCs were then lysed for Western blot analyses. The following primary Abs were used: polyclonal rabbit Abs to caspase-8, caspase-3, Bcl-xL, Bad (Cell Signaling), Bax (Santa Cruz Biotechnology), Bcl-2, Bak (Upstate Biotechnology), McI-1 (Fitzgerald), Bim (Stressgen), Bid (Imgenex), Blk or Bmf (Biovision), or mouse mAb to caspase-9 (MLB), Noxa (Imgenex), or XIAP (BD Bioscience). The blots were then probed with HRP-conjugated secondary Abs and developed using the chemiluminescent method (Pierce). CD11c+CD11b+ DCs, CD3+ T cells, or CD19+ B cells sorted from the spleen of DC-DKO and control mice were also used for Western blot analyses of Bax and Bak, as above. The blots were also probed with anti-α-tubulin (Santa Cruz Biotechnology) to ensure equal loading.

**Intracellular staining for cytokines**

CD11c+ BMDCs (10^9/ml) were cultured in the absence or presence of 1 μg/ml LPS (Sigma) or 0.5 μM phosphorothioate-stabilized CpG oligonucleotide (5’-TCCATGACGTTCCCTGATGCT-3’) for 24 h. Brefeldin A (1 μg/ml) and monensin (2 μM) were added during the last 6 h to inhibit cytokine 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), PE-conjugated IL-6 (BD Biosciences), or PE-conjugated rat IgG1 as isotype control. The cells were then analyzed by flow cytometry.

**Statistical analysis**

Data are presented as mean ± SD. The p values were determined by two-tailed Student t test using GraphPad Prism software version 4.0 for Macintosh; p < 0.05 was considered statistically significant.

**Results**

**Accumulation of DCs because of deficiencies in Bax and Bak**

To determine the functions of mitochondrion-dependent apoptosis in immune regulation, we generated double knockouts of Bax and Bak in DCs (DC-DKO) by crossing CD11c+cre mice with BaxfloxBak−/− mice. Specific deletion of Bax in DCs was observed in DC-DKO mice (Supplemental Fig. 1A). Because Bax and Bak are functionally redundant of each other (34), deletion of Bak alone is not expected to affect apoptosis in other cell types. DC-DKO mice, but not Bak−/− or DC-Bax−/− mice, displayed enlargement of the spleen and lymph nodes (Supplemental Fig. 1B). Terminally differentiated DCs have low proliferative potentials; pulsing with BrdU in vivo can be used to measure the rate of DC turnover by the appearance of newly generated BrdU+ DCs (52). We found that DCs were labeled more slowly with BrdU in DC-DKO mice (Supplemental Fig. 1C), indicating a reduced DC turnover rate in these mice. Consistently, the percentages and total numbers of CD11c+I-Aβ−/− or CD11c+CD40+ conventional DCs were increased in DC-DKO mice (Fig. 1A, 1B). CD11c+low PDCA-1+ plasmacytoid DCs increased to a lesser extent in DC-DKO mice (Fig. 1A, 1B). The percentage of T, B, or NK cells was not elevated in DC-DKO mice (Supplemental Fig. 1D). DCs from DC-DKO mice, but not Bak−/−, DC-Bax−/−, Fas-deficient lpr, CD11c+ or CD19+ cells, showed defects in spontaneous cell death (Fig. 1C). However, splenic DCs from DC-DKO mice and WT controls expressed similar levels of CD40, MHC class II (MHC-II), B7.1, B7.2, and ICAM-1 (Supplemental Fig. 1E), suggesting that deficiencies in Bax and Bak cause DC accumulation but not abnormal DC activation.

**Spontaneous T cell activation and systemic autoimmunity in DC-DKO mice**

T cells from DC-DKO mice showed increased expression of an activation marker, CD69 (Supplemental Fig. 2A). In particular, more than half of CD4+ T cells were CD69+ (Supplemental Fig. 2A). CD69 was also increased on CD8+ T cells and CD19+ B cells in DC-DKO mice (Supplemental Fig. 2A). In addition, similar to lpr mice, T cells with the CD44+CD62Llow phenotype were increased in DC-DKO mice compared with controls (Supplemental Fig. 2A). These data suggested that deficiencies in Bax and Bak in DCs lead to DC expansion and abnormal lymphocyte activation.

We determined whether deficiencies in mitochondrion-dependent cell death induce autoimmune responses in DC-DKO mice. DC expansion and enlargement of lymphocyte areas were observed in spleen sections of 6-mo-old DC-DKO mice (Supplemental Fig. 1F). Severe perivascular lymphocyte infiltration was found in the liver, lung, and kidney of DC-DKO mice but not DC-Bax−/−, Bak−/− or WT controls (Supplemental Fig. 1G). IgG deposits were also observed in the glomeruli of kidneys in DC-DKO mice (Supplemental Fig. 1H). We also detected autoantibodies, including anti-dsDNA, anti-ssDNA, and ANAs, in the sera of 3- and 6-mo-old DC-DKO mice, but not in controls, by ELISA (Fig. 2A). Consistent with the production of ANAs, sera from DC-DKO mice showed nuclear staining of Hep2 cells (Fig. 2B). Together, these observations suggested that deficiencies in Bax and Bak in DCs lead to the development of systemic autoimmunity. Normal levels of CD4+Foxp3+ natural Tregs were detected in DC-DKO mice (Supplemental Fig. 2A). Moreover, natural Tregs from DC-DKO and control mice showed comparable activities in inhibiting the proliferation of Teffs (Supplemental Fig. 2B), indicating that there is no intrinsic defect in natural Tregs in DC-DKO mice.

**Increased immunogenicity of Bax−/− Bak−/− DCs**

We found that cells from the draining lymph nodes of immunized DC-DKO mice responded better to Ag restimulation than those of
Although natural Foxp3+ Tregs were not reduced or dysfunctional in vivo (Fig. 3E), CD4⁺Foxp3⁺ Tregs suppressed the proliferation of OT2 Teffs induced by WT, Bax−/−, or Bak−/− DCs in vivo, as measured by CFSE dilution (Fig. 3F). In contrast, Bax−/−Bak−/− DC-induced T cell proliferation was less susceptible to inhibition by Tregs in vivo (Fig. 3F). This supports the conclusion that Bax−/−Bak−/− DCs are refractory to suppression by Tregs.

**Induction of Bax- and Bak-dependent cell death in DCs by Tregs**

We found that activated Tregs acquired cytotoxicity against DCs (Fig. 4A). We examined whether activated Tregs could directly induce cell death in DCs in a Bax- and Bak-dependent manner. We observed that DCs were more susceptible than activated B or T cells to killing by CD4⁺Foxp3⁺ Tregs (Fig. 4B). WT, but not Bax−/−Bak−/− DCs were sensitive to killing by Tregs (Fig. 4C, Supplemental Fig. 3A, 3B), suggesting that Tregs induce DC cell death in a Bax- and Bak-dependent manner. Treg-mediated killing of DCs is not affected by treating DCs with LPS (Supplemental Fig. 3C), suggesting that Treg-mediated killing of DCs is not affected by DC maturation. It was shown that Tregs can negatively regulate DCs by restricting DC development (54), inhibiting the expression of costimulatory molecules on DCs or competing with Teffs in the interaction with DCs (36–38, 40). Apoptotic DCs may promote further induction of Tregs (55). Inducing Bax- and Bak-dependent cell death in DCs may provide another mechanism for DC regulation by Tregs.

Blocking MHC-II inhibited the killing of DCs by polyclonal Tregs (Fig. 4D). This suggests that the polyclonal Tregs are potentially autoreactive. By using OVA-specific OT2 Foxp3⁺ Tregs, we found that Foxp3⁺ Tregs killed Ag-pulsed, but not unpulsed, DCs (Fig. 4E). Using Foxp3⁺ Tregs expressing a transgenic TCR specific for a foreign Ag may not recuperate the action of natural Tregs carrying TCRs that tend to be autoreactive. Nevertheless, our data obtained by using OT2 Foxp3⁺ Tregs suggested that recognition of Ags on DCs is important for killing by Tregs. In contrast to Tregs, polyclonal Tafs did not efficiently kill DCs in the absence of anti-CD3 (Fig. 4C), whereas OT2 Tafs showed killing of DCs in the presence of OVA Ag at a higher T:DC ratio (3:1; Fig. 4E).

**Bax- and Bak-dependent clearance of DCs in the draining lymph nodes after adoptive transfer**

We consistently detected more Bax−/−Bak−/− DCs in the draining lymph nodes after adoptive transfer (Fig. 5A). Moreover, cotransfer of Tregs led to the loss of WT, but not Bax−/−Bak−/− DCs in the draining lymph nodes (Fig. 5A). This is consistent with the conclusion that Bax and Bak regulate spontaneous cell death of DCs, as well as Treg-dependent DC killing. Tregs can induce Bax- and Bak-dependent cell death in DCs. In addition, we also observed that Tregs formed conjugates with both WT and Bax−/−Bak−/− DCs in the draining lymph nodes (Fig. 5A). Conjugate formation between Tregs and DCs was also detected in vitro (Supplemental Fig. 3D–H). WT and DKO DCs were similar in forming conjugates with Tregs after 1 h of incubation in vitro (Supplemental Fig. 3E, 3H). Consistent with confocal microscopy analyses, more Bax−/−Bak−/− DCs were found in the draining lymphocytes than WT DCs, as determined by flow cytometry (Fig. 5B). Cotransfer of Tregs also promoted the clearance of WT, but not Bax−/−Bak−/− DCs (Fig. 5B). These data suggested that Bax and Bak do not affect conjugate for-

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**Figure 2**. Systemic autoimmunity in DC-DKO mice. A, ELISA for autoantibodies in the sera of 3- or 6-mo-old DC-cre mice (WT control), DC-DKO, Bak−/−, and DC-Bax−/− mice. *p < 0.05, **p < 0.01. B, Hep2 cell slides were incubated with sera (1:640 dilution) from 6-mo-old DC-DKO or control mice, followed by staining with FITC-anti-IgG. Scale bar, 30 μm. Data are representative results of five mice/group analyzed.
mation between Tregs and DCs. Rather, Bax and Bak regulate spontaneous cell death of DCs, as well as Treg-mediated clearance of DCs in vivo.

Autoimmune potential of Bax<sup>-/-</sup>Bak<sup>-/-</sup> DCs in adoptive transfer

To directly test whether Bax<sup>-/-</sup>Bak<sup>-/-</sup> DCs can induce autoimmune responses, we performed adoptive transfer of WT and Bax<sup>-/-</sup>Bak<sup>-/-</sup> DCs, with or without Tregs. It was shown that transfer of excessive activated DCs can trigger the production of autoantibodies in recipient mice (51, 56). Adoptive transfer of Bax<sup>-/-</sup>Bak<sup>-/-</sup> DCs at a low dose (1 × 10<sup>6</sup> cells/mouse) induced the production of anti-dsDNA, anti-ssDNA, and ANAs, whereas a higher dose (5 × 10<sup>6</sup> cells/mouse) triggered more autoantibody production (Fig. 6). Cotransfer of Tregs did not significantly suppress autoantibody production induced by Bax<sup>-/-</sup>Bak<sup>-/-</sup> DCs (Fig. 6). In contrast, WT DCs induced detectable levels of autoantibody production only at the high dose (5 × 10<sup>6</sup> cells/mouse), which was efficiently suppressed by cotransfer of Tregs (Fig. 6). These data provided direct evidence to support the conclusion that Bax<sup>-/-</sup>Bak<sup>-/-</sup> DCs have the propensity for triggering autoantibody production. Moreover, Bax<sup>-/-</sup>Bak<sup>-/-</sup>, but not WT, DCs are resistant to suppression by Tregs in the induction of autoimmune responses.

Molecules involved in the killing of DCs by activated Tregs

We next investigated apoptosis signaling in DCs induced by Tregs. After incubation with Tregs, WT DCs lost ΔΨm, whereas DCs were relatively resistant to the loss of ΔΨm (Fig. 7A). Consistent with the activation of mitochondrion-dependent cell death in WT DCs, Tregs induced the activation of caspase-9 and caspase-3 in WT, but not Bax<sup>-/-</sup>Bak<sup>-/-</sup> DCs (Fig. 7B). It was shown that cleavage of Bid into active truncated Bid by caspases can trigger mitochondrial apoptosis (57). Caspase-dependent cleavage may inactivate antiapoptotic Bcl-2 family proteins to promote mitochondrion-dependent cell death (58). However, we did not find processing of Bcl-2 family proteins in DCs after incubation with Tregs (Supplemental Fig. 4A). Tregs also did not activate Bax and Bak in DCs after encountering Tregs. Although we did not find cleavage of Bid into active truncated Bid by caspases, we did find cleavage of Bid into active truncated Bid by caspases, which triggers mitochondrion-dependent cell death (58). However, we did not find processing of Bcl-2 family proteins in DCs after incubation with Tregs (Supplemental Fig. 4A). Tregs also did not activate Bax and Bak in DCs after encountering Tregs. Although
the precise mechanism for the activation of Bim is not resolved, Bim potentially transmits cell death signaling by sequestering antiapoptotic molecules or directly activating Bax and Bak (59, 60).

We then investigated which effector molecules in Tregs could induce cell death in DCs. The killing of DCs by Teffs involves Fas and perforin (22, 61, 62) but does not appear to require Bax and Bak (Supplemental Fig. 4B, 4C), suggesting that Tregs and Teffs use different mechanisms to kill DCs. Interestingly, Tregs in tumor tissues can kill DCs in a perforin/granzyme-dependent manner (41), whereas Treg-induced cell death in Teffs is independent of Fas or perforin (63). We found that Treg-mediated killing of DCs did not involve Fas but required prior activation of Tregs (Fig. 4A, Supplemental Fig. 4D). Also, Tregs did not induce proteolytic activation of caspase-8 in DCs (Fig. 7B), consistent with the possibility that Tregs do not engage Fas on DCs to activate caspase-8. In addition, deficiencies in perforin or granzymes A/B in Tregs did not affect the killing of DCs (Supplemental Fig. 4E).

**FIGURE 4.** Susceptibility of DCs to killing by Tregs. A, Sorted CD4+Foxp3+ Tregs were cultured in vitro with anti-CD3- and anti-CD28–coated Dynabeads in the presence of IL-2 for 0, 1, 2, or 3 d. BMDCs were labeled with CFSE and incubated with Tregs for 6 h. Killing of DCs by Tregs was determined as described in Materials and Methods. **p < 0.01, day 3 versus day 0 at 0.3:1 and 1:1 Treg:DC ratios (n = 3). B, CD4+Foxp3+ Tregs were sorted and stimulated with anti-CD3- and anti-CD28–coated Dynabeads in the presence of IL-2 for 3 d. Treg-mediated killing (n = 5) of BMDCs, splenic DCs, or activated B or T cells was determined as described in Materials and Methods. C, CD4+Foxp3+ Tregs or CD4+Foxp3+ Teffs, sorted and expanded as in Figure 3, were incubated with WT or Bax−/−Bak−/− (DKO) BMDCs for 6 h, followed by staining with 7-AAD and analyses by flow cytometry. Loss of 7-AAD− DCs was quantitated. **p < 0.01, WT versus DKO (n = 3). D, Killing of DCs by CD4+Foxp3+ Tregs, as in A, in the presence of anti–MHC-II or control IgG. **p < 0.01 (n = 3). E, CD4+Foxp3+ Tregs or CD4+Foxp3+ Teffs were sorted from OT2/Foxp3GFP mice and expanded with OVA323–339 peptide-pulsed BMDCs for 3 d. OT2 Tregs or Teffs were then isolated by removing DCs with anti-CD11c MACS beads (Miltenyi Biotec) and used for killing of BMDCs, with or without pulsing with OVA323–339 peptide. **p < 0.01, WT versus DKO (n = 3).

**FIGURE 5.** Clearance of DCs by Tregs after adoptive transfer. A, WT and Bax−/−Bak−/− BMDCs were labeled with CMTMR (red) and injected s.c. into footpad. In some groups, CFSE (green)-labeled Tregs were also injected into footpad. Draining (popliteal) lymph nodes were harvested 24 h later. Frozen sections were prepared and analyzed by confocal microscopy. Scale bar, 20 μm. Quantification of DCs per focal section field (0.02 mm²) in the draining lymph nodes (right panel). **p < 0.01 (n = 9). B, CD11c+ WT or DKO BMDCs (10⁶ or 5 × 10⁵) labeled with CFSE were injected with or without Tregs (Treg:DC ratio 0.5:1) into syngeneic C57BL/6 recipient mice at the footpad. Twenty-four hours later, the draining lymph nodes (LN) were harvested. CFSE+ DCs were analyzed by flow cytometry, and total CFSE+ DCs in the draining lymph nodes were calculated. **p < 0.01 (n = 6).

**FIGURE 6.** Suppression of DC-induced autoantibody production by Tregs after adoptive transfer. CD11c+ WT or DKO BMDCs (10⁶ or 5 × 10⁵) were injected, with or without Tregs (Treg:DC ratio 0.5:1), into syngeneic C57BL/6 recipient mice i.p. (six mice/group), followed by injection of LPS i.p. (30 μg/mouse) 24 h later. Sera were collected from recipient mice 1 wk later for quantitation of ANA, anti-ssDNA, and anti-dsDNA by ELISA. **p < 0.01 (n = 6).
suggesting that these molecules are not required for the killing of DCs by natural Tregs.

We observed that blocking LFA-1 inhibited Treg-mediated killing of DCs (Fig. 7D). The conjugate formation between Tregs and DCs was inhibited by blocking LFA-1 (Supplemental Fig. 4G, 4H), suggesting that cell adhesion promotes the killing of DCs by Tregs. Molecules highly expressed by Tregs were suggested to be critical for Treg functions (11, 40, 64, 65). We observed that inhibition of LAG3 partially suppressed the killing of DCs by Tregs (Fig. 7D), whereas neutralization Abs to soluble factors, including IL-10 and TGF-β, had no effect (data not shown). LAG3 is a potent ligand for MHC-II molecules and can trigger negative signaling in DCs to suppress maturation and immunostimulatory capacity of DCs (66). On unstimulated Tregs, the expression of LAG3 was low (Fig. 7E). Tregs acquired cytotoxicity against DCs after activation (Fig. 4A), whereas LAG3 was also significantly upregulated on activated Tregs (Fig. 7E). Increased LAG3 on activated Tregs may enable Tregs to trigger cell death in DCs by engaging MHC-II. Indeed, we observed that engagement of MHC-II on DCs with an agonist Ab triggered the loss of ΔΨm in WT, but not Bax−/− Bax−/− DCs (Fig. 7F). Engagement of MHC-II or other molecules on DCs by activated Tregs may transmit signals into DCs through BH3-only molecules, such as Bim, to induce the activation of Bax and Bak, leading to mitochondrial disruption and cell death.

**Discussion**

DCs harbor an active mitochondrion-dependent apoptosis pathway regulated by BCL-2 family members (27–29). In this study, we determined the functions of mitochondrion-dependent cell death in DCs in immune regulation using mice with DC-specific knockout of Bax and Bak. Bax and Bak deficiencies in DCs resulted in DC expansion, spontaneous T cell activation, and development of systemic autoimmunity. In addition to regulating spontaneous cell death by Bax and Bak, our data suggested another level of regulation of Treg-induced cell death in DCs through Bax and Bak. Expression of LAG3 may enable Tregs to trigger mitochondrion-dependent cell death in DCs. Adoptive-transfer experiments provide direct evidence to show that Bax- and Bak-deficient DCs have increased propensity for inducing the production of autoantibodies. Moreover, Tregs inhibited WT, but not Bax- and Bak-deficient, DCs in the induction of autoantibodies after adoptive transfer. Our data suggested that the Bax- and Bak-dependent pathway is involved in both spontaneous cell death and Treg-mediated killing of DCs, and both of these mechanisms are important for maintaining DC homeostasis and preventing autoimmunity.

Bax- and Bak-dependent spontaneous cell death in DCs may occur throughout the courses of immune responses. In contrast, only preactivated Tregs expressed higher levels of LAG3 (Fig. 7E) and showed the capacity to kill DCs (Fig. 4A), suggesting that Bax- and Bak-dependent killing of DCs may happen only after Tregs are activated. We and other investigators previously demonstrated that DCs are susceptible to Fas-mediated apoptosis (22, 23). Fas-dependent killing of DCs may take place when activated T cells that express high levels of FasL are present. This potentially provides a negative-feedback mechanism for the suppression of DC-dependent activation of T cells at late stages of immune responses, possibly after significant T cell activation induced by DCs has taken place. It was reported that Tregs can kill autologous...
CD8+ T cells or LPS-induced monocytes through FasL/Fas interactions (67, 68). Other studies showed that the suppressive effect of Tregs is not inhibited by neutralization of FasL, or using FasL-deficient Tregs (69, 70). We also observed that the killing of DCs by Tregs was independent of Fas (Supplemental Fig. 4). Such differences in the involvement of Fas/L-Fas interactions could be due to different target cells used in these studies.

Fas-mediated killing is usually dependent on activated T cells that express high levels of FasL. Spontaneous cell death in DCs through mitochondrion-dependent intrinsic apoptosis pathway is regulated by Bcl-2 family members (28, 71). Different from Fas-dependent cell death, Bax- and Bak-dependent spontaneous cell death in DCs may have a more broad influence at the initiation, expansion, and contraction phases of immune responses. In contrast, killing of DCs by activated Tregs through Bax and Bak may only function at the contraction phase of immune responses.

Recognition of Ags by TCRs on Tregs and LFA-1–dependent Treg-DC conjugate formation may be critical for Tregs to kill DCs. Interestingly, activated Tregs expressed elevated LAG3 and acquired killing activities toward DCs, and blocking of LAG3 partially inhibited the killing of DCs by Tregs (Fig. 7). It was shown that LAG3 can trigger negative signaling to inhibit the maturation and immunostimulatory capacity of DCs (66). Interestingly, cross-linking of MHC-II triggered the loss of ΔΨm in DCs in a Bax- and Bak-dependent manner (Fig. 7H). Our data suggested that LAG3 on activated Tregs can engage MHC-II on DCs to induce mitochondrial disruption and cell-death signaling in DCs.

Certain DC subsets can induce the generation of Tregs in vitro and in vivo (20, 72–77). In contrast, Tregs also restrict the development of Tregs in vivo (10, 54). Our data suggested that LAG3 on activated Tregs can engage MHC-II on DCs in a Bax- and Bak-dependent manner (Fig. 7).

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Analyses of DC-DKO mice. (A) Western blot for Bax and Bak in DCs, T cells in DC-DKO mice or controls. Bax is deleted in DCs, but not in lymphocytes in DC-DKO mice. (B) Enlargement of the spleen and inguinal lymph nodes in six-month-old DC-DKO mice (N=7). (C) Splenocytes from mice labeled with BrdU for 3 or 6 days were stained with antibodies to CD11c and CD11b, followed by intracellular staining with FITC-anti-BrdU. Percentages of BrdU labeling of CD11c+DC11b+ DCs (mean ± SD) were determined by flow cytometry (6 mice/group). (D) Splenocytes of WT and DC-DKO mice were stained with APC-anti-CD11c and PE-conjugates anti-TCRj, anti-DC5a or anti-CD19, followed by flow cytometry. The expression of CD11c or GFP under the control of the transgenic CD11c promoter (CD11c-cre-IRES-GFP) versus other cell surface markers was plotted. (E) Expression of surface molecules on DCs in DC-DKO vs WT mice (N=5). CD40, I-A<sup>+</sup>, CD80 CD86 and ICAM-1 on CD11c<sup>+</sup>CD11b<sup>+</sup> DCs (solid lines) were examined by flow cytometry. Dotted lines: isotype control. (F) Immunohistochemistry analyses for T cells (CD3<sup>+</sup>), B cells (B220<sup>+</sup>) and DCs (CD11c<sup>+</sup>) in the spleens of 6-month-old DC-DKO and control mice (N=6). Scale bar: 50 μm. (G) Sections of lungs, livers and kidneys of 6-month-old DC-DKO or control mice (N=6) were stained with H&E. Scale bar: 50 μm. (H) Kidney sections of 6-month-old DC-DKO or control mice were stained with Alexa Fluor 594-conjugated anti-mouse IgG. Scale bar: 50 μm.
**Supplemental Figure 2.** Activation of T cells and cytokine production by DCs. (A) Splenocytes from 6-month-old DC-DKO and control mice were stained with antibodies to different markers and analyzed by flow cytometry. CD69 expression on CD4+ T cells, CD8+ T cells and CD19+ B cells and CD44 and CD62L expression on TCRαβ+ T cells were analyzed. Splenocytes were also stained with FITC-anti-CD4, followed by intracellular staining with PE-anti-FoxP3. Data are representative of 5 mice per genotype. (B) CD4+CD25high Treg cells from DC-DKO and control mice were incubated with CD4+CD25low Teff cells at different ratios, and cultured in the presence of 0.25 μg/ml soluble anti-CD3 and irradiated T-cell-depleted spleen cells. Proliferation was quantified by 3H-thymidine incorporation 4 days later. (C) CD4+FoxP3+ Treg cells expanded in vitro were mixed with freshly sorted CD4+FoxP3low Teff cells from GFP-FoxP3 mice at different ratios in the presence of 0.25 μg/ml soluble anti-CD3 and irradiated spleen cells depleted of T cells and DCs (5x10^4/well). Cell proliferation were quantitated 4 days later by 3H-thymidine incorporation. Statistical comparison to control with no Treg cells: *P<0.05, **P<0.01. (D) WT or DKO BMDCs were unpulsed or pulsed with OVASIINFEKL peptide. Various numbers of DCs were then incubated with MACS beads purified OVA-specific CD8+ transgenic OT1 T cells (5x10^4/well). Cell proliferation was measured by 3H-thymidine incorporation 3 days later. WT+OVA versus DKO+OVA: **P<0.01. (E) CFSE-labeled CD8+ OT1 T cells (5x10^4/well) were labeled with CFSE and incubated with DCs pulsed with OVASIINFEKL peptide (300 DCs/well). Cell proliferation was measured by CFSE dilution 4 days later. Average numbers of cell cycle ± SD are shown. WT versus DKO: P=0.03. (F) WT or DKO BMDCs were stimulated with LPS or CpG for 24 h, followed by intracellular staining of IL-12 or IL-6. The percentage of IL-12- or IL-6-producing cells and the mean fluorescence staining (MFI) (means ± SD) for IL-12 or IL-6 staining were plotted. The statistic difference between WT and DKO: not significant.
Supplemental Figure 3. T\textsubscript{reg} cells in the killing of and conjugate formation with DCs. (A) BMDCs were incubated in the absence or presence of Treg cells. An example of forward scattering (FSC) versus 7-AAD staining of DCs was plotted. (B) T\textsubscript{reg} cells in the killing of BMDCs or splenic DCs from wild type or DC-DKO mice. WT versus DKO: *P<0.05, **P<0.01. (C) T\textsubscript{reg} cells in the killing of BMDCs with or without LPS stimulation. (D) OT2 FoxP3\textsuperscript{+} Treg cells and DCs labeled with CFSE and CMTMR, respectively, were mixed (1:1) and centrifuged at 500 g for 5 min, followed by incubation at 37 °C for different time. The cells were collected and analyzed by flow cytometry. Percentage of T\textsubscript{reg} cells that formed conjugates with DCs was plotted. Groups with OVA versus no OVA at 1, 2 and 3 h, P<0.01. (E) Percentages of OT2 FoxP3\textsuperscript{+} Treg cells forming conjugates with OVA-pulsed WT or DKO DCs after 1 h of incubation. (F) OT2 FoxP3\textsuperscript{+} T\textsubscript{reg} cells and OVA-pulsed DCs were labeled with CFSE and CMTMR, respectively, and cultured as in (D) in the presence of 10 μg/ml of different antibodies for 1 h. Anti-LFA-1 versus IgG: **P<0.01. Each group with DC-OVA versus no OVA: P<0.01. (G) CD4\textsuperscript{+}FoxP3\textsuperscript{+} T\textsubscript{reg} cells expanded in vitro were mixed with DCs in the presence of rat IgG, anti-LAG3 or anti-LFA-1 for 1 h, followed by quantification of cell conjugates. Anti-LFA-1 versus IgG: **P<0.01. (H) Percentages of CD4\textsuperscript{+}FoxP3\textsuperscript{+} T\textsubscript{reg} cells as in (G) forming conjugates with WT or DKO DCs.
Supplemental Figure 4. T\textsubscript{reg}-mediated cell death in DCs. (A) Wild type or DKO BMDCs were incubated with or without T\textsubscript{reg} cells (T\textsubscript{reg}:DC at 0.5:1) for 6 h. CD11\textsuperscript{+} DCs were isolated using MACS beads and lysed for Western blot. Data are representative of 2 independent experiments. (B, C) CD4\textsuperscript{+}FoxP3\textsuperscript{-} T effector cells were activated with anti-CD3/anti-CD28 Dynabeads and incubated with wild type or lpr (B) or Bax\textsuperscript{-/-}Bak\textsuperscript{-/-} (C) BMDCs in the presence of 1 \mu g/ml anti-CD3 for 4 h. Killing of DCs was measured by flow cytometry. WT versus lpr: **P<0.01. (D) Killing of wild type or lpr BMDCs by activated CD4\textsuperscript{+}FoxP3\textsuperscript{+} T\textsubscript{reg} cells. (E) T\textsubscript{reg}-mediated killing of DCs. WT and PFP\textsuperscript{-/-} (left panel) or granzyme A\textsuperscript{+/+}/granzyme B\textsuperscript{+/+} (right panel) T\textsubscript{reg} cells were incubated with CFSE-labeled DCs at a ratio of 0.1:1, 0.3:1 or 1:1 for 6 h, followed by measurement of killing of DCs.