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

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Abstract

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. Here we show that DC-specific knockout of pro-apoptotic 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, $Bax^{-/-}Bak^{-/-}$ DCs were resistant to killing by $CD4^{+}FoxP3^{+}$ T regulatory (T_{reg}) cells compared to wild type DCs. T_{reg} cells inhibited the activation of T effector cells by wild type, but not $Bax^{-/-}Bak^{-/-}$ DCs. $Bax^{-/-}Bak^{-/-}$ DCs showed increased propensity for inducing autoantibodies. Moreover, the autoimmune potential of $Bax^{-/-}Bak^{-/-}$ DCs was resistant to suppression by T_{reg} cells. Our data suggest that Bax and Bak not only mediate intrinsic spontaneous cell death in DCs, but also regulate DC killing triggered by T_{reg} cells. 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.

INTRODUCTION

During T cell development in the thymus, T cells that recognize self MHC molecules presenting autoantigens can survive by positive selection, while highly self-reactive T cells are subsequently deleted by negative selection (1–4). However, T cells that undergo successful maturation and eventually populate the peripheral lymphoid organs carry certain degree of autoreactivity. It is essential to keep these mature T cells in check to maintain peripheral tolerance. Programmed cell death of mature lymphocytes is a major mechanism for the maintenance of lymphocyte homeostasis and peripheral tolerance (5–7). In addition, natural T_{reg} cells that express FoxP3 have been established to play an essential role in the protection of immune tolerance (8–14).

DCs, the most efficient antigen presenting cells, are important regulators of both innate and adaptive immune responses (15–19). DCs may also play important roles in the maintenance of immune tolerance (20, 21). We have previously observed that DC-specific expression of the baculoviral caspase inhibitor, p35, leads to inhibition of Fas-mediated apoptosis in DCs and the development of systemic autoimmune symptoms (22). Consistently, knockout of Fas in DCs also induces the onset of autoimmunity in mice (23). Interestingly, interactions of antigen-pulsed DCs with the antigen-specific T cells may lead to accelerated loss of DCs *in vivo* (24). It is possible that Fas-dependent killing of DCs by activated T cells provides a negative feedback mechanism that helps to terminate the activation of lymphocytes by antigen-bearing DCs (25).

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It has been recognized that DCs have a short lifespan since the original discovery of this cell type (26). The short half-life of DCs *in vivo* has been linked to an active mitochondrion-dependent cell death pathway regulated by Bcl-2 family members (27–29). The Bcl-2 family members are upstream regulators of mitochondrion-dependent apoptosis pathway (30, 31). They share the Bcl-2 homology (BH) domains and are divided into three subfamilies (30, 31), including pro-apoptotic Bax and Bak; the anti-apoptotic subfamily proteins, such as Bcl-2, Bcl-xL and Mcl-1; and the pro-apoptotic BH3-only subfamily, such as Bim, Bid and Bad. BH3-only proteins are the upstream sensors for different apoptosis signaling in specific cell types (32). BH3-only proteins initiate mitochondrion-dependent apoptosis by either inhibiting the anti-apoptotic molecules, or directly activating pro-apoptotic Bax or Bak to induce apoptosis (32, 33). While deficiency in either Bax or Bak has no salient phenotypes, knockout of both Bax and Bak abolishes mitochondrion-dependent apoptosis (34), indicating that Bax and Bak are essential for mitochondrion-dependent apoptosis but functionally redundant of each other. Both negative selection of developing T cells in the thymus and apoptosis of mature T cells are defective in $Bax^{-/-}Bak^{-/-}$ mice (35), suggesting that Bax- and Bak-dependent mitochondrial apoptosis in T cells is involved in the regulation of lymphocyte homeostasis and immune tolerance.

The rapid turnover rates of DCs *in vivo* could be attributed to the active mitochondrion-dependent cell death pathway in DCs (27). Indeed, transgenic expression of anti-apoptotic Bcl-2 or deletion of pro-apoptotic Bim can inhibit spontaneous cell death in DCs (28, 29). In contrast, Fas-mediated signaling is not required for spontaneous cell death in DCs (29). It is therefore possible that mitochondrion-dependent and Fas-mediated cell death pathways regulate DC turnover at different phases of immune responses. However, the function for the active mitochondrion-dependent cell death pathway of DCs in immune regulation has not been well characterized. Furthermore, whether mitochondrion-dependent cell death in DCs plays a role in the regulation of immune tolerance is not known.

T_{reg} cells can interact with DCs to inhibit the activation of antigen-specific T cells *in vivo* (36–42). T_{reg} cells may down-regulate important co-stimulatory molecules on DCs (40, 43–48). Interestingly, one report has suggested that T_{reg} cells may cause the disappearance of DCs in the draining lymph nodes (38). Whether T_{reg} cells can induce cell death in DCs, and whether such interactions help to protect immune tolerance have not be determined. Using mice with deficiencies of Bax and Bak in DCs, we show that $CD4^+FoxP3^+$ T_{reg} cells efficiently induce mitochondrion-dependent cell death in DCs. Different from the killing of DCs by T effector cells through Fas, our current study suggests that T_{reg} cells exploit the active mitochondrion-dependent apoptosis pathway in DCs for immune regulation. Such interactions between T_{reg} cells and DCs potentially play a fundamental role in the regulation of initiation and expansion of antigen-specific immune responses, and in the protection of immune tolerance.

Materials and Methods

Mice

$Bak^{-/-}$, Bax^{flox}, $Bim^{-/-}$, $perforin^{-/-}$, *lpr*, CD11c-cre transgenic, OVA-specific-OT1 or -OT2 transgenic mice were obtained from the Jackson Laboratory and maintained on the C57BL/6 background. Granzyme $A^{-/-}$granzyme $B^{-/-}$ mice and wild type controls on the 129X1/SvJ background were also obtained from the Jackson Laboratory. $FoxP3^{GFP}$ knock-in mice were provided by Dr. Alexander Rudensky (49). $FoxP3^{GFP}$ mice were crossed with $perforin^{-/-}$ mice to generate $perforin^{-/-}FoxP3^{GFP}$ mice, and with OT2 mice to generate OT2-$FoxP3^{GFP}$ mice. OT2 mice were also crossed with CD45.1 congenic mice. The mice were maintained in a specific-pathogen-free facility and used with the approval of the Institutional Animal Care and Use Committee at Baylor College of Medicine.

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 bromodeoxyuridine (BrdU) labeling, and detection of autoantibodies in mice were preformed as described (22, 29).

Proliferation assays

Mice were immunized with ovalbumin (OVA; 50 μg/mouse) emulsified in complete Freud adjuvant (CFA) at the footpad. Ten days later, total cells (2×10^5/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 [^{3}H]-thymidine for the last 12 h and harvested to measure [^{3}H]-thymidine incorporation.

$CD4^+FoxP3^+$ T_{reg} cells were sorted from $FoxP3^{GFP}$ mice and expanded *in vitro* with anti-CD3-and anti-CD28-coated Dynabeads (Invitrogen; 10 μl beads/10^6 cells) in the presence of 1000 U/ml IL-2 for 3 days. Wild type or $Bax^{-/-}Bak^{-/-}$ DCs were either unpulsed or pulsed with $OVA_{323–339}$ peptide. DCs (10^3/well) were mixed with freshly sorted $CD4^+CD25^-$ T cells from OT2 mice (10^4/well) in 96-well U-bottom plates, in the presence of different numbers of expanded T_{reg} cells. The proliferation of OT2 T cells was measured by [^{3}H]-thymidine incorporation 4 days later. Alternatively, $CD4^+CD25^-$ T_{eff} cells were sorted from wild type mice by flow cytometry. Different numbers of T_{reg} cells expanded as above were added to $CD4^+CD25^-$ T_{eff} cells (5×10^4/well) at different ratios in the presence of 0.25 μg/ml soluble anti-CD3 (2C11; BD Biosciences) and irradiated (3000 rads) T cell- and DC-depleted syngeneic splenocytes (5×10^4/well) in 96-well U-bottom plates. [^{3}H]-thymidine incorporation was measured 3 days later.

$CD4^+$ T cells from OT2 mice (CD45.1 × CD45.2) were either sorted and labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) at room temperature for 10 min. The CFSE-labeled OT2 T cells were injected into C57BL/6 mice retro-orbitally (2×10^6/mouse). DCs (2×10^5) with or without pulsing with $OVA_{323–339}$ peptide were injected intradermally at footpad 24 h later with or without T_{reg} cells. Four days later, popliteal lymph nodes were collected and $CD4^+CD45.1^+$ T cells were analyzed by flow cytometry to determine CFSE dilution.

Spontaneous cell death in DCs and Killing of DCs or other target cells by T_{reg} cells

$CD11c^+I\text{-}Ab^+$ DCs were sorted from the spleens of DC-DKO or control mice. DCs were cultured in RPMI complete medium for 0 or 24 h. Spontaneous cell death of DCs were quantitated as described (29). BMDCs or $CD11c^+CD11b^+$ splenic DCs from wild type, DC-DKO or $Bim^{-/-}$ mice were labeled with 1 μM CFSE (Invitrogen) for 10 min at 37 °C. The killing of DCs was analyzed similar to the previously described protocol (50). T_{reg} cells were expanded as above. In some experiments, $CD4^+CD25^{high}$ T_{reg} cells were sorted from granzyme $A^{-/-}$granzyme $B^{-/-}$ mice or wild type controls and expanded as above. T_{reg} cells were mixed with DCs (2×10^4/well) at different ratios in 96-well U-bottomed plates for 6 h. the cells were incubated with 5 ng/ml 7-amino-actinomycin D (7-AAD; BD Biosciences) at room temperature for 10 min, followed by flow cytometry. Induction of cell death in DCs was quantified essentially as described (50) with the following formula: percentages of killing of DCs by T cells = $100\% \times (DC_{control}-DC_T)/DC_{control}$, with $DC_{control}$ and DC_T representing $CFSE^+7\text{-}AAD^-$ DCs in the absence or presence of T cells, respectively. In some experiments, 10 μg/ml blocking antibody to LAG3 (clone C9B7W; BD Biosciences), LFA-1 (clone M17/4; BD Biosciences) or I-A/I-E (clone M5/114.15.2; Biolegend), or rat IgG were added to the culture.

To measure mitochondrial membrane potential, DCs with or without incubation with T_{reg} cells above were incubated with 10 μM etramethylrhodamine ethyl ester (TMRE; Invitrogen) at 37 °C for 20 min, followed by flow cytometry. In some experiments, DCs were incubated with 10μg/ml agonist anti-I-A/I-E (clone 2G9; BD Bioscience) or control rat IgG for 6 h, followed with TMRE staining and flow cytometry.

Splenic $CD11c^+$ DCs were purified 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 100 U/ml IL-2 for 3 days. B cells were purified with anti-CD19 MACS beads and stimulated with 1 μg/ml LPS and 100 μM CpG for 48 h. T_{reg}-mediated killing of splenic DCs and activated T or B cells were measured as the killing of BMDCs above.

$CD4^+FoxP3^+$ T_{reg} cells or $CD4^+FoxP3^-$ T_{eff} cells sorted from OT2-$FoxP3^{GFP}$ mice were stimulated with $OVA_{323–339}$ peptide-pulsed DCs (T:DC=2:1) for 3 days. OT2 T_{reg} or T_{eff} cells were then isolated by removing DCs with anti-CD11c-MACS beads (Miltenyi Biotec) and used for killing of DCs with or without pulsing with $OVA_{323–339}$ peptide. Percentages of killing of DCs by T cells were measured as above.

Analyses of interaction between Treg cells and DCs

To detect interactions between T_{reg} cells and DCs *in vivo*, DCs and T_{reg} cells were labeled at 37 °C for 10 min with 10 μM 5-(and -6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR) and CFSE (Invitrogen), respectively. Wild type or DKO DCs (1×10^6) were injected into the footpad of recipient mice. T_{reg} cells (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 a LSM 510 confocal microscope (Zeiss).

To determine the conjugate formation between DCs and T_{reg} cells *in vitro*, DCs and T_{reg} cells 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.

Adoptive transfer of DCs

To determine autoantibody production after adoptive transfer, wild type or $Bax^{-/-}Bak^{-/-}$ BMDCs (10^6 or 5×10^6/mouse) were injected into 8-week-old C57BL/6 mice (6 mice/group) with or without T_{reg} cells (with T_{reg}:DC at 0.5:1) intraperitoneally essentially as described (29, 51). The mice were then injected with LPS (30 μg/mouse) intraperitoneally one day later. Sera were collected from the recipient mice 1 week after DC transfer. ANAs, anti-ssDNA and anti-dsDNA were measured as above. In parallel experiments, CFSE-labeled wild type or $Bax^{-/-}Bak^{-/-}$BMDCs (10^6 or 5×10^6/mouse) were injected into 8-week-old C57BL/6 mice (6 mice/group) with or without T_{reg} cells (with T_{reg}:DC at 0.5:1) at 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 nodes were calculated.

Western blot

To determine T_{reg}-mediated signaling in DCs, wild type or DKO DCs were incubated with T_{reg} cells 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 antibodies were used: polyclonal rabbit antibodies to caspase-8, caspase-3, Bcl-xL, Bad (Cell Signaling), Bax (Santa Cruz Biotechnology), Bcl-2, Bak (Upstate Biotechnology), Mcl-1 (Fitzgerald), Bim (Stressgen), Bid (Imgenex), Blk or Bmf (Biovision), or mouse monoclonal antibody to caspase-9 (MBL), Noxa (Imgenex) or XIAP (BD Bioscience). The blots were then probed with HRP-conjugated secondary antibodies 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

$CD11c^+$ bone marrow–derived DCs (10^6/ml) were cultured in the absence or presence of 1 μg/ml LPS (Sigma) or 0.5 μM phosphorothioate-stabilized CpG oligonucleotide (TCCATGACGTTCCTGATGCT) for 24 hours. Brefeldin A (1 μg/mL) and monensin (2 μM) were added during the last 6 hours 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 than analyzed by flow cytometry.

Statistical analysis

Data were presented as mean ± SD. *P* values were determined by two-tailed Student's *t*-test using GraphPad Prism software version 4.0 for Macintosh. $P<0.05$ is considered to be statistically significant.

RESULTS

Accumulation of DCs due to 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 Bax^{flox} and $Bak^{-/-}$ mice. Specific deletion of Bax in DCs were 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, 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 bromodeoxyuridine (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 for DCs in these mice. Consistently, the percentages and total numbers of $CD11c^{high}I\text{-}Ab^+$ or $CD11c^{high}CD40^+$ conventional DCs were increased in DC-DKO mice (Fig. 1A and B). $CD11c^{low}PDCA\text{-}1^+$ plasmacytoid DCs increased to a lesser extent in DC-DKO mice (Fig. 1A and B). The percentages of T, B or NK cells were not elevated in DC-DKO mice (Supplemental Fig. 1D). DCs from DC-DKO, but not $Bak^{-/-}$, DC-$Bax^{-/-}$, Fas-deficient *lpr*, CD11c-cre or C57BL/6 control mice, showed defects in spontaneous cell death (Fig. 1C). However, splenic DCs from DC-DKO mice and wild type controls express similar levels of CD40, MHC-II, B7.1, B7.2 and ICAM-1 (Supplemental Fig. 1E), suggesting that deficiencies of 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 $CD44^+CD62L^-$ activated/memory phenotype were increased in DC-DKO mice compared to controls (Supplemental Fig. 2A). These data suggest that deficiencies of 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-month-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 wild type 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 antinuclear antibodies (ANAs) in the sera of 3- and 6-month-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 suggest that deficiencies of Bax and Bak in DCs lead to the development of systemic autoimmunity. Normal levels of $CD4^+FoxP3^+$ natural T_{reg} cells were detected in DC-DKO mice (Supplemental Fig. 2A). Moreover, natural T_{reg} cells from DC-DKO and control mice showed comparable activities in inhibiting the proliferation of T effector (T_{eff}) cells (Supplemental Fig. 2B), indicating that there is no intrinsic defect in natural T_{reg} cells 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 antigen re-stimulation than those of wild type, $Bak^{-/-}$ or DC-$Bax^{-/-}$ mice (Fig. 3A). Consistently, $Bax^{-/-}Bak^{-/-}$ DCs induced more robust proliferation of antigen-specific T cells than control DCs *in vivo* as assayed by CFSE dilution (Fig. 3B). This suggests that $Bax^{-/-}Bak^{-/-}$DCs are more efficient in priming antigen-specific T cells *in vivo*. However, $Bax^{-/-}Bak^{-/-}$ DCs did not produce more IL-6 or IL-12 (Supplemental Fig. 2F), suggesting that $Bax^{-/-}Bak^{-/-}$ DCs do not over-produce these cytokines to induce more activation of T_{eff} cells. Interestingly, the increase in the capacity for $Bax^{-/-}Bak^{-/-}$ DCs to stimulate antigen-specific T_{eff} cells *in vitro* was detectable but less dramatic (Fig. 3C, D; Supplemental Fig. 2D, E). This is reminiscent of previous observations that cell death in DCs has a more profound effect on the immunogenicity of DCs *in vivo* than *in vitro* (53). Such differences between *in vivo* and *in vitro* observations could be due to the presence of other cell types that regulate the interactions between DCs and T cells *in vivo*.

Deficiencies in Bax and Bak enable DCs to overcome the suppression by T_{reg} cells

Although natural $FoxP3^+$ T_{reg} cells were not reduced or dysfunctional in DC-DKO mice (Supplemental Fig. 2A, Supplemental Fig. 2B), it remains possible that $Bax^{-/-}Bak^{-/-}$ DCs might be refractory to suppression by T_{reg} cells to induce uncontrolled T cell activation. We investigated whether deficiencies of Bax and Bak might enable DCs to overcome the inhibitory effect of T_{reg} cells. We sorted and expanded $CD4^+FoxP3^+$ natural T_{reg} cells from $FoxP3^{GFP}$ knock-in mice (49). To the culture of antigen-pulsed DCs and ovalbumin (OVA)-specific OT2 T_{eff} cells (with DCs:T_{eff} at a fixed ratio of 0.1:1), we added varied numbers of the expanded T_{reg} cells (with T_{reg}:T_{eff} ratios ranging from 0.01:1 to 1:1). When $Foxp3^+$ T_{reg} cell numbers were low (T_{reg}:T_{eff} from 0.01:1 to 0.1:1), we detected significant suppression of antigen-specific T cell proliferation induced by wild type, $Bax^{-/-}$, and $Bak^{-/-}$ but not

$Bax^{-/-}Bak^{-/-}$ DCs (Fig. 3E). When T_{reg}:T_{eff} ratios were higher (0.1:1 to 1:1), significant suppression of $Bax^{-/-}Bak^{-/-}$ DC-induced proliferation of OT2 T_{eff} cells was detected (Fig. 3E). However, this could at least be partially attributed to a direct suppression of T_{eff} cells by T_{reg} cells at higher T_{reg}: T_{eff} ratios (Supplemental Fig. 2C).

Consistent with *in vitro* observations (Fig. 3E), $CD4^{+}FoxP3^{+}$ T_{reg} cells suppressed the proliferation of OT2 T_{eff} cells induced by wild type, $Bax^{-/-}$ 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 T_{reg} cells *in vivo* (Fig. 3F). This supports the conclusion that $Bax^{-/-}Bak^{-/-}$ DCs are refractory to suppression by T_{reg} cells.

Induction of Bax- and Bak-dependent cell death in DCs by T_{reg} cells

We found that activated T_{reg} cells acquired cytotoxicity against DCs (Fig. 4A). We examined whether activated T_{reg} cells 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 $CD4^{+}FoxP3^{+}$ T_{reg} cells (Fig. 4B). Wild type, but not $Bax^{-/-}Bak^{-/-}$ DCs were sensitive to killing by T_{reg} cells (Fig. 4C; Supplemental Fig. 3A, B), suggesting that T_{reg} cells induce DC cell death in a Bax- and Bak-dependent manner. T_{reg}-mediated killing of DCs is not affected by treating DCs with LPS (Supplemental Fig. 3C), suggesting that T_{reg}-mediated killing of DCs is not affected by DC maturation. It has been shown that T_{reg} cells can negatively regulate DCs by restricting DC development (54), inhibiting the expression of co-stimulatory molecules on DCs or competing with T_{eff} cells in the interaction with DCs (36–38, 40). Apoptotic DCs may promote further induction of T_{reg} cells (55). Inducing Bax- and Bak-dependent cell death in DCs may provide another mechanism for DC regulation by T_{reg} cells.

Blocking MHC-II inhibited the killing of DCs by polyclonal T_{reg} cells (Fig. 4D). This suggests that the polyclonal T_{reg} cells are potentially autoreactive. By using OVA-specific OT2 $FoxP3^{+}$ T_{reg} cells, we found that $FoxP3^{+}$ T_{reg} cells killed antigen-pulsed, but not unpulsed DCs (Fig. 4E). Using $FoxP3^{+}$ T_{reg} cells expressing a transgenic TCR specific for a foreign antigen may not recuperate the action of natural T_{reg} cells carrying TCRs that tend to be autoreactive. Nevertheless, our data obtained by using OT2 $FoxP3^{+}$ T_{reg} cells suggest that recognition of antigens on DCs is important for killing by T_{reg} cells. In contrast to T_{reg} cells, polyclonal T_{eff} cells did not efficiently kill DCs in the absence of anti-CD3 (Fig. 4C), while OT2 T_{eff} cells showed killing of DCs in the presence of OVA antigen 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 detect more $Bax^{-/-}Bak^{-/-}$ DCs in the draining lymph nodes after adoptive transfer (Fig. 5A). Moreover, co-transfer of T_{reg} cells led to the loss of wild type 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 T_{reg}-dependent DC killing. T_{reg} cells can induce Bax- and Bak-dependent cell death in DCs. In addition, we also observed that T_{reg} cells formed conjugates with both wild type and $Bax^{-/-}Bak^{-/-}$ DCs in the draining lymph nodes (Fig. 5A). Conjugate formation between T_{reg} cells and DCs was also detected *in vitro* (Supplemental Fig. 3D–H). Wild type and DKO DCs were similar in forming conjugates with T_{reg} cells after 1 hour of incubation *in vitro* (Supplemental Fig. 3E, H). Consistent with confocal microscopy analyses, more $Bax^{-/-}Bak^{-/-}$ DCs were found in the draining lymphocytes than wild type DCs as determined by flow cytometry (Fig. 5B). Co-transfer of T_{reg} cells also promoted the clearance of wild type but not $Bax^{-/-}Bak^{-/-}$ DCs (Fig. 5B). These data suggest that Bax and

Bak do not affect conjugate formation between T_{reg} cells and DCs. Rather, Bax and Bak regulate spontaneous cell death of DCs, as well as T_{reg}-mediated clearance of DCs *in vivo*.

Autoimmune potential of $Bax^{-/-}Bak^{-/-}$ DCs in adoptive transfer

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

Molecules involved in the killing of DCs by activated Treg cells

We next investigated apoptosis signaling in DCs induced by T_{reg} cells. After incubation with T_{reg} cells, wild type DCs lost mitochondrial membrane potential ($\Delta\Psi m$), while DKO DCs were relatively resistant to the loss of $\Delta\Psi m$ (Fig. 7A). Consistent with the activation of mitochondrion-dependent cell death in wild type DCs, T_{reg} cells induced the activation of caspase-9 and caspase-3 in wild type, but not $Bax^{-/-}Bak^{-/-}$ DCs (Fig. 7B). It has been shown that cleavage of Bid into active tBid by caspases can trigger mitochondrial apoptosis (57). Caspase-dependent cleavage may inactivate anti-apoptotic 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 T_{reg} cells (Supplemental Fig. 4A). T_{reg} cells also did not change the expression of Bcl-2 family proteins in DCs (Fig. 7B, Supplemental Fig. 4A). Interestingly, $Bim^{-/-}$ DCs showed resistance to killing by T_{reg} cells compared to wild type DCs, while deletion of either Bax or Bak in DCs did not affect their killing by T_{reg} cells (Fig. 7C). This suggests that Bim serves as a mediator to activate Bax and Bak in DCs after encountering T_{reg} cells. Although the precise mechanism for the activation of Bim is not resolved, Bim potentially transmits cell death signaling by sequestering anti-apoptotic molecules or directly activating Bax and Bak (59, 60).

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

We observed that blocking LFA-1 inhibited T_{reg}-mediated killing of DCs (Fig. 7D). The conjugate formation between T_{reg} cells and DCs was inhibited by blocking LFA-1 (Supplemental Fig. 4G, H), suggesting that cell adhesion promotes the killing of DCs by

T_{reg} cells. Molecules highly expressed by T_{reg} cells have been suggested to be critical for T_{reg} functions (11, 40, 64, 65). We observed that inhibition of LAG3 partially suppressed the killing of DCs by T_{reg} cells (Fig. 7D), while neutralization antibodies to soluble factors, including IL-10 and TGF-β, have 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 un-stimulated T_{reg} cells, the expression of LAG3 was low (Fig. 7E). T_{reg} cells acquired cytotoxicity against DCs after activation (Fig. 4A), while LAG3 was also significantly up-regulated on activated T_{reg} cells (Fig. 7E). Increased LAG3 on activated T_{reg} cells may enable T_{reg} cells to trigger cell death in DCs by engaging MHC-II. Indeed, we observed that engagement of MHC-II on DCs with an agonist antibody triggered the loss of ΔΨm in wild type, but not $Bax^{-/-}Bak^{-/-}$ DCs (Fig. 7F). Engagement of MHC-II or other molecules on DCs by activated T_{reg} cells 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. Deficiencies of Bax and Bak 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 suggest another level of regulation of T_{reg}-induced cell death in DCs through Bax and Bak. Expression of LAG3 may enable T_{reg} cells 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, T_{reg} cells inhibited wild type, but not Bax- and Bak-deficient DCs in the induction of autoantibodies after adoptive transfer. Our data suggest that Bax- and Bak-dependent pathway is involved in both spontaneous cell death and T_{reg}-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 pre-activated T_{reg} cells 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 T_{reg} cells are activated. We and others have 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 Fas ligand are present. This potentially provide 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 has been reported that Treg cells can kill autologous $CD8^+$ T cells or LPS-induced monocytes through FasL/Fas interactions (67, 68). Other studies have shown that the suppressive effect of T_{reg} cells is not inhibited by neutralization of FasL or using FasL-deficient Treg cells (69, 70). We also observed that the killing of DCs by Treg cells was independent of Fas (Supplemental Fig. 4). Such differences in the involvement of FasL/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. On the

other hand, killing of DCs by activated T_{reg} cells through Bax and Bak may only function at the contraction phase of immune responses.

Recognition of antigens by TCRs on T_{reg} cells and LFA-1-dependent T_{reg}-DC conjugate formation may be critical for T_{reg} cells to kill DCs. Interestingly, activated T_{reg} cells expressed elevated LAG3 and acquired killing activities towards DCs, and blocking of LAG3 partially inhibited the killing of DCs by T_{reg} cells (Fig. 7). It has been shown that LAG3 can trigger negative signaling to inhibit the maturation and immunostimulatory capacity of DCs (66). Interestingly, crosslinking of MHC-II triggered the loss of $\Delta\Psi m$ in DCs in a Bax- and Bak-dependent manner (Fig. 7H). Our data suggest that LAG3 on activated T_{reg} cells can engage MHC-II on DCs to induce mitochondrial disruption and cell death signaling in DCs.

Certain DC subsets can induce the generation of T_{reg} cells *in vitro* and *in vivo* (20, 72–77). On the other hand, T_{reg} cells also restrict the development of DCs *in vivo* (10, 54). Our data suggest that T_{reg} cells can restrict DC expansion through Bax- and Bak-dependent killing, and deficiency in the killing of DCs by T_{reg} cells contributes to DC accumulation and induction of autoimmunity in DC-$Bax^{-/-}Bak^{-/-}$ mice. Therefore, dynamic interplays between DCs and T_{reg} cells are important for the maintenance of a balanced immune system. Induction of mitochondrion-dependent cell death in DCs by T_{reg} cells may serve as one important mechanism for immune regulation. Promoting the interactions between T_{reg} cells and DCs may provide effective avenues to prevent and treat autoimmune diseases, whereas inhibiting the killing of DCs by T_{reg} cells may help to boost immune responses to infections and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

7-AAD	7-amino-actinomycin D
ANA	antinuclear antibodies
BMDCs	bone marrow-derived DCs
CMTMR	5-(and -6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine
TMRE	etramethylrhodamine ethyl ester
T_{eff}	T effector
T_{reg}	T regulatory
$\Delta\Psi m$	mitochondrial membrane potential

References

1. von Boehmer H, Aifantis I, Gounari F, Azogui O, et al. Thymic selection revisited: how essential is it? Immunol Rev. 2003; 191:62–78. [PubMed: 12614352]
2. Marrack P, Kappler J. Positive selection of thymocytes bearing alpha beta T cell receptors. Curr Opin Immunol. 1997; 9:250–255. [PubMed: 9099796]
3. Nossal GJ. Negative selection of lymphocytes. Cell. 1994; 76:229–239. [PubMed: 8293461]
4. Mathis D, Benoist C. Aire. Annu Rev Immunol. 2009; 27:287–312. [PubMed: 19302042]
5. Rathmell JC, Thompson CB. Pathways of apoptosis in lymphocyte development, homeostasis, and disease. Cell. 2002; 109(Suppl):S97–107. [PubMed: 11983156]
6. Lenardo M, Chan KM, Hornung F, McFarland H, et al. Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment. Annu Rev Immunol. 1999; 17:221–253. [PubMed: 10358758]
7. Nagata S, Suda T. Fas and Fas ligand: lpr and gld mutations. Immunol Today. 1995; 16:39–43. [PubMed: 7533498]
8. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet. 2001; 27:68–73. [PubMed: 11138001]
9. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol. 2010; 11:7–13. [PubMed: 20016504]
10. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol. 2007; 8:191–197. [PubMed: 17136045]
11. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity. 2009; 30:636–645. [PubMed: 19464986]
12. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol. 2008; 9:239–244. [PubMed: 18285775]
13. Schwartz RH. Natural regulatory T cells and self-tolerance. Nat Immunol. 2005; 6:327–330. [PubMed: 15785757]
14. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003; 4:330–336. [PubMed: 12612578]
15. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998; 392:245–252. [PubMed: 9521319]
16. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell. 2001; 106:259–262. [PubMed: 11509173]
17. Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. Cell. 2001; 106:263–266. [PubMed: 11509174]
18. Pulendran B. Variegation of the immune response with dendritic cells and pathogen recognition receptors. J Immunol. 2005; 174:2457–2465. [PubMed: 15728447]
19. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol. 2005; 23:975–1028. [PubMed: 15771591]
20. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol. 2003; 21:685–711. [PubMed: 12615891]
21. Banchereau J, Pascual V, Palucka AK. Autoimmunity through cytokine-induced dendritic cell activation. Immunity. 2004; 20:539–550. [PubMed: 15142523]
22. Chen M, Wang YH, Wang Y, Huang L, et al. Dendritic cell apoptosis in the maintenance of immune tolerance. Science. 2006; 311:1160–1164. [PubMed: 16497935]
23. Stranges PB, Watson J, Cooper CJ, Choisy-Rossi CM, et al. Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity. Immunity. 2007; 26:629–641. [PubMed: 17509906]
24. Ingulli E, Mondino A, Khoruts A, Jenkins MK. In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. J Exp Med. 1997; 185:2133–2141. [PubMed: 9182685]
25. Chen M, Wang J. Programmed cell death of dendritic cells in immune regulation. Immunol Rev. 2010; 236:11–27. [PubMed: 20636805]

26. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. J Exp Med. 1974; 139:380–397. [PubMed: 4589990]
27. Hou WS, Van Parijs L. A Bcl-2-dependent molecular timer regulates the lifespan and immunogenicity of dendritic cells. Nat Immunol. 2004; 5:583–589. [PubMed: 15133508]
28. Nopora A, Brocker T. Bcl-2 controls dendritic cell longevity in vivo. J Immunol. 2002; 169:3006–3014. [PubMed: 12218115]
29. Chen M, Huang L, Wang J. Deficiency of Bim in dendritic cells contributes to overactivation of lymphocytes and autoimmunity. Blood. 2007; 109:4360–4367. [PubMed: 17227827]
30. Adams JM, Huang DC, Puthalakath H, Bouillet P, et al. Control of apoptosis in hematopoietic cells by the Bcl-2 family of proteins. Cold Spring Harb Symp Quant Biol. 1999; 64:351–358. [PubMed: 11232307]
31. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 1999; 13:1899–1911. [PubMed: 10444588]
32. Strasser A. The role of BH3-only proteins in the immune system. Nat Rev Immunol. 2005; 5:189–200. [PubMed: 15719025]
33. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol. 2005; 17:617–625. [PubMed: 16243507]
34. Lindsten T, Ross AJ, King A, Zong WX, et al. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell. 2000; 6:1389–1399. [PubMed: 11163212]
35. Rathmell JC, Lindsten T, Zong WX, Cinalli RM, Thompson CB. Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. Nat Immunol. 2002; 3:932–939. [PubMed: 12244308]
36. Oldenhove G, de Heusch M, Urbain-Vansanten G, Urbain J, et al. CD4+ CD25+ regulatory T cells control T helper cell type 1 responses to foreign antigens induced by mature dendritic cells in vivo. J Exp Med. 2003; 198:259–266. [PubMed: 12874259]
37. Tang Q, Adams JY, Tooley AJ, Bi M, et al. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. Nat Immunol. 2006; 7:83–92. [PubMed: 16311599]
38. Tadokoro CE, Shakhar G, Shen S, Ding Y, et al. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. J Exp Med. 2006; 203:505–511. [PubMed: 16533880]
39. DiPaolo RJ, Brinster C, Davidson TS, Andersson J, Glass D, Shevach EM. Autoantigen-specific TGFbeta-induced Foxp3+ regulatory T cells prevent autoimmunity by inhibiting dendritic cells from activating autoreactive T cells. J Immunol. 2007; 179:4685–4693. [PubMed: 17878367]
40. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science. 2008; 322:271–275. [PubMed: 18845758]
41. Boissonnas A, Scholer-Dahirel A, Simon-Blancal V, Pace L, et al. Foxp3+ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes. Immunity. 2010; 32:266–278. [PubMed: 20137985]
42. Tran DQ, Glass DD, Uzel G, Darnell DA, et al. Analysis of adhesion molecules, target cells, and role of IL-2 in human FOXP3+ regulatory T cell suppressor function. J Immunol. 2009; 182:2929–2938. [PubMed: 19234188]
43. Misra N, Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV. Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. J Immunol. 2004; 172:4676–4680. [PubMed: 15067041]
44. Houot R, Perrot I, Garcia E, Durand I, Lebecque S. Human CD4+CD25high regulatory T cells modulate myeloid but not plasmacytoid dendritic cells activation. J Immunol. 2006; 176:5293–5298. [PubMed: 16621995]
45. Bayry J, Triebel F, Kaveri SV, Tough DF. Human dendritic cells acquire a semimature phenotype and lymph node homing potential through interaction with CD4+CD25+ regulatory T cells. J Immunol. 2007; 178:4184–4193. [PubMed: 17371975]
46. Oderup C, Cederbom L, Makowska A, Cilio CM, Ivars F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. Immunology. 2006; 118:240–249. [PubMed: 16771859]

47. Veldhoen M, Moncrieffe H, Hocking RJ, Atkins CJ, Stockinger B. Modulation of dendritic cell function by naive and regulatory CD4+ T cells. J Immunol. 2006; 176:6202–6210. [PubMed: 16670330]
48. Hanig J, Lutz MB. Suppression of mature dendritic cell function by regulatory T cells in vivo is abrogated by CD40 licensing. J Immunol. 2008; 180:1405–1413. [PubMed: 18209035]
49. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity. 2005; 22:329–341. [PubMed: 15780990]
50. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity. 2004; 21:589–601. [PubMed: 15485635]
51. Hanada T, Yoshida H, Kato S, Tanaka K, et al. Suppressor of cytokine signaling-1 is essential for suppressing dendritic cell activation and systemic autoimmunity. Immunity. 2003; 19:437–450. [PubMed: 14499118]
52. Kamath AT, Henri S, Battye F, Tough DF, Shortman K. Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. Blood. 2002; 100:1734–1741. [PubMed: 12176895]
53. Choi BK, Kim YH, Kwon PM, Lee SC, et al. 4-1BB functions as a survival factor in dendritic cells. J Immunol. 2009; 182:4107–4115. [PubMed: 19299708]
54. Liu K, Victora GD, Schwickert TA, Guermonprez P, et al. In vivo analysis of dendritic cell development and homeostasis. Science. 2009; 324:392–397. [PubMed: 19286519]
55. Kushwah R, Oliver JR, Zhang J, Siminovitch KA, Hu J. Apoptotic dendritic cells induce tolerance in mice through suppression of dendritic cell maturation and induction of antigen-specific regulatory T cells. J Immunol. 2009; 183:7104–7118. [PubMed: 19917707]
56. Roskrow MA, Dilloo D, Suzuki N, Zhong W, Rooney CM, Brenner MK. Autoimmune disease induced by dendritic cell immunization against leukemia. Leuk Res. 1999; 23:549–557. [PubMed: 10374848]
57. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 1998; 94:491–501. [PubMed: 9727492]
58. Cheng EH, Kirsch DG, Clem RJ, Ravi R, et al. Conversion of Bcl-2 to a Bax-like death effector by caspases. Science. 1997; 278:1966–1968. [PubMed: 9395403]
59. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell. 1999; 3:287–296. [PubMed: 10198631]
60. Zhu Y, Swanson BJ, Wang M, Hildeman DA, et al. Constitutive association of the proapoptotic protein Bim with Bcl-2-related proteins on mitochondria in T cells. Proc Natl Acad Sci U S A. 2004; 101:7681–7686. [PubMed: 15136728]
61. Matsue H, Edelbaum D, Hartmann AC, Morita A, et al. Dendritic cells undergo rapid apoptosis in vitro during antigen-specific interaction with CD4+ T cells. J Immunol. 1999; 162:5287–5298. [PubMed: 10228004]
62. Yang J, Huck SP, McHugh RS, Hermans IF, Ronchese F. Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific CD8+ T cells in vivo. Proc Natl Acad Sci U S A. 2006; 103:147–152. [PubMed: 16373503]
63. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. Nat Immunol. 2007; 8:1353–1362. [PubMed: 17982458]
64. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. Immunity. 2009; 30:616–625. [PubMed: 19464984]
65. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol. 2008; 8:523–532. [PubMed: 18566595]
66. Liang B, Workman C, Lee J, Chew C, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. J Immunol. 2008; 180:5916–5926. [PubMed: 18424711]

67. Venet F, Pachot A, Debard AL, Bohe J, et al. Human CD4+CD25+ regulatory T lymphocytes inhibit lipopolysaccharide-induced monocyte survival through a Fas/Fas ligand-dependent mechanism. J Immunol. 2006; 177:6540–6547. [PubMed: 17056586]
68. Strauss L, Bergmann C, Whiteside TL. Human circulating CD4+CD25highFoxp3+ regulatory T cells kill autologous CD8+ but not CD4+ responder cells by Fas-mediated apoptosis. J Immunol. 2009; 182:1469–1480. [PubMed: 19155494]
69. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol. 1998; 10:1969–1980. [PubMed: 9885918]
70. Mohamood AS, Trujillo CJ, Zheng D, Jie C, et al. Gld mutation of Fas ligand increases the frequency and up-regulates cell survival genes in CD25+CD4+ TR cells. Int Immunol. 2006; 18:1265–1277. [PubMed: 16769751]
71. Hon H, Rucker EB 3rd, Hennighausen L, Jacob J. bcl-xL is critical for dendritic cell survival in vivo. J Immunol. 2004; 173:4425–4432. [PubMed: 15383573]
72. Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. Immunity. 2003; 18:605–617. [PubMed: 12753738]
73. Sato K, Yamashita N, Baba M, Matsuyama T. Modified myeloid dendritic cells act as regulatory dendritic cells to induce anergic and regulatory T cells. Blood. 2003; 101:3581–3589. [PubMed: 12511411]
74. Watanabe N, Wang YH, Lee HK, Ito T, Cao W, Liu YJ. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. Nature. 2005; 436:1181–1185. [PubMed: 16121185]
75. Yamazaki S, Dudziak D, Heidkamp GF, Fiorese C, et al. CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. J Immunol. 2008; 181:6923–6933. [PubMed: 18981112]
76. Travis MA, Reizis B, Melton AC, Masteller E, et al. Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. Nature. 2007; 449:361–365. [PubMed: 17694047]
77. Sharma MD, Baban B, Chandler P, Hou DY, et al. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. J Clin Invest. 2007; 117:2570–2582. [PubMed: 17710230]

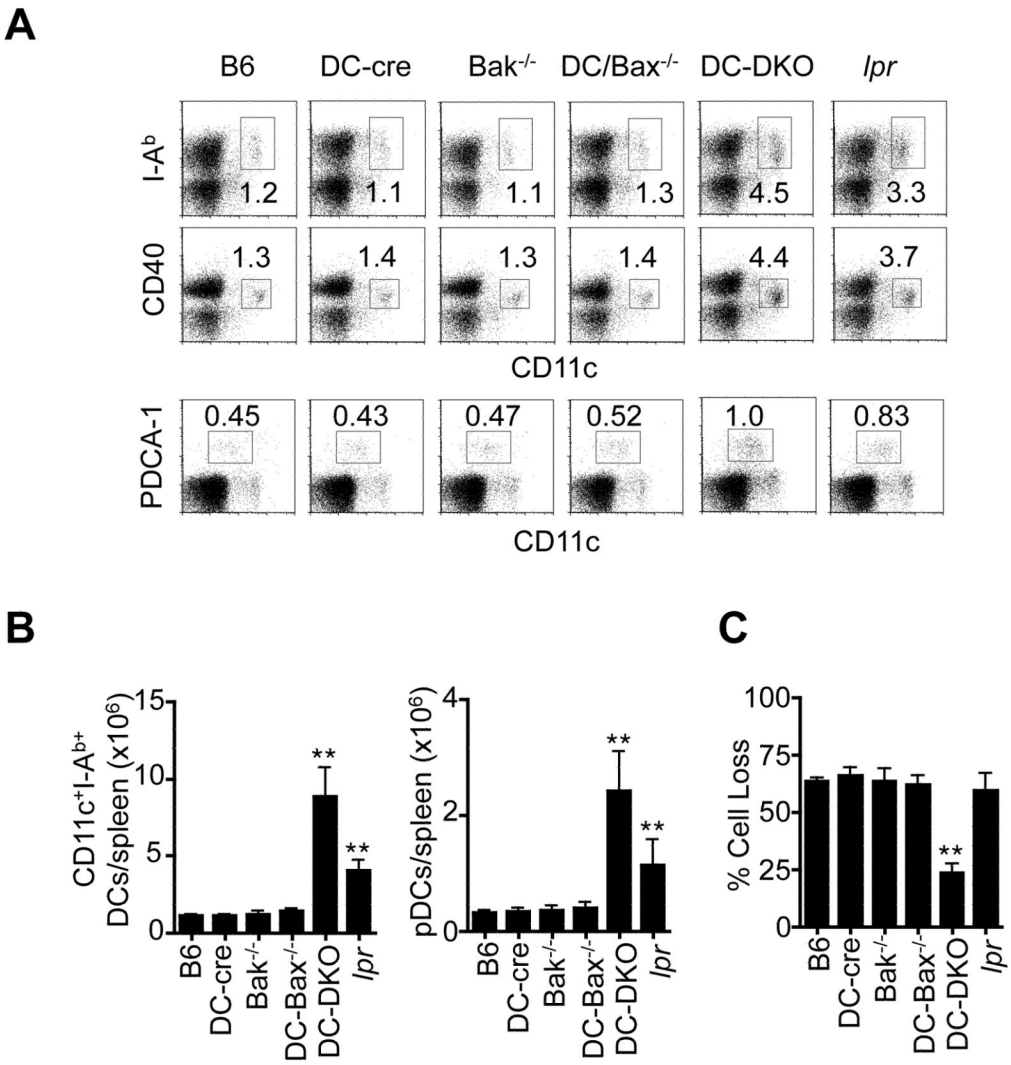

Figure 1. Reduced spontaneous cell death of DCs, DC accumulation and spontaneous lymphocyte activation in DC-DKO mice

(A) Splenocytes from 6-month-old DC-DKO and control mice were stained with antibodies to different markers. The cells were analyzed by flow cytometry of $CD11c^{high}I\text{-}Ab^{+}$ or $CD11c^{high}CD40^{+}$ conventional DCs and $CD11c^{low}PDCA\text{-}1^{+}$ pDCs. (B) $CD11c^{high}I\text{-}Ab^{+}$ DCs in the spleen of 6-month-old wild type C57BL/6 (B6), CD11c-cre (DC-cre), $Bak^{-/-}$, DC-$Bax^{-/-}$, DC- $Bax^{-/-}Bak^{-/-}$ (DC-DKO) and *lpr* mice. ***P*<0.01, n=5. (C) Splenic $CD11c^{+}CD11b^{+}$ DCs from DC-DKO, *lpr* or control mice were cultured *in vitro* for 0 or 24 h. Cell death after 24 h of culture was determined. ***P*<0.01.

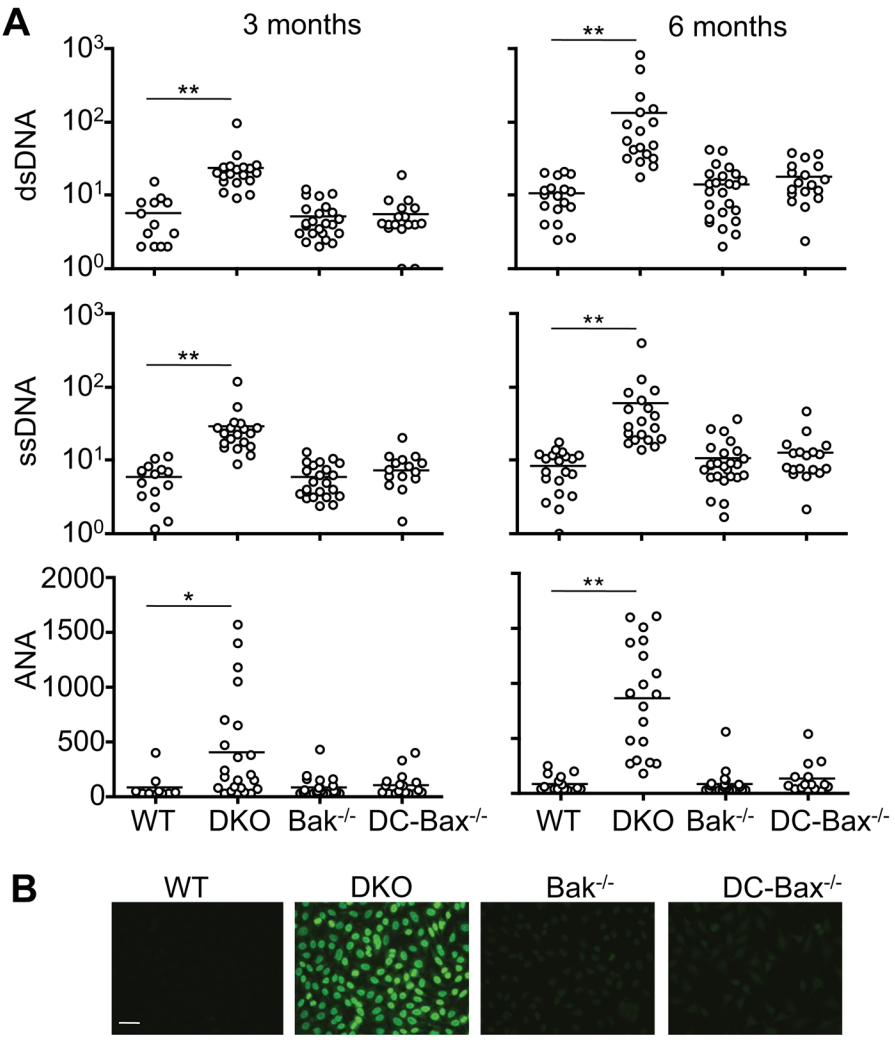

Figure 2. Systemic autoimmunity in DC-DKO mice

(A) ELISA for autoantibodies in the sera of 3- or 6-month-old DC-cre mice as wild type (WT) control, DC-DKO, Bak$^{-/-}$, DC-Bax$^{-/-}$mice. *P<0.05, **P<0.01. (B) Hep2 cell slides were incubated with sera (1:640 dilution) from 6-month-old DC-DKO or control mice, followed by staining with FITC-anti-IgG. Scale bar: 30 μm. Data are representative results of 5 mice per group analyzed.

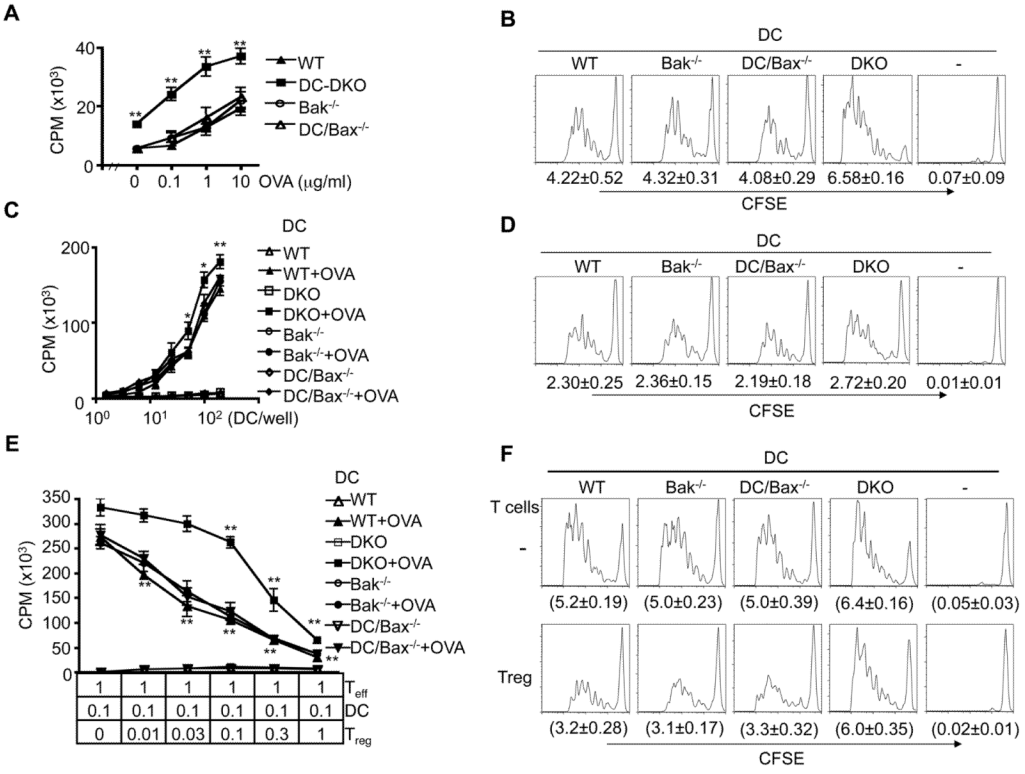

Figure 3. Increased immunogenicity of Bax$^{-/-}$Bak$^{-/-}$ DC

(A) Draining lymph node cells from 6-week-old DC-cre mice as wild type (WT) control, DC-DKO, Bak$^{-/-}$, DC-Bax$^{-/-}$ mice immunized with OVA (5 mice per group) were re-stimulated *in vitro* with OVA. Cell proliferation was measured by ^{3}H-thymidine incorporation 4 days later. WT versus DKO: ***P*<0.01. (B) CD4^{+}CD25^{-} OT2 T cells from OT2 transgenic mice crossed with CD45.1 congenic mice (CD45.1×CD45.2 OT2 F1) were sorted, labeled with CFSE and injected intravenously into recipient C57BL6 mice. Recipient mice (5 mice/group) were then immunized with bone marrow-derived DCs (BMDCs) from DC-DKO or control mice pulsed with OVA$_{323–339}$ peptide the next day. Proliferation of transferred CD4^{+}CD45.1^{+} T cells by CFSE dilution in draining lymph nodes was analyzed 4 days later. The numbers of cell cycle (average ± SD) are shown. WT versus DKO: *P*<0.01, with 65% increased in cell cycles induced by DKO DCs. (C) BMDCs from DC-DKO or control mice with or without OVA$_{323–339}$ pulsing were incubated with CD4^{+}CD25^{-} OT2 T cells. ^{3}H-thymidine incorporation was measured on day 4. WT versus DKO: **P*<0.05, ***P*<0.01. (D) CFSE-labeled OT2 T cells (5×10^{4}/well) were incubated with BMDCs from DC-DKO or control mice pulsed with OVA$_{323–339}$ peptide (300 DCs/well) in 96-well plates. Cell proliferation was measured by CFSE dilution 4 days later. The numbers of cell cycle (average ± SD) are shown. WT versus DKO: *P*<0.05 (n=4), with 13.9% increase in cell cycles induced by DKO DCs. (E) BMDCs from DC-DKO or control mice pulsed with OVA$_{323–339}$ peptide were cultured with CD4^{+}CD25^{-} T cells from OT2 mice, in the absence or presence of T$_{reg}$ cells sorted and expanded from FoxP3^{GFP} mice. Proliferation of OT2 T cells was measured by ^{3}H-thymidine incorporation 4 days later. Statistic comparison with control group containing no T$_{reg}$ cells: ***P*<0.01 (n=3). (F) CD4^{+}CD25^{-} OT2 T cells as in Fig. 3B were labeled with CFSE and injected intravenously into recipient C57BL6 mice. Recipient mice were then immunized at the footpad with BMDCs from DC-DKO or control mice pulsed with OVA$_{323–339}$ peptide together with T$_{reg}$ cells stimulated with anti-CD3-anti-CD28-coated Dynabeads in the presence of IL-2 for 3 days (T$_{reg}$:DC: 0.3:1). The

proliferation of CD45.1$^+$ OT2 T cells in the draining popliteal lymph nodes was quantitated by CFSE dilution 4 days later. The numbers of cell cycle (average ± SD) are shown (n=4). Statistic comparison between groups with or without T_{reg} cells: $P<0.01$ (WT DC), not significant (DKO DC).

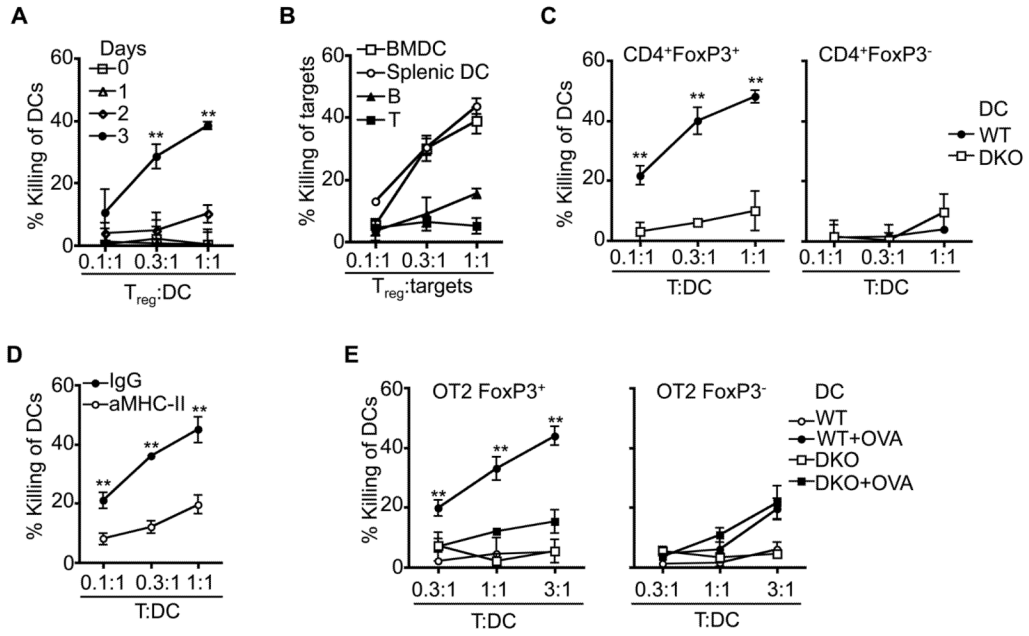

Figure 4. Susceptibility of DCs to killing by T_{reg} cells

(A) Sorted $CD4^+FoxP3^+$ T_{reg} cells were cultured in vitro with anti-CD3- anti-CD28-coated Dynabeads in the presence of IL-2 for 0, 1, 2 or 3 days. BMDCs were labeled with CFSE and incubated with T_{reg} cells for 6 h. Killing of DCs by T_{reg} cells was determined as described in Materials and Methods. Day 3 versus day 0 at 0.3: and 1:1 T_{reg}: DC ratios: $^{**}P<0.01$ (n=3). (B) $CD4^+FoxP3^+$ Treg cells were sorted and stimulated with anti-CD3-anti-CD28-coated Dynabeads in the presence of IL-2 for 3 days. Treg-mediated killing (n=5) of BMDCs, splenic DCs, or activated B or T cells were was determined as described in Materials and Methods. (C) $CD4^+FoxP3^+$ T_{reg} cells or $CD4^+FoxP3^-T_{eff}$ cells sorted and expanded as in Fig. 3 were incubated with wild type or $Bax^{-/-}Bak^{-/-}$ (DKO) BMDCs for 6 h, followed by staining with 7-AAD and analyses by flow cytometry. Loss of $7\text{-}AAD^-$ DCs was quantitated. WT versus DKO: $^{**}P<0.01$ (n=3). (D) Killing of DCs by $CD4^+FoxP3^+$ T_{reg} cells as in (A) in the presence of anti-MHC-II or control IgG. $^{**}P<0.01$ (n=3). (E) $CD4^+FoxP3^+$ T_{reg} cells or $CD4^+FoxP3^-$ T_{eff} cells were sorted from OT2/$FoxP3^{GFP}$ mice and expanded with $OVA_{323–339}$ peptide-pulsed BMDCs for 3 days. OT2 T_{reg} or T_{eff} cells were then isolated by removing DCs with anti-CD11c-MACS beads (Miltenyi Biotec) and used for killing of BMDCs with or without pulsing with $OVA_{323–339}$ peptide. WT versus DKO: $^{**}P<0.01$ (n=3).

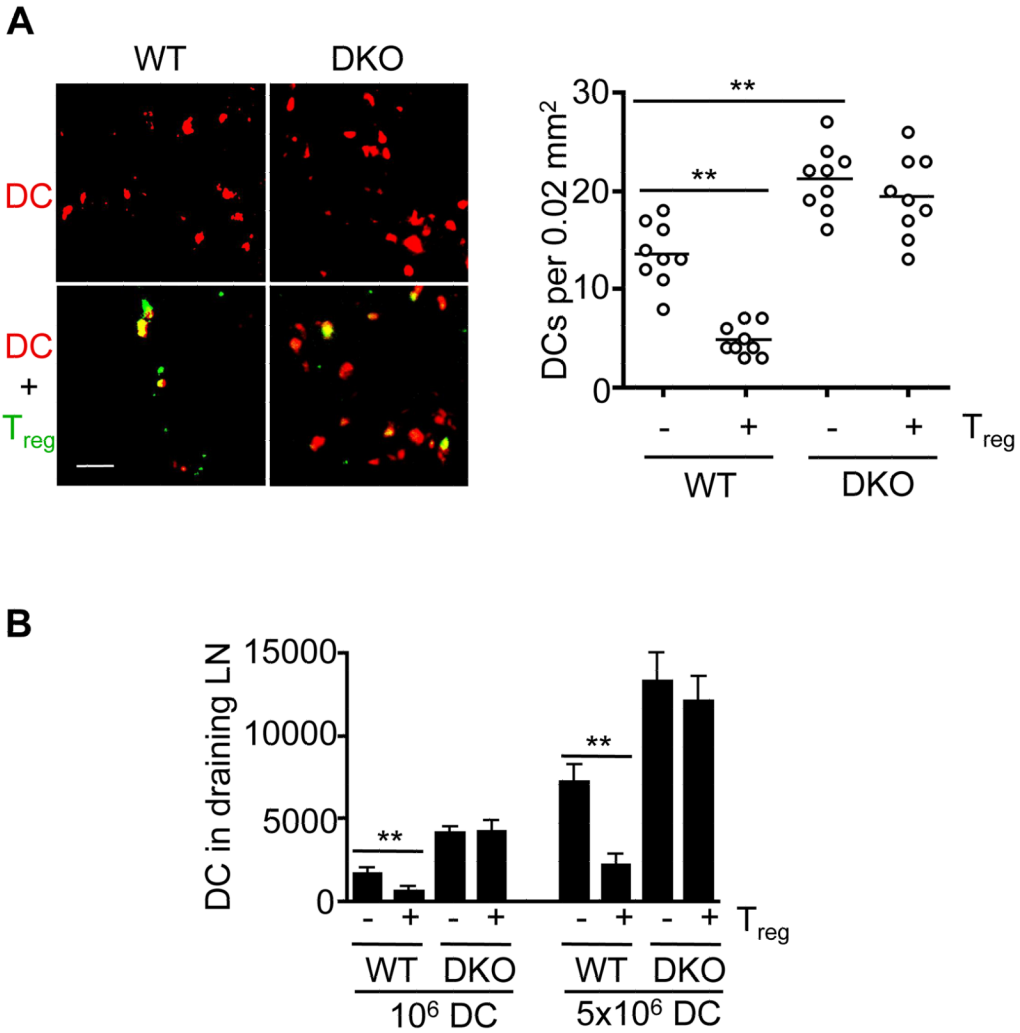

Figure 5. Clearance of DCs by Treg cells after adoptive transfer

(A) Wild type and $Bax^{-/-}Bak^{-/-}$ BMDCs were labeled with CMTMR (red) and injected subcutaneously into footpad. In some groups, CFSE (green)-labeled T_{reg} cells 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 mm^2) in the draining lymph nodes is shown (right panel). ***P*<0.01 (n=9). (B) $CD11c^+$ WT or DKO BMDCs (10^6 or 5×10^6) labeled with CFSE were injected with or without T_{reg} cells (T_{reg}:DC=0.5:1) into syngenic C57BL/6 recipient mice at the footpad. Twenty-four hours later, the draining (popliteal) lymph nodes (LN) were harvested. $CFSE^+$ DCs were analyzed by flow cytometry and total $CFSE^+$ DCs in the draining LN were calculated. ***P*<0.01 (n=6).

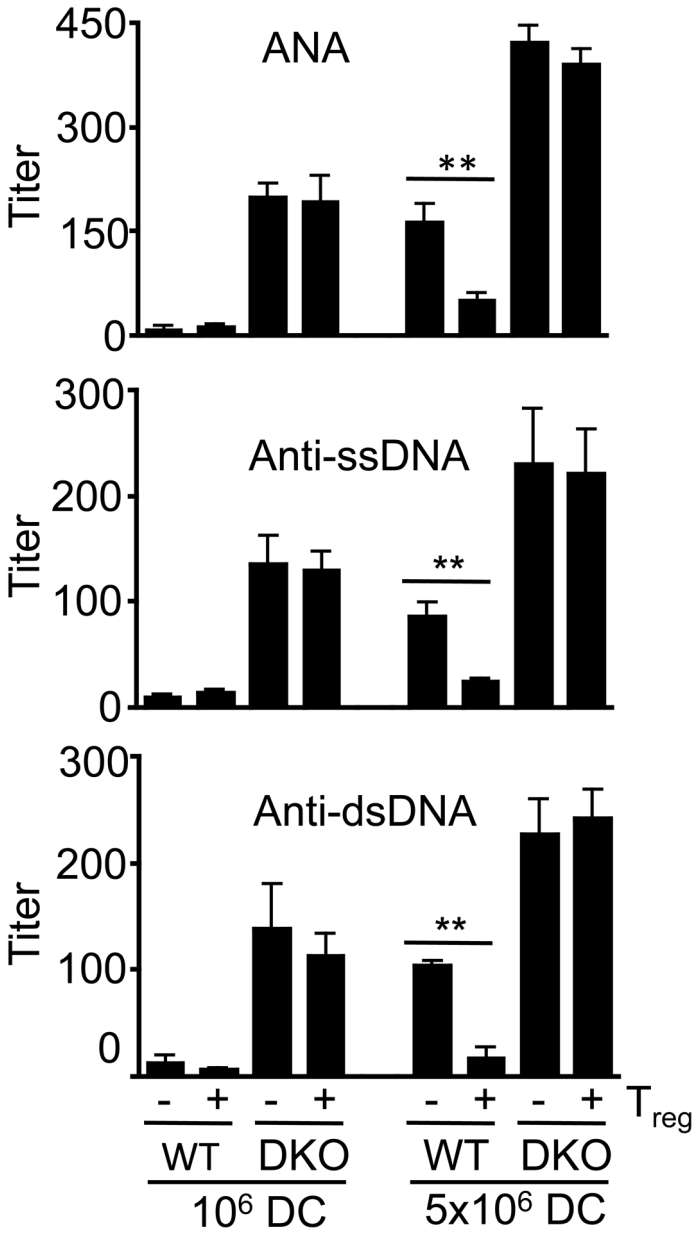

Figure 6. Suppression of DC-induced autoantibody production by Treg cells after adoptive transfer

$CD11c^+$ WT or DKO BMDCs (10^6 or 5×10^6) were injected with or without T_{reg} cells (T_{reg}:DC=0.5:1) into syngenic C57BL/6 recipient mice intraperitoneally (6 mice/group), followed by injection of LPS intraperitoneally (30 μg/mouse) 24 hours later. Sera were collected from recipient mice 1 week later for quantitation of ANA, anti-ssDNA, and anti-dsDNA by ELISA. ***P*<0.01 (n=6).

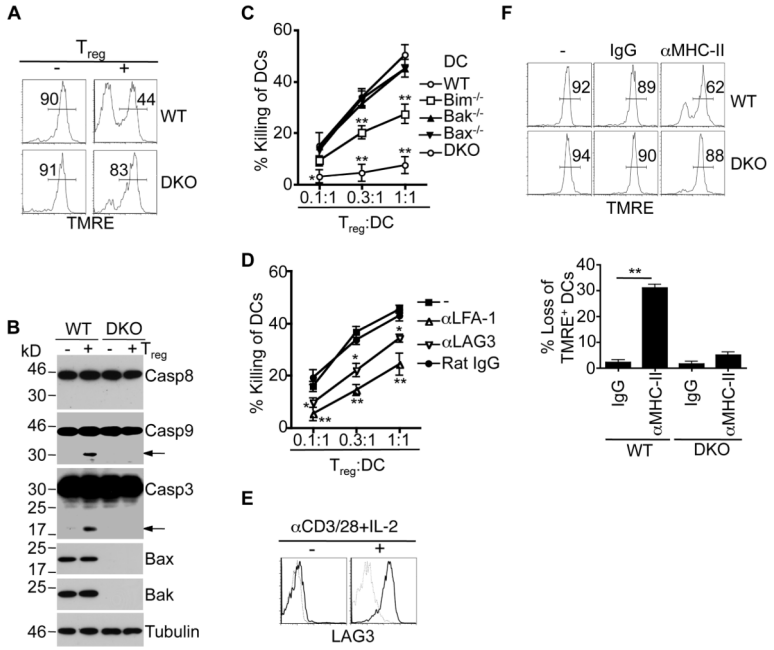

Figure 7. Induction of mitochondrion-dependent cell death in DCs by T_{reg} cells

(A) Wild type or DKO BMDCs with or without incubation with T_{reg} cells for 6 h were labeled with TMRE, followed by flow cytometry. Percentages of $TMRE^+$ cells (mean ± SD): wild type, 89.4 ± 2.1 (no T_{reg}), 44.9 ± 5.0 (with T_{reg}), P=0.0001; DKO, 91.2 ± 1.5 (no T_{reg}), 83.2 ± 5.8 (with T_{reg}), P=0.081 (n=3). (B) Wild type or DKO BMDCs with or without incubation with T_{reg} cells for 6 h were lysed for Western blotting of caspase-8 (Casp8), Casp9, Casp3, Bax or Bak. (C) T_{reg} cells were incubated with wild type, $Bak^{-/-}$, $Bax^{-/-}$, $Bim^{-/-}$ or DKO BMDCs, followed by analysis of killing of DCs as in Fig. 4. $Bim^{-/-}$ or DKO versus WT DCs: *P<0.05, **P<0.01 (n=3). (D) T_{reg} cells were incubated with wild type BMDCs for 6 h in the presence of various antibodies of control IgG, followed by quantification of killing of DCs as in Fig. 6A. Antibody treatments versus IgG control: *P<0.05; **P<0.01 (n=3). (E) $CD4^+FoxP3^+$ T_{reg} cells with or without stimulation with anti-CD3/anti-CD28 Dynal beads plus IL-2 for 3 days were stained with PE-conjugated anti-LAG3 (solid line) or an isotype control (dashed line), followed by analyses by flow cytometry. (F) WT or DKO DCs were incubated with anti-I-A^b or control IgG for 6 h, followed by incubation with TMRE. The cells were analyzed by flow cytometry and the loss of $TMRE^+$ cells were calculated. **P<0.01 (n=3).