Epithelial and dendritic cells in the thymic medulla promote CD4+Foxp3+ regulatory T cell development via the CD27–CD70 pathway

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CD4+Foxp3+ regulatory T cells (T_{reg} cells) are largely autoreactive yet escape clonal deletion in the thymus. We demonstrate here that CD27–CD70 co-stimulation in the thymus rescues developing T_{reg} cells from apoptosis and thereby promotes T_{reg} cell generation. Genetic ablation of CD27 or its ligand CD70 reduced T_{reg} cell numbers in the thymus and peripheral lymphoid organs, whereas it did not alter conventional CD4+Foxp3− T cell numbers. The CD27–CD70 pathway was not required for pre-T_{reg} cell generation, Foxp3 induction, or mature T_{reg} cell function. Rather, CD27 signaling enhanced positive selection of T_{reg} cells within the thymus in a cell-intrinsic manner. CD27 signals promoted the survival of thymic T_{reg} cells by inhibiting the mitochondrial apoptosis pathway. CD70 was expressed on Aire− and Aire+ medullary thymic epithelial cells (mTECs) and on dendritic cells (DCs) in the thymic medulla. CD70 on both mTECs and DCs contributed to T_{reg} cell development as shown in BM chimera experiments with CD70-deficient mice. In vitro experiments indicated that CD70 on the CD8α+ subset of thymic DCs promoted T_{reg} cell development. Our data suggest that mTECs and DCs form dedicated niches in the thymic medulla, in which CD27–CD70 co-stimulation rescues developing T_{reg} cells from apoptosis, subsequent to Foxp3 induction by TCR and CD28 signals.

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Abbreviations used: cDC, conventional DC; CLSM, confocal laser-scanning microscopy; EAE, experimental autoimmune encephalomyelitis; FTOC, fetal thymic organ culture; MLPA, multiplex ligation-dependent probe amplification; mTEC, medullary TEC; pDC, plasmacytoid DC; rT, room temperature; sAg, superantigen; TEC, thymic epithelial cell.

To achieve immunological tolerance, self-reactive T cells are either eliminated by clonal deletion in the thymus or actively suppressed by regulatory T cells (T_{reg} cells) in the periphery. The best characterized T_{reg} cells are $CD4^+$ cells that express Foxp3 and CD25 (Sakaguchi et al., 2008). These T_{reg} cells can inhibit the response of self-reactive T cells and curtail T cell responses to foreign antigens by various mechanisms (Shevach, 2009). The transcription factor Foxp3 is the master switch for T_{reg} cell formation (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Its loss of function in mice and humans is associated with severe autoimmune syndromes, which highlights the importance of T_{reg} cells for immunological tolerance (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001).

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The Rockefeller University Press $30.00
J. Exp. Med. 2013 Vol. 210 No. 4 715-728
www.jem.org/cgi/doi/10.1084/jem.20112061

The Journal of Experimental Medicine

Discovery of T_{reg} cells was based on the observation that neonatal thymectomy in mice led to severe autoimmunity, which could be prevented by transfer of $CD4^+CD25^+$ T cells (Sakaguchi et al., 1995). T_{reg} cells develop in the thymus in the first weeks after birth, after the peripheral lymphoid organs have been populated with conventional $CD4^+$ and $CD8^+$ T cells (Fontenot et al., 2005a). T_{reg} cells appear relatively late because their development depends on the medullary region of the thymus that is not yet fully established at birth (Liston and Rudensky, 2007). Foxp3 induction can occur in the thymic cortex (Liston et al., 2008; Nunes-Cabaço et al., 2010), but Foxp3 expression is most evident in the thymic medulla. This is where the great majority of T_{reg} cells arise from $CD4^+$ thymocytes (Fontenot et al., 2003). Foxp3 expression can also be induced in mature, conventional $CD4^+$ T cells, particularly in the TGFβ-rich environment of the gut (Atarashi et al., 2011).

After rearrangement of TCRβ and TCRα genes, developing thymocytes are positively selected for functional TCR expression at the $CD4^+CD8^+$ stage on MHC class I– and MHC class II–expressing epithelial cells in the thymic cortex. The resulting $CD4^+$ and $CD8^+$ (single positive) mature thymocytes are subsequently negatively selected against autoreactivity in the thymic medulla (von Boehmer, 2004). Certain medullary thymic epithelial cells (TECs [mTECs]) express many otherwise tissue-restricted antigens, largely driven by the Aire transcriptional regulator (Anderson et al., 2002). In this way, mTECs can present a great variety of autoantigens and enable negative selection of potentially autoreactive thymocytes. Negative selection involves the induction of apoptosis in medullary thymocytes that express a TCR with a high affinity for self-peptide–MHC complexes (von Boehmer, 2004). In contrast to conventional $CD4^+$ T cells, T_{reg} cells have a TCR repertoire that is primarily autoreactive (Romagnoli et al., 2002; Hsieh et al., 2006; Pacholczyk et al., 2006). This implies that T_{reg} cells can somehow escape negative selection in the thymus. Indeed, it has been observed that certain $CD4^+$ thymocytes acquire Foxp3 expression upon contact with Aire-expressing mTECs, survive selection against autoreactivity, and exit to peripheral lymphoid organs as $CD4^+Foxp3^+$ T_{reg} cells (Aschenbrenner et al., 2007).

Foxp3 induction relies on TCRαβ signaling that results from interaction with MHC class II^+ antigen-presenting cells (Fontenot et al., 2003; Aschenbrenner et al., 2007; Liston et al., 2008; Proietto et al., 2008; Román et al., 2010). Whereas deletion would be expected, there is evidence that $CD4^+CD25^+$ T_{reg} cell precursors are positively selected by moderate- to high-affinity TCR ligands (Jordan et al., 2001; Apostolou et al., 2002; Kawahata et al., 2002; Ribot et al., 2006) and can survive high level TCR signaling much better than $CD4^+CD25^-$ conventional T cell precursors (van Santen et al., 2004; Taylor et al., 2007). Moreover, Foxp3 induction and thymic T_{reg} cell development are highly dependent on CD28 co-stimulation (Tai et al., 2005), whereas CD28 signaling promotes the deletion of autoreactive $CD4^+$ thymocytes (McKean et al., 2001). The question has been raised therefore which signals enable T_{reg} cells to survive TCR/CD28 triggering in the thymic medulla (Liu, 2006). We here report that the CD27–CD70 co-stimulatory pathway fulfills this function, most likely within dedicated thymic niches.

In both mouse and human, certain cells in the thymic medulla constitutively express the TNF family member CD70. There is evidence that these are mTECs, but this has not been firmly established (Hintzen et al., 1994; Tesselaar et al., 2003; Derbinski et al., 2005). The receptor for CD70, CD27, is expressed on thymocytes: in the mouse from the pro-T cell stage onwards (Gravestein et al., 1996; Igarashi et al., 2002) and in humans on positively selected $CD4^+CD8^+$ thymocytes (Martorell et al., 1990). In mature peripheral T cells, CD27–CD70 co-stimulation promotes $CD8^+$ effector and memory T cell formation (Hendriks et al., 2003, 2005) and T-helper 1 differentiation (Soares et al., 2007; Xiao et al., 2008), whereas it suppresses T-helper 17 effector functions (Coquet et al., 2013). This translates into improved T cell responsiveness to protein antigens, acute virus infections, and tumors and reduced autoimmunity in an experimental autoimmune encephalomyelitis (EAE) model (Nolte et al., 2009; Coquet et al., 2013). Our initial work suggested that CD27–CD70 co-stimulation in the mouse thymus promotes generation of the $CD4^+CD8^+$ thymocyte compartment (Gravestein et al., 1996). However, development of $CD4^+$ and $CD8^+$ αβ T cells proved normal in *Cd27*$^{-/-}$ mice (Hendriks et al., 2000), raising the question of what role CD27–CD70 interactions play in the thymus. Recently, we discovered that they are essential for the thymic development of IFN-γ–producing γδ T cells (Ribot et al., 2009).

We report here that the CD27–CD70 pathway makes an important contribution to the thymic development of T_{reg} cells. We found CD70 exclusively in the thymic medulla, where it was expressed on mTECs, including those that expressed Aire, and on DCs. By using CD70-deficient mice (Coquet et al., 2013) in BM chimera experiments, we demonstrate that CD70 on both mTECs and DCs contributed to T_{reg} cell development. CD27–CD70 interactions in the thymus enhanced positive selection of T_{reg} cells and rescued developing T_{reg} cells from apoptosis without quantitatively affecting the survival or development of conventional αβ T cells. Our findings suggest that mTECs and DCs create cellular niches within the thymic medulla where CD27–CD70 signals promote the survival of developing T_{reg} cells.

RESULTS

The CD27–CD70 pathway is important for the development of T_{reg} cells but not conventional αβ T cells

The development of αβ T cells was examined in CD27-deficient mice (Hendriks et al., 2000) and CD70-deficient (*Cd70*$^{Cre/Cre}$) mice (Coquet et al., 2013). Flow cytometric analysis of CD27- and CD70-deficient mice identified a reduction in the frequency and absolute number of $CD4^+Foxp3^+$ T_{reg} cells in these mice (Fig. 1, A and B). In contrast, numbers of conventional, $Foxp3^-CD4^+$ T cells in the thymus and spleen were similar (Fig. 1 B). In addition, the sizes of the various immature thymocyte subpopulations and the size of the $CD4^-CD8^+$ populations in thymus and spleen were comparable between all

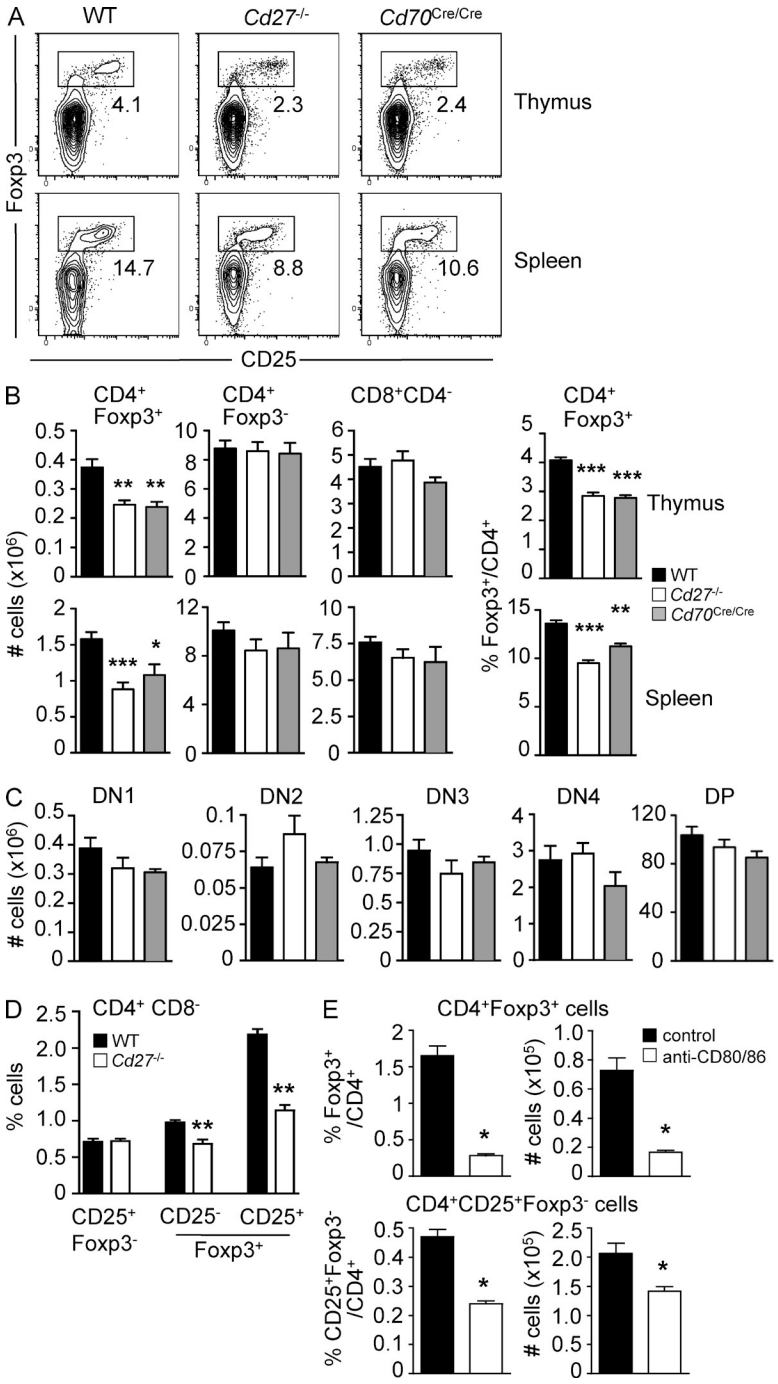

Figure 1. T_{reg} cell development is compromised in *Cd27*$^{-/-}$ and *Cd70*$^{Cre/Cre}$ mice. Thymocytes and splenocytes were isolated from WT and CD27- and CD70-deficient mice, enumerated, stained with antibodies to CD4, CD8, CD25, CD44, and Foxp3 and analyzed by flow cytometry. (A) Representative analysis of CD25 versus Foxp3 staining on gated CD4$^+$CD8$^-$ cells in thymus and spleen. Numbers indicate the percentage of Foxp3$^+$ cells present in the indicated gate. (B) Absolute number and percentage of CD4$^+$Foxp3$^+$ T_{reg} cells among total CD4$^+$ T cells and absolute numbers of conventional CD4$^+$Foxp3$^-$ T cells and CD8$^+$CD4$^-$ T cells in the thymus and spleen of WT, *Cd27*$^{-/-}$, and *Cd70*$^{Cre/Cre}$ mice. (C) Absolute number of cells in the double negative (DN; CD4$^-$CD8$^-$) subsets, based on CD44 and CD25 expression, and in the double positive (DP; CD4$^+$CD8$^+$) subset in the thymus of WT, *Cd27*$^{-/-}$, and *Cd70*$^{Cre/Cre}$ mice. Data in B and C are derived from 10–16 mice per genotype, pooled from at least three independent analyses. Numbers (mean + SEM) from WT versus *Cd27*$^{-/-}$ and *Cd70*$^{Cre/Cre}$ mice were significantly different according to one-way analysis of variance, followed by a post-Dunn's test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (D) Thymic CD4$^+$CD8$^-$ cells were analyzed for the percentage of CD25$^+$Foxp3$^-$ pre-T_{reg} cells and CD25$^+$ or CD25$^-$Foxp3$^+$ T_{reg} cells. Data are from nine mice pooled from three experiments. Values from WT and *Cd27*$^{-/-}$ mice were compared by Mann–Whitney U rank test. **, $P < 0.01$. See also Fig. S1 A. (E) *Cd27*$^{-/-}$ mice were administered 100 µg anti-CD80 and anti-CD86 or the appropriate isotype control antibody i.p. every other day for 2 wk. 2 d after the last administration, T_{reg} cell frequency in the thymus was analyzed by flow cytometry. Percentage and absolute number of T_{reg} and pre-T_{reg} cells in treated *Cd27*$^{-/-}$ mice are shown. Data shown are from one representative of two independent experiments with three mice per group. Results were analyzed by Student's *t* test. *, $P < 0.05$. See also Fig. S1 B. Error bars are SEM.

genotypes (Fig. 1, B and C). Thus, in mice lacking CD27 or CD70, generation of T_{reg} cells was specifically impaired, whereas the development of conventional T cells appeared normal.

During T_{reg} cell development in the thymus, T_{reg} cell precursors contact self-antigens presented in MHC class II molecules with their TCR. This induces expression of the IL-2 receptor α chain (CD25), before Foxp3 expression enables further T_{reg} cell development (Burchill et al., 2008; Lio and Hsieh, 2008). Thymic CD4$^+$Foxp3$^-$CD25$^+$ cells are referred to as pre-T_{reg} cells and include a large proportion of cells destined to become mature T_{reg} cells (Lio and Hsieh, 2008). CD27 signals were not required to generate this cell population, as the frequency of pre-T_{reg} cells was normal in the thymus of *Cd27*$^{-/-}$ mice (Fig. 1 D and Fig. S1 A). Generation of pre-T_{reg} cells as well as mature T_{reg} cells depends on TCR signals, as well as CD28 co-stimulation (Lio et al., 2010; Vang et al., 2010). We determined whether CD28 co-stimulation still made a contribution to T_{reg} cell development in the absence of CD27. For this purpose, *Cd27*$^{-/-}$ mice were administered neutralizing antibodies to CD80 and CD86 over a period of 10 d, after which T_{reg} cells

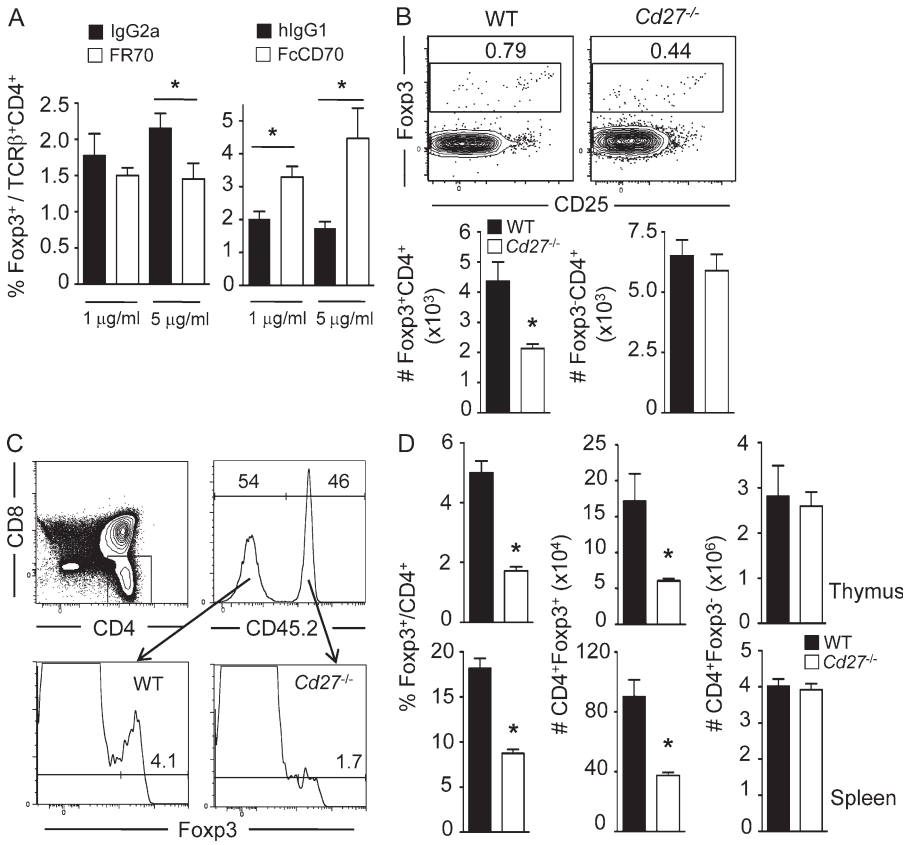

Figure 2. CD27–CD70 co-stimulation promotes thymic T_{reg} cell development in vitro and in vivo. (A) Independent FTOCs were performed with either 1 or 5 μg/ml CD70-blocking antibody FR70 and an internal control of 1 or 5 μg/ml of control IgG2a antibody (left). Alternatively, cultures were performed in the presence of either 1 or 5 μg/ml CD27-agonist FcCD70 or control IgG1 antibody (right). After 14–16 d, T_{reg} cell development was assessed by disrupting the lobes and performing flow cytometric analyses for Foxp3, TCRβ, and CD4. Bar diagrams depict the percentage of Foxp3+ cells (T_{reg} cells) among TCRβ+CD4+cells. Data are from two to three separate experiments comprising four to six independent wells. Data were analyzed using the Mann–Whitney U rank test (*, $P < 0.05$). (B) Thymocytes were isolated from WT and *Cd27*$^{-/-}$ neonates, stained with antibodies to CD4, CD8, CD25, and Foxp3, and analyzed by flow cytometry. Representative plots of CD25 and Foxp3 expression on gated CD4+CD8− thymocytes are shown, and graphs depict the absolute number of Foxp3+ T_{reg} cells and Foxp3−CD4+ thymocytes. Data in histograms are mean + SEM from five WT and six *Cd27*$^{-/-}$ mice and were analyzed by Mann–Whitney U rank test (*, $P < 0.05$). (C and D) Mixed BM chimeras were established by lethally irradiating B6 mice of the CD45.2 allotype and reconstituting them the next day with a 1:1 mixture of CD45.1+ WT and CD45.2+ *Cd27*$^{-/-}$ BM cells. 8 wk later, reconstitution of recipient thymus and spleen was assessed by flow cytometry. (C) The dot plot (top left) depicts a representative analysis of the thymus and the histogram (top right) depicts the expression of CD45.2 on gated CD4+ cells. The histograms in the bottom panels depict the expression of Foxp3 within the gated (arrows) CD45.2− WT (left) or CD45.2+ *Cd27*$^{-/-}$ (right) CD4+ cell populations. Numbers in each plot represent percentages of the gated population. (D) The percentage of T_{reg} cells among total CD4+ cells and the absolute number of conventional CD4+Foxp3− T cells and CD4+Foxp3+ T_{reg} cells in the thymus and spleen of WT and *Cd27*$^{-/-}$ mice. Data are mean + SEM from four mice per group and are representative of three separate experiments. The Mann–Whitney U rank sum test was used to calculate significance between groups (*, $P < 0.05$).

were enumerated. Anti-CD80/CD86 treatment drastically reduced the frequency and absolute numbers of CD4+Foxp3+ T_{reg} cells in the thymus of *Cd27*$^{-/-}$ mice (Fig. 1 E and Fig. S1 B), and in the spleen (not depicted), demonstrating that the CD28–CD80/86 and the CD27–CD70 co-stimulatory pathways played nonredundant roles in T_{reg} cell development. Although absence of CD27 or CD70 did not affect the generation of pre-T_{reg} cells, blockade of CD28 signaling did (Fig. 1 E), strongly suggesting that CD27 co-stimulation acts subsequent to CD28 co-stimulation in thymic T_{reg} cell development.

Stimulation of CD27 on T cell progenitors in the thymus promotes T_{reg} cell development

To examine the impact of the CD27–CD70 pathway on thymic T_{reg} cell development, fetal thymic organ cultures (FTOCs) were performed using embryonic day (E) 15 fetal thymic lobes. The addition of a blocking antibody directed at CD70 (FR70) reduced the output of CD4+Foxp3+ T_{reg} cells from these cultures (Fig. 2 A), whereas the addition of an agonistic soluble form of CD70 (FcCD70; Peperzak et al., 2010b) enhanced T_{reg} cell output (Fig. 2 A). This suggested that CD27 stimulation on thymic progenitors by CD70 promoted T_{reg} cell development. To further confirm the impact of CD27–CD70 co-stimulation on T_{reg} cell development in the thymus, we enumerated T_{reg} cells in neonatal mice. Directly after birth, T_{reg} cell levels are very low because the thymic medullary niches that are essential for T_{reg} cell development are incompletely formed (Liston and Rudensky, 2007). In the thymus of *Cd27*$^{-/-}$ neonates, the frequency and number of thymic T_{reg} cells was reduced as compared with WT, whereas numbers of CD4+Foxp3− cells were normal (Fig. 2 B), confirming our earlier results. T_{reg} cells were virtually absent in the periphery of neonatal mice (not depicted), making it highly unlikely that the reduction in thymic T_{reg} cell numbers observed in *Cd27*$^{-/-}$ mice was caused by a recirculation defect of mature peripheral T_{reg} cells.

To address whether CD27 played a cell-intrinsic role in T_{reg} cell development, BM chimeras were created. WT mice were lethally irradiated, reconstituted with a 1:1 mixture of BM from WT and *Cd27*$^{-/-}$ mice and analyzed after 8 wk for the development of T_{reg} cells and conventional CD4+ T cells

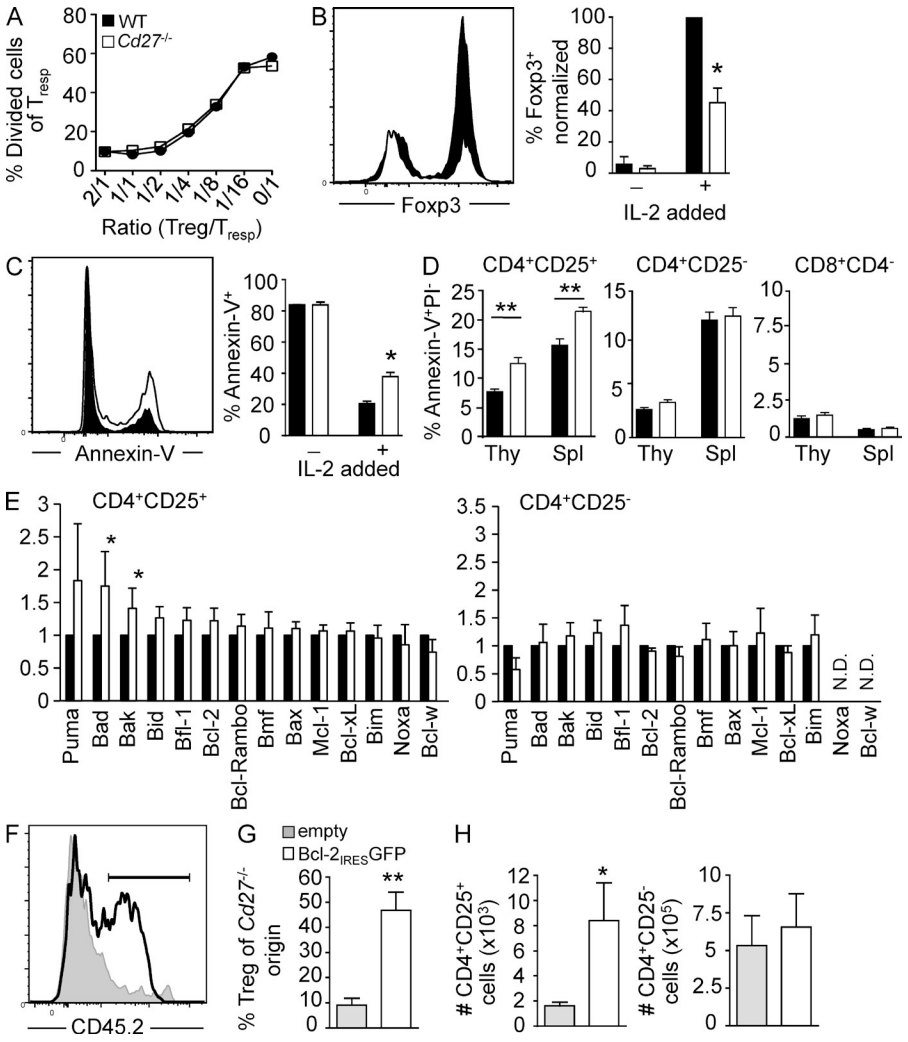

Figure 3. CD27–CD70 co-stimulation promotes T_{reg} cell survival but not function. (A) $CD4^+CD25^+$ (T_{reg}) cells were sorted from naive spleens of WT or *Cd27*$^{-/-}$ mice and co-cultured with sorted CFSE-labeled $CD4^+CD25^-$ responder T (T_{resp}) cells from WT mice of the CD45.1 allotype on plates coated with anti-CD3 mAb, in the presence of irradiated splenocytes. After 3 d, flow cytometry was performed and gated $CD45.1^+$ cells were analyzed for dilution of CFSE. Plotted is the percentage of divided responder T cells after culture in the presence of various numbers of T_{reg} cells. One representative experiment of four is shown. Genotype labeling indicated in A also applies to B–E. (B and C) $CD4^+CD8^-CD25^+CD69^+HSA^+$ (pre-T_{reg}) cells were sorted by flow cytometry and cultured with or without 10 ng/ml IL-2 for 3 d and diagnosed by flow cytometry for Foxp3 expression (B) and Annexin-V binding (C). See also Fig. S1 C. Data in the bar graph in B were normalized to the percentage of $Foxp3^+$ cells derived from WT cultures in the presence of IL-2. Bar diagrams depict mean + SEM of three to four individual experiments. Data were analyzed using the Mann–Whitney U rank test (*, $P < 0.05$). (D) $CD4^+CD8^-CD25^+$, $CD4^+CD8^-CD25^-$, and $CD8^+CD4^-$ cells from thymus or spleen of WT or *Cd27*$^{-/-}$ mice were analyzed for Annexin-V binding and propidium iodide (PI) exclusion and the proportion of apoptotic cells (Annexin-V^+PI^-) in each population is depicted. See also Fig. S1 D. Bar diagrams depict mean + SEM of four individual mice. Data were analyzed by Student's *t* test (**, $P < 0.01$). (E) mRNA was isolated from purified $CD4^+CD8^-CD25^+$ and $CD4^+CD8^-CD25^-$ cells from WT or *Cd27*$^{-/-}$ mice in four independent experiments each with five pooled thymi and analyzed by MLPA with primers specific for the indicated genes. Levels of apoptosis-related genes were calculated relative to the housekeeping gene *Gusb*, and in each experiment the level of gene expression in the *Cd27*$^{-/-}$ sample was normalized to that of the WT sample. Bar graphs depict the level of mRNA expression in relative units in WT (closed bars) and *Cd27*$^{-/-}$ (open bars) cells. Data were analyzed according to one-way analysis of variance, followed by a post-Dunn's test (*, $P < 0.05$). (F–H) CD45.1$^+$ WT and CD45.2$^+$ *Cd27*$^{-/-}$ BM cells were harvested and T cell depleted. *Cd27*$^{-/-}$ cells were transduced with retroviral vectors expressing GFP alone (gray-filled histogram) or Bcl-2_{IRES}GFP (black line), and WT cells were transduced with a vector expressing GFP alone. Empty GFP vector–expressing WT and *Cd27*$^{-/-}$ cells or GFP vector–expressing WT cells and Bcl-2_{IRES}GFP–expressing *Cd27*$^{-/-}$ cells were injected at a 1:1 ratio into irradiated recipients, and T_{reg} cell development was analyzed 8 wk later. (F) Expression of the *Cd27*$^{-/-}$ cell marker CD45.2 on gated $GFP^+CD4^+CD25^+$ T_{reg} cells. (G) Percentage of T_{reg} cells arising from the *Cd27*$^{-/-}$ donor cells when cells were transduced to express GFP alone or Bcl-2 and GFP. (H) Absolute number of $CD4^+CD25^+$ T_{reg} cells or $CD4^+CD25^-$ conventional T cells arising from the *Cd27*$^{-/-}$ donor cells when cells were transduced with GFP alone (empty) or Bcl-2_{IRES}GFP. Results in F–H are from one representative of two independent experiments with five mice per group and were analyzed by Mann–Whitney U rank test (*, $P < 0.05$; **, $P < 0.01$). Error bars are SEM.

(Fig. 2 C). Within $CD4^+$ cells, the percentage of $Foxp3^+$ cells originating from WT precursors was much higher than those originating from *Cd27*$^{-/-}$ precursors (Fig. 2, C and D). This difference was also reflected in the total number of T_{reg} cells originating from WT or *Cd27*$^{-/-}$ precursors. In contrast, conventional $CD4^+Foxp3^-$ T cell development in the thymus and spleen was comparable (Fig. 2 D). These results indicate that CD27 expression by thymic precursors is important for T_{reg} cell development but not required for development of conventional $CD4^+$ T cells.

CD27 signaling prevents apoptosis of T_{reg} cells but does not affect their functional differentiation

To determine whether CD27 co-stimulation affected the functional programming of T_{reg} cells, their suppressive activity was assessed. T_{reg} cells were purified from WT and *Cd27*$^{-/-}$ mice and co-cultured for 3 d with CFSE-labeled conventional $CD4^+CD25^-$ responder T cells. WT and *Cd27*$^{-/-}$ T_{reg} cells dampened responder $CD4^+$ T cell proliferation in a highly similar, dose-dependent fashion (Fig. 3 A). This finding indicates that the CD27–CD70 pathway is not important for

acquisition of suppressive function by T_{reg} cells, as assessed in this in vitro assay. Furthermore, Foxp3 was expressed at a similar level in T_{reg} cells from WT, *Cd27*$^{-/-}$, and *Cd70*$^{Cre/Cre}$ mice (Fig. 1 A and not depicted), suggesting that, contrary to CD28 co-stimulation (Tai et al., 2005), CD27 co-stimulation did not regulate Foxp3 expression in developing T_{reg} cells.

We next determined whether CD27–CD70 co-stimulation affected the development and survival of T_{reg} cells. For this purpose, pre-T_{reg} cells were purified from the thymus of WT and *Cd27*$^{-/-}$ mice on basis of a CD69$^+$HSA$^+$CD25$^+$CD4$^+$CD8$^-$ phenotype (Fig. S1 C) and cultured in vitro with or without IL-2, as described previously (Lio and Hsieh, 2008). In the absence of IL-2, the pre-T_{reg} cells hardly gave rise to Foxp3$^+$ cells (Fig. 3 B) and most cells died (Fig. 3 C). In the presence of IL-2, pre-T_{reg} cells developed into Foxp3$^+$ T_{reg} cells, with *Cd27*$^{-/-}$ precursors giving rise to significantly less Foxp3$^+$ cells than WT precursors (Fig. 3 B). Moreover, significantly more Annexin-V$^+$ apoptotic cells were observed in the *Cd27*$^{-/-}$ cultures as compared with the WT cultures (Fig. 3 C), suggesting that CD27 provided important survival signals to developing T_{reg} cells. Direct ex vivo analysis of Annexin-V binding also indicated that CD4$^+$CD25$^+$ cells from *Cd27*$^{-/-}$ mice underwent more apoptosis than their WT counterparts (Fig. 3 D and Fig. S1 D). This effect was selective for T_{reg} cells because there was no difference in the proportion of apoptotic cells within the conventional CD4$^+$CD8$^-$CD25$^-$ or CD8$^+$CD4$^-$ populations of WT and *Cd27*$^{-/-}$ mice when analyzed directly ex vivo (Fig. 3 D).

To gain molecular insight into survival signaling by the CD27–CD70 system, we determined the mRNA expression level of a comprehensive set of key apoptosis regulators in WT and *Cd27*$^{-/-}$ CD4$^+$CD25$^+$ and CD4$^+$CD8$^-$CD25$^-$ thymocytes. Although antiapoptotic Bcl-2 family members were similarly expressed, the proapoptotic Bcl-2 family members Bad and Bak were expressed at significantly higher levels in *Cd27*$^{-/-}$ CD25$^+$CD4$^+$ thymocytes than in the WT population, while Puma showed a similar trend (Fig. 3 E). These proapoptotic molecules were not differentially expressed in CD25$^-$CD4$^+$ thymocytes from WT or *Cd27*$^{-/-}$ mice (Fig. 3 E), suggesting that CD27 specifically counteracts the mitochondrial apoptosis pathway in developing T_{reg} cells. To further assess whether CD27 signaling promoted the survival of developing T_{reg} cells by inhibiting the mitochondrial apoptosis pathway, we examined whether deliberate expression of the antiapoptotic Bcl-2 protein could rescue the survival of *Cd27*$^{-/-}$ T_{reg} cells. CD45.2$^+$ *Cd27*$^{-/-}$ BM cells were transduced with a vector encoding Bcl-2 and GFP in an IRES configuration or with the empty vector encoding GFP only. Each population was mixed in a 1:1 ratio with CD45.1$^+$ WT BM cells that had been transduced with empty GFP vector and injected into lethally irradiated CD45.2 WT recipients. 8 wk later, the thymus was harvested and analyzed by flow cytometry for the contribution of *Cd27*$^{-/-}$ cells (CD45.2$^+$) among gated GFP$^+$CD25$^+$CD4$^+$ T_{reg} cells (Fig. 3 F). *Cd27*$^{-/-}$ BM cells contributed only ∼10% of the total GFP$^+$ T_{reg} cell population when they had been transduced with empty GFP vector, confirming the superiority of WT precursors over *Cd27*$^{-/-}$ precursors to develop into T_{reg} cells. However, the proportion of *Cd27*$^{-/-}$ cells contributing to the total GFP$^+$ T_{reg} cell pool was significantly enhanced to almost 50% when *Cd27*$^{-/-}$ BM cells had been transduced with the Bcl-2$_{IRES}$GFP vector (Fig. 3 G). The contribution of Bcl-2 to T_{reg} cell development from *Cd27*$^{-/-}$ BM cells was also reflected in absolute numbers of T_{reg} cells that were significantly higher in the Bcl-2–transduced population than in the empty vector–transduced population (Fig. 3 H). Importantly, *Bcl-2* gene transduction did not affect the development of conventional CD25$^-$CD4$^+$ cells from *Cd27*$^{-/-}$ BM cells (Fig. 3 H). The fact that thymic *Cd27*$^{-/-}$ T_{reg} cell numbers but not conventional CD4$^+$ T cell numbers increased upon apoptosis inhibition by Bcl-2 lends further support to our findings that CD27 deficiency enhances apoptosis of developing T_{reg} cells but not conventional CD4$^+$ T cells.

CD70 is expressed on Aire$^+$ and Aire$^-$ mTECs

In the mouse, CD70 is expressed on DEC205$^+$ cells in the thymic medulla (Tesselaar et al., 2003). DEC205 marks both epithelial cells and DCs (Jiang et al., 1995), but we suggested that CD70 was expressed by mTECs because *Cd70* mRNA was found in murine mTECs (Derbinski et al., 2005) and CD70 was detected on human TEC lines (Hintzen et al., 1994). To precisely define CD70 expression on epithelial cells in the mouse thymus, we here performed immunohistochemistry combined with confocal laser-scanning microscopy (CLSM), using thymi from *Cd70*$^{Cre/Cre}$ mice as negative controls. It was confirmed that CD70 localizes exclusively to the thymic medulla, as defined by nuclear staining with DAPI and detection of mTECs with anti–Keratin-5 mAb (Fig. 4, II and III; Lomada et al., 2007) or ER-TR5 mAb (Fig. 4, I, II, VII, and VIII; van Ewijk et al., 1988). Using both mTEC markers in conjunction with nuclear staining, we consistently found that a large proportion of Keratin-5$^+$ and ER-TR5$^+$ mTECs expressed CD70, typically at different locations in the same cell (Fig. 4, I–IV). This is consistent with CD70 residing in late endosomal compartments and the cell surface (Keller et al., 2007) and Keratin-5 forming a network within the cytoplasm. Interestingly, CD70 was found on Aire$^-$ mTECs but also on the great majority of (if not all) Aire$^+$ mTECs (Fig. 4, V and VI). These data firmly establish that CD70 is expressed on mTECs, including those that express Aire and are implicated in T_{reg} cell selection (Aschenbrenner et al., 2007; Lei et al., 2011).

CD70 on TECs promotes positive selection of T_{reg} cells

To address whether CD27–CD70-dependent survival signals were required for positive selection of thymic T_{reg} cells, we used a model of superantigen (sAg)-driven T_{reg} cell selection in C57BL/6 (B6) versus DBA/2 hosts (Ribot et al., 2006). Both strains express sAgs encoded by endogenous mouse mammary tumor virus 8 (Mtv-8) and Mtv-9. In DBA/2 mice, these sAgs bind to I-E^d MHC class II molecules and form high-affinity ligands for Vβ5 TCRs. In B6 mice, however, the sAgs bind to I-A^b MHC class II molecules and form low-affinity ligands for Vβ5 TCRs (Luther and Acha-Orbea, 1997).

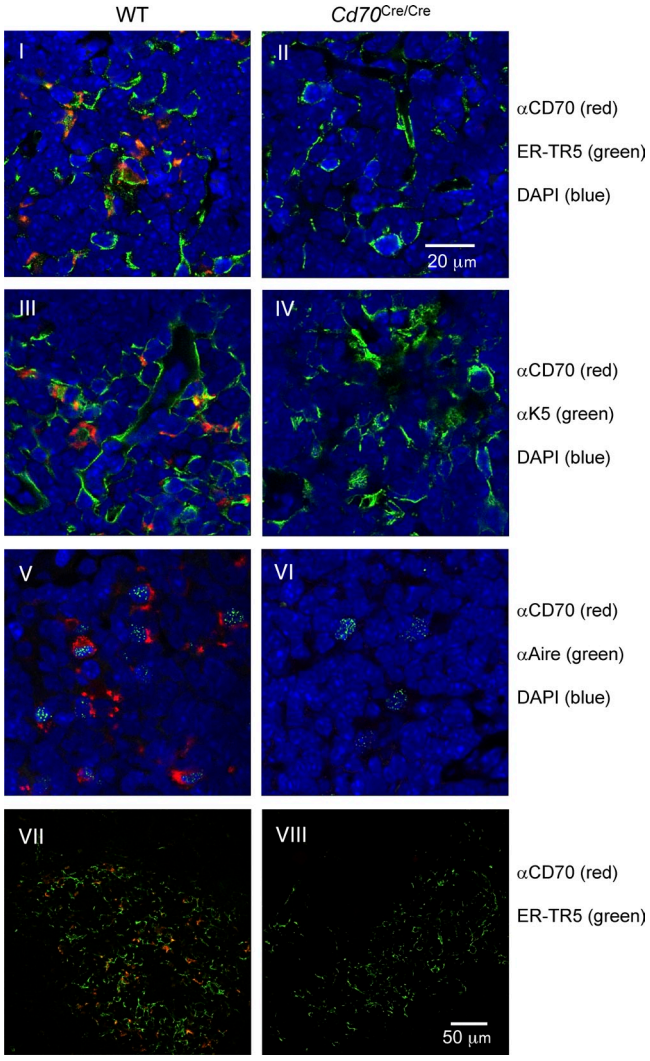

Figure 4. CD70 is expressed by Aire$^+$ and Aire$^-$ epithelial cells in the thymic medulla. (I–IV, VII, and VIII) Thymic sections of WT and *Cd70*$^{Cre/Cre}$ mice were stained to detect CD70 on mTECs using sequential staining with ER-TR5 mAb, Alexa Fluor 488–conjugated anti-IgG, anti-CD70 mAb, and Alexa Fluor 568–conjugated anti-IgG (I, II, VII, and VIII) or sequential staining with anti–Keratin-5 (K5) mAb, Alexa Fluor 488–conjugated anti-IgG, anti-CD70 mAb, and Alexa Fluor 568–conjugated anti-IgG (III and IV). (V and VI) Thymic sections of WT and *Cd70*$^{Cre/Cre}$ mice were stained to detect CD70 and Aire using sequential staining with anti-CD70 mAb, Alexa Fluor 568–conjugated anti-IgG, and Alexa Fluor 488–conjugated anti-Aire mAb. Sections I–VI were subsequently stained with DAPI to detect the nuclei of all cells present. The same magnification is shown for all panels. High magnification (I–VI) and lower magnification (VII–VIII) images of the thymus are shown. Data for each staining are representative of at least three to four different sections that were stained and analyzed independently.

Based on this, we created BM chimeras in DBA/2 and B6 mice by irradiating mice and reconstituting them with a 1:1 mixture of WT and *Cd27*$^{-/-}$ BM. In both DBA/2 and B6 recipients, CD27 co-stimulation significantly promoted the development of T_{reg} cells, without affecting conventional CD4$^+$ T cell development (Fig. 5 A), thus corroborating the aforementioned results. To assess whether CD27 promoted positive selection of sAg-specific T_{reg} cells, Vβ5 expression was analyzed in the T_{reg} cell compartment (Fig. 5 B). In DBA/2 recipient mice, the frequency of WT Vβ5$^+$ T_{reg} cells was significantly higher than that of *Cd27*$^{-/-}$ Vβ5$^+$ T_{reg} cells (Fig. 5 C). In B6 mice, the frequencies were not significantly different. The frequencies of control Vβ4$^+$ T_{reg} cells, which do not recognize these sAgs (Luther and Acha-Orbea, 1997), were similar among WT and *Cd27*$^{-/-}$ T_{reg} cells (Fig. 5 C), Furthermore, the frequencies of Vβ5$^+$ and Vβ4$^+$ conventional CD4$^+$ T cells were comparable between WT and *Cd27*$^{-/-}$ cells in B6 and DBA/2 mice (not depicted). These results suggest that CD27 co-stimulation promotes the positive selection of Vβ5$^+$ T_{reg} cells.

To determine whether CD70 expression by epithelial cells alone sufficed to promote positive selection of T_{reg} cells, irradiated DBA/2 mice were reconstituted either with a 1:1 mixture of WT (*Cd27*$^{+/+}$) and *Cd27*$^{-/-}$ BM cells or with a 1:1 mixture of *Cd27*$^{+/+}$;*Cd70*$^{Cre/Cre}$ and *Cd27*$^{-/-}$;*Cd70*$^{Cre/Cre}$ BM cells. In this way, we created environments in which CD70 was expressed by both TECs and hematopoietic cells or by TECs only. In these DBA/2 chimaeras, the positive selection of Vβ5$^+$ T_{reg} cells was significantly impaired by CD27 deficiency, both when CD70 was present or absent on hematopoietic cells (Fig. 5 D). These data suggest a scenario in which T_{reg} cell precursors recognize MHC class II–sAg complexes on TECs and are supported in their positive selection by concomitant CD27–CD70 co-stimulation. Intriguingly, we also noted that total T_{reg} cell development in this system was most efficient when both hematopoietic and radio-resistant cells expressed CD70 (Fig. 5 E). This suggested that CD70 on hematopoietic cells also played a role in thymic T_{reg} cell selection.

CD70 is present on DCs in the thymic medulla, and CD8α$^+$ conventional DCs (cDCs) support T_{reg} cell development in vitro

The BM chimera studies in DBA/2 mice suggested that not only CD70 on epithelial cells but also CD70 on hematopoietic cells contributed to T_{reg} cell selection. Previously, it has been shown that DCs play a role in thymic T_{reg} cell development (Proietto et al., 2008; Lei et al., 2011). Because CD70 is expressed on activated DCs in the periphery, we examined whether it was present on DCs in the thymus. For this purpose, thymus sections were stained with antibodies to CD70 and the DC marker CD11c, in conjunction with nuclear staining (not depicted) or staining for CD8α (Fig. 6 A). Both analyses indicated that a small fraction of medullary DCs expressed CD70. CD70 localized primarily intracellularly, consistent with its storage in late endosomal MHC class II compartments in mature peripheral DCs (Keller et al., 2007). CD70 was not readily detectable on thymic DCs by ex vivo flow cytometry (not depicted), and therefore, we confirmed its expression at the transcriptional level. *Cd70* gene transcription was analyzed by RT-PCR on thymic CD11c$^+$B220^{lo} DC purified from WT and *Cd70*$^{Cre/Cre}$ mice on the basis of CD8α and SIRPα expression (Fig. 6 B). *Cd70* mRNA was

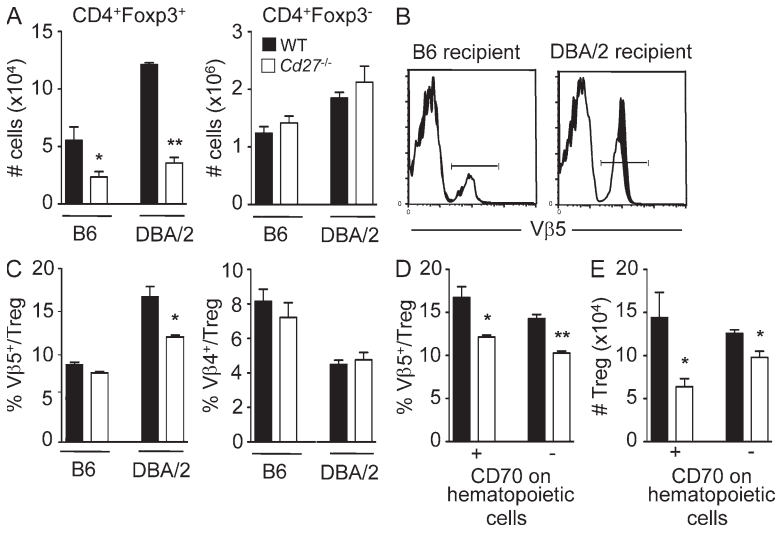

Figure 5. CD27–CD70 co-stimulation promotes positive selection of sAg-specific T_{reg} cells. (A–C) Mixed BM chimeras were established by lethally irradiating recipient DBA/2 or B6 mice and reconstituting them the next day with a 1:1 mixture of T cell–depleted CD45.1+ WT and CD45.2+ *Cd27*$^{-/-}$ BM cells. 8 wk later, thymocytes were analyzed by flow cytometry for CD4 and Foxp3 expression within the CD45.1+ and CD45.1− populations. (A) Bar diagrams depict the numbers of WT (CD45.1+) or *Cd27*$^{-/-}$ (CD45.1−) CD4+Foxp3+ or CD4+Foxp3− thymocytes in B6 or DBA/2 recipients. (B) Representative histograms of Vβ5 expression in gated WT and *Cd27*$^{-/-}$ CD4+Foxp3+ thymocytes from B6 or DBA/2 recipient mice. (C) Percentages of Vβ5+ and Vβ4+ cells among WT and *Cd27*$^{-/-}$ CD4+Foxp3+ thymocytes in B6 and DBA/2 mice. Data are derived from one experiment with three to four mice per group and are representative of two independent experiments. (D and E) Mixed BM chimeras were established by lethally irradiating recipient DBA/2 mice and reconstituting them the next day with a 1:1 mixture of WT (*Cd27*$^{+/+}$) and *Cd27*$^{-/-}$ BM cells or *Cd27*$^{+/+}$;*Cd70*$^{Cre/Cre}$ and *Cd27*$^{-/-}$;*Cd70*$^{Cre/Cre}$ BM cells, resulting in expression of CD70 on hematopoietic cells or not, as indicated. After 8 wk, the development of T_{reg} cells was analyzed as outlined for A–C. Bar diagrams indicate the percentage of Vβ5+ T_{reg} cells (D) and the absolute number of T_{reg} cells (E) within WT and *Cd27*$^{-/-}$ cells or *Cd70*$^{Cre/Cre}$ and *Cd27*$^{-/-}$;*Cd70*$^{Cre/Cre}$ cells. Data are from two independent experiments with six to eight mice per group. All data were analyzed by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). Error bars are SEM.

detected in both CD8α+ and SIRPα+ WT DCs but not in *Cd70*$^{Cre/Cre}$ DCs (Fig. 6 C). To specify which thymic DCs may offer CD70 to promote T_{reg} cell development, we performed in vitro experiments. CD8α+ and SIRPα+ CD11c^{hi}B220^{lo} cDCs and CD11c^{int}B220^{int} plasmacytoid DCs (pDCs) were purified from WT and *Cd70*$^{Cre/Cre}$ thymus and co-cultured with purified WT CD4+CD25− thymocytes. After 5 d, CD4+ cells were analyzed for Foxp3 and CD25 expression. In cultures with WT SIRPα+ and CD8α+ cDCs, but not in cultures with pDCs, T_{reg} cell development was observed after 5 d (Fig. 6, D and E), consistent with published data (Proietto et al., 2008). Importantly, CD70 deficiency significantly reduced the capacity of CD8α+ cDCs to induce T_{reg} cells, whereas *Cd70*$^{Cre/Cre}$ SIRPα+ cDCs could induce T_{reg} cells equally well as their WT counterparts (Fig. 6, D and E). These data suggest that CD8α+ cDCs in the thymic medulla, which are thymus-resident cells (Li et al., 2009), present CD70 and promote the development of T_{reg} cells.

CD70 on DCs as well as CD70 on epithelial cells supports T_{reg} cell development in vivo

To test whether CD70 on hematopoietic cells supports T_{reg} cell development in vivo, we created BM chimeras. *Cd70*$^{Cre/Cre}$ mice were irradiated and reconstituted with a 1:1 mixture of WT and *Cd27*$^{-/-}$ BM. In this setting, CD70 was present on hematopoietic cells including DCs but not on epithelial cells of the thymus. After 8 wk, the number of CD4+Foxp3+ and CD4+Foxp3− cells was determined in thymus and spleen. Although the number of CD4+Foxp3− cells of WT and *Cd27*$^{-/-}$ origin was similar, CD4+Foxp3+ cells were more efficiently generated from WT donor cells than from *Cd27*$^{-/-}$ donor cells (Fig. 7 A). This indicates that interaction between CD27 on T_{reg} cell precursors and CD70 on a BM-derived cell type (most likely the DCs identified above) contributes to T_{reg} cell development.

To test the relative contributions of CD70 on BM-derived versus epithelial cells in T_{reg} cell development, we performed a comparative series of BM chimera experiments. As a control, WT BM was transferred into WT mice. To exclude a role for CD70 on BM-derived cells, *Cd70*$^{Cre/Cre}$ BM was transferred into WT mice. To exclude a role for CD70 on epithelial cells, WT BM was transferred into *Cd70*$^{Cre/Cre}$ mice. The fourth setting, in which *Cd70*$^{Cre/Cre}$ BM was introduced into *Cd70*$^{Cre/Cre}$ mice, excluded CD70 on both epithelial and hematopoietic cells. Analysis of the thymus and spleen after reconstitution revealed that elimination of CD70 on either BM-derived or epithelial cells each slightly reduced T_{reg} cell output but not to a significant extent (Fig. 7 B). When CD70 was lacking on both cell types, however, T_{reg} cell development was significantly impaired. These data indicate that CD70 on both DCs and epithelial cells contributes to T_{reg} cell development by engaging CD27 on developing T_{reg} cells.

DISCUSSION

CD4+ T cell precursors with specificity for self-antigens undergo selection processes in the thymus that prevent autoimmunity. They can be negatively selected (clonally deleted), which is the basis of recessive tolerance. Alternatively, they can be positively selected into the T_{reg} cell lineage that mediates tolerance by suppressing the remaining autoreactive T cells in the periphery. Despite the expression of various co-stimulatory molecules in the thymic medulla, conventional CD4+ and CD8+ T cells appear to develop normally in their absence, and the consequence of defective co-stimulation is only apparent in peripheral T cell responses. In contrast, thymic T_{reg} cell development is quantitatively impaired by lack of co-stimulatory

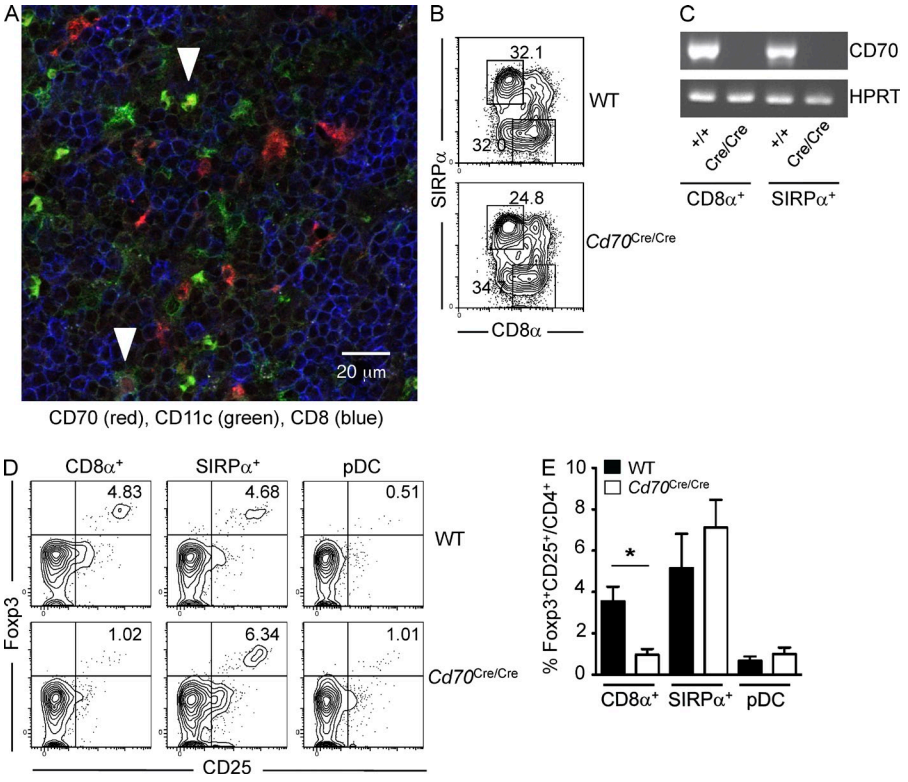

Figure 6. CD70 on CD8α⁺ thymic cDCs contributes to T_{reg} cell development. (A) Thymic sections were stained sequentially using anti-CD70 mAb, Alexa Fluor 568–conjugated anti-IgG, FITC-conjugated anti-CD11c mAb, and APC-conjugated anti-CD8α mAb. Arrowheads indicate cells with double staining for CD70 and CD11c. (B and C) DCs were enriched from WT or *Cd70*$^{Cre/Cre}$ thymi and analyzed by flow cytometry. (B) Shown are representative plots of CD8α versus SIRPα expression on gated CD11c^{+}B220^{lo} DCs. (C) CD8α^{+} and SIRPα^{+} DC subsets were purified from WT and *Cd70*$^{Cre/Cre}$ thymi, RNA was isolated, and RT-PCR was performed on cDNA. Shown are PCR products from cDNA samples amplified with primers specific for CD70 and HPRT. (D) CD8α^{+} and SIRPα^{+} CD11c^{+}B220$^{low/-}$ cDCs and CD11c^{+}B220^{+} pDCs were purified from the pooled thymi of two WT or *Cd70*$^{Cre/Cre}$ mice. The purified DC subsets were co-cultured for 5 d with purified CD4^{+}CD25^{-} WT thymocytes. After culture, gated CD4^{+} cells were analyzed for expression of Foxp3 and CD25. Shown is one representative plot of Foxp3 and CD25 expression on gated CD4^{+} cells in the presence of the indicated DC population. (E) Data from D, expressed as the percentage of Foxp3^{+}CD25^{+} cells among gated CD4^{+} cells in the presence of the indicated DC populations. Results are from four to five separate wells over two independent experiments. The Mann–Whitney U rank sum test was used to analyze results (*, $P < 0.05$). Error bars are SEM.

input, as shown previously for co-stimulation by CD28 (Kumanogoh et al., 2001; Tai et al., 2005; Proietto et al., 2008; Spence and Green, 2008) and in this study for co-stimulation by CD27.

Although cortical T_{reg} cell development has been described (Liston et al., 2008), it is generally accepted that T_{reg} cell precursors find the optimal signals to differentiate into mature T_{reg} cells in the thymic medulla. The niche for T_{reg} cell development is easily saturable because T_{reg} cell precursors most efficiently develop into mature T_{reg} cells when they are present at a low frequency (Bautista et al., 2009). Thus, even within the thymic medulla, the possibility for a T_{reg} cell precursor to become a mature T_{reg} cell is limited. It is becoming increasingly clear in mouse and human that subtle interplay between mTECs and DCs creates the appropriate niches for deletion of autoreactive thymocytes and induction of T_{reg} cells. Aire-expressing mTECs that have been implicated in T_{reg} cell selection (Aschenbrenner et al., 2007) can directly present a great variety of tissue-restricted antigens, but thymic DCs can also cross-present these (Gallegos and Bevan, 2004). In the human thymus, clusters of terminally differentiated mTECs called Hassall's corpuscles produce thymic stromal lymphopoietin (TSLP), which activates DCs to express high levels of CD80 and CD86 and induce T_{reg} cell development (Watanabe et al., 2005). More recently, XCL-1 production by mTECs in mice was shown to attract XCR1^{+} thymic DCs into clusters within the thymic medulla, and this XCL-1 production by mTECs was shown to be Aire dependent. Mice lacking XCL-1 or Aire displayed a twofold reduction in T_{reg} cell numbers in the thymus, strongly suggesting that the clusters of mTECs and DCs are important for T_{reg} cell development (Lei et al., 2011).

Our data strongly support the scenario that mTECs and DCs in the thymic medulla jointly create the appropriate environment for T_{reg} cell development and implicate CD70 on both of these cell types in T_{reg} cell development. CD70 was exclusively found in the thymic medulla, with evident expression on a large proportion of mTECs. Most, if not all Aire^{+} mTECs expressed CD70, but Aire^{-} mTECs also expressed CD70. It is known that mTECs develop from intrathymic progenitor cells and gradually mature to terminally differentiated cells that express high levels of Aire, CD80, and MHC class II (Gray et al., 2007; Yano et al., 2008). Intermediate developmental stages express CD80 in the absence of Aire, and therefore CD70^{+}Aire^{-} mTECs are most likely the precursors of CD70^{+}Aire^{+} mTECs. CD70 expression on thymic DCs was less evident, but incidental DCs with primarily intracellular CD70 expression were detected by CLSM. CD70 expression on the surface of thymic DCs was not detectable by flow cytometry. This is perhaps not surprising because CD70 expression in DCs is tightly regulated, both at the transcriptional

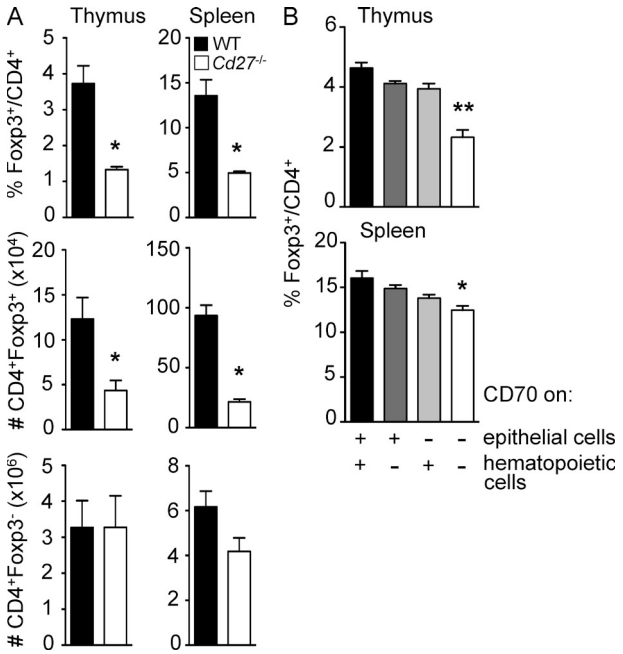

Figure 7. CD70 expression by both epithelial and hematopoietic cells is required for T_{reg} cell development. (A) CD45.2$^+$ *Cd70*$^{Cre/Cre}$ mice were lethally irradiated and reconstituted with a 1:1 mixture of BM cells from CD45.1$^+$ WT and CD45.2$^+$ *Cd27*$^{-/-}$ mice. 8 wk later, mice were analyzed for the presence of CD4$^+$Foxp3$^+$ T_{reg} cells and CD4$^+$Foxp3$^-$ conventional T cells in thymus and spleen by flow cytometry. Bar diagrams represent the percentage of T_{reg} cells within the total CD4$^+$ population and the absolute numbers of T_{reg} cells and conventional CD4$^+$ T cells for WT and *Cd27*$^{-/-}$ cells. Results are from one experiment with four mice per group and are representative of two independent experiments. The Mann–Whitney U rank sum test was used to calculate significance between groups (*, $P < 0.05$). (B) WT and *Cd70*$^{Cre/Cre}$ mice were lethally irradiated and reconstituted with BM cells from WT or *Cd70*$^{Cre/Cre}$ mice. 8 wk later, mice were analyzed for the presence of Foxp3$^+$ T_{reg} cells among total CD4$^+$CD8$^-$ cells in thymus and spleen by flow cytometry. Bar diagrams represent the percentage of T_{reg} cells within the total CD4$^+$ population for the indicated combinations. Results are pooled from two independent experiments with three to eight mice per group, except for the setting with *Cd70*$^{Cre/Cre}$ BM into *Cd70*$^{Cre/Cre}$ recipients which was included in only one experiment. For the comparison of multiple groups, a one-way analysis of variance followed by a post-Dunn's test was used to assess significance of the difference with the WT control setting (*, $P < 0.05$; **, $P < 0.01$). Error bars are SEM.

level and at the level of intracellular transport (Keller et al., 2007). CD70 is stored in MHC class II compartments and is brought to the cell surface in the immune synapse upon cognate interaction between T cells and DCs. Possibly, it is regulated in a similar manner within the thymus and only brought to the cell surface when developing T_{reg} cell contact the appropriate DCs.

T_{reg} cell generation in vitro relied on CD70 in co-cultures with CD8α^+ cDCs but not with SIRPα^+ cDCs. CD8α^+ cDCs express high levels of XCR1 (Dorner et al., 2009), which may serve to attract DCs to Aire$^+$ mTECs (Lei et al., 2011). This fits in with our hypothesis that mTECs and DCs form niches to promote T_{reg} cell development, in which CD80/86 and CD70 play an important role. However, SIRPα^+ cDCs could also induce T_{reg} cell development in vitro, in a CD70-independent manner. SIRPα^+ cDCs are a circulatory population and express higher levels of CD80, CD86, and MHC II than thymus-resident CD8α^+ cDCs (Proietto et al., 2008; Li et al., 2009; Lei et al., 2011). The ability of SIRPα^+ cDCs to promote T_{reg} cell development independently of CD70 may therefore be caused by their high expression of other co-stimulatory molecules. Alternatively, SIRPα^+ cDCs may present different autoantigens in MHC class II than CD8α^+ cDCs and positively select a different pool of T_{reg} cell precursors.

Interestingly, it was previously demonstrated that like CD70, CD80 and/or CD86 on both TECs and DCs contributes to T_{reg} cell development (Proietto et al., 2008). Furthermore, CD40 on epithelial cells or hematopoietic cells can also contribute to T_{reg} cell development (Spence and Green, 2008). It is likely that CD40 engagement promotes T_{reg} cell development in part by inducing the expression of CD70 on the surface of mTECs or thymic DCs because it does so on DCs in lymphoid organs (Tesselaar et al., 2003; Sanchez et al., 2007).

We found that CD28 co-stimulation promoted T_{reg} cell development in the absence of CD27. During the priming of naive T cells, CD28 and CD27 also perform complementary functions. CD28 amplifies the TCR signal and lowers the threshold for entry into cell cycle (Acuto and Michel, 2003), whereas CD27 provides survival signals throughout successive cell cycles (Hendriks et al., 2003). During T_{reg} cell development, however, CD28 does not merely act as a signal amplifier for the TCR. It contributes to Foxp3 induction over a range of TCR affinities, rather than supporting the development of cells in which Foxp3 is already switched on (Tai et al., 2005). Specifically, CD28 signaling via the Lck tyrosine kinase, but not via the PKB survival pathway, drives pre-T_{reg} cell development to the next stage. In contrast, CD27 deficiency did not impact pre-T_{reg} cell numbers, Foxp3 induction, or programming of suppressive function in T_{reg} cells. Rather, CD27 signaling counteracted the mitochondrial apoptosis pathway in developing T_{reg} cells, as demonstrated by the increased exposure of phosphatidylserine and increased expression of proapoptotic Bcl-2 molecules in CD27-deficient T_{reg} cells, as well as the capacity of ectopic Bcl-2 to rescue the development of CD27-deficient T_{reg} cells. Several lines of evidence argue that T_{reg} cells rely on survival signals for their thymic development. First, CD4$^+$CD25$^+$ thymocytes are more resistant to negative selection than CD4$^+$CD25$^-$ thymocytes (van Santen et al., 2004; Taylor et al., 2007). Furthermore, deficiency in the pro-apoptotic Bcl-2 protein Bim leads to a large increase specifically in T_{reg} cell numbers (Ouyang et al., 2010). Our data lend further support to the idea that T_{reg} cell development relies on signals that inhibit the intrinsic apoptosis pathway and implicate the CD27–CD70 pathway in providing such signals, as it does in peripheral T cells (Peperzak et al., 2010a).

The question remains as to why CD27 signals enhance the numerical output of T_{reg} cells without affecting that of

conventional αβ T cells. Thymic γδ T cells are the only other cells that have been shown to benefit from CD70 in the thymus (Ribot et al., 2009). Both T_{reg} and γδ T cells express CD27 at approximately twofold higher levels than conventional αβ T cells (not depicted), and perhaps they profit more from CD27–CD70 co-stimulation for this reason. Higher expression of CD27 on γδ T cells and T_{reg} cells may be explained by the higher-affinity TCR signals they receive in the thymus (Ciofani and Zúñiga-Pflücker, 2010).

Another critical factor in T_{reg} cell development is signaling by the common γ chain of cytokine receptors, as provided largely through the IL-2 receptor, which stabilizes Foxp3 expression via STAT-5 signaling (Burchill et al., 2007, 2008). IL-2 is provided to T_{reg} cell precursors in a paracrine fashion by other thymocytes. Accordingly, the co-transfer of WT precursors can correct the inability of IL-2–deficient precursors to become T_{reg} cells (Fontenot et al., 2005b; Tai et al., 2005). Importantly, CD27 as well as CD28 contribute to T_{reg} cell development independently of paracrine IL-2 because co-transfer of WT donor cells together with CD27- or CD28-deficient donor cells in BM chimeras did not correct their defect in T_{reg} cell generation (Tai et al., 2005; this study).

T_{reg} cell numbers in *Cd27*$^{-/-}$ or *Cd70*$^{Cre/Cre}$ mice or chimeras thereof are reduced to maximally about half of control numbers in the periphery (Fig. 1 B). We have not observed signs of spontaneous autoimmunity in these mice. The same applies to *Cd28*$^{-/-}$ mice, in which T_{reg} cell numbers are reduced to about one third of control (Tai et al., 2005). In *Cd28*$^{-/-}$ mice, there are also no reported signs of autoimmunity. Therefore, it is possible that an ∼50–70% reduction in T_{reg} cell numbers does not affect self-tolerance. Alternatively though, self-tolerance may not be affected in these mice because conventional T cells also lack CD27 or CD28 and therefore have a higher threshold for responsiveness.

It was recently shown that immune surveillance to tumors with CD70$^+$ infiltrating lymphocytes was greater in *Cd27*$^{-/-}$ mice than in WT mice. This was caused by impaired expansion and survival of peripheral T_{reg} cells (Claus et al., 2012). Therefore, the regulation of T_{reg} cell numbers via the CD27–CD70 pathway can play an important physiological role. Furthermore, we have recently shown that *Cd27*$^{-/-}$ and *Cd70*$^{Cre/Cre}$ mice develop more severe EAE. The CD27–CD70 pathway functionally disabled T-helper 17 cells that play a dominant role in this disease (Coquet et al., 2013). Possibly, apart from increased numbers of T-helper 17 cells, reduced expansion of peripheral myelin-specific T_{reg} cells also contributed to enhanced EAE in *Cd27*$^{-/-}$ and *Cd70*$^{Cre/Cre}$ mice. The use of MHC II tetramers or TCR sequencing technology to detect antigen-specific T_{reg} cells in cancer or autoimmune models may help to shed light on the role of the CD27–CD70 pathway in peripheral T_{reg} cell responses.

In conclusion, although T_{reg} cell development abides by many of the rules applicable to conventional αβ T cell development, T_{reg} cells are unique in their requirement for co-stimulation and IL-2 in dedicated niches in the thymic medulla. Our data demonstrate that the CD28 and CD27 co-stimulatory pathways make specific and nonredundant contributions to T_{reg} cell development. TCR and CD28 signals kick start the developmental process by induction of Foxp3 expression. Thereafter, IL-2/IL-2 receptor signaling stabilizes T_{reg} cell differentiation, whereas CD27–CD70 co-stimulation rescues developing T_{reg} cells from apoptosis and thereby allows them to take part in the peripheral T_{reg} cell population.

MATERIALS AND METHODS

Mice. B6 WT, *Cd27*$^{-/-}$, *Cd70*$^{Cre/Cre}$, DBA/2 mice, and congenic CD45.1 mice were bred at the animal facilities of the Netherlands Cancer Institute (NKI) and Instituto de Medicina Molecular (IMM). *Cd27*$^{-/-}$ mice were made on a 129J/Ola background and backcrossed to B6 for eight generations (Hendriks et al., 2000). *Cd70*$^{Cre/Cre}$ mice were generated as described below. All animal experiments were performed according to national and institutional guidelines and were approved by the respective experimental animal committees of the NKI and IMM.

Generation of *Cd70*$^{Cre/Cre}$ mice. *Cd70*$^{Cre/Cre}$ mice are deficient for CD70 expression because exon I of the *Cd70* locus has been replaced by coding sequences of the Cre recombinase. The genetic modification was performed in E14 (129P2/Ola) embryonic stem cells (Coquet et al., 2013). Using speed congenics, germline competent chimeras were crossed for five generations onto a B6 background (>98% B6 contribution) and subsequently maintained as inbred strain. Note that we have thus far no indication that the Cre gene is efficiently expressed under control of the endogenous *Cd70* gene promoter.

BM chimaeras. Mice were irradiated with two doses of 5 Gy, 3 h apart, and the next day injected intravenously with a total of 10^7 whole BM cells in 200 µl PBS. The hematopoietic compartment was allowed to reconstitute for 8 wk before organs were harvested for analysis by flow cytometry. In Fig. 5 (D and E), CD27 was used as a marker for T_{reg} cell generation from *Cd70*$^{Cre/Cre}$ donor cells because both *Cd70*$^{Cre/Cre}$ and *Cd27*$^{-/-}$;*Cd70*$^{Cre/Cre}$ donor cells were of CD45.2$^+$ origin. In the *Bcl2* transduction experiments, c-Kit$^+$ BM cells were enriched by magnetic cell sorting (Miltenyi Biotech) and infected overnight with pMig.IRES-GFP retroviral empty vector or containing *Bcl2* (cloned from Addgene plasmid no. 8750) in the presence of 0.8 mg/ml polybrene (Sigma-Aldrich). c-Kit$^+$GFP$^+$ cells were sorted by flow cytometry, and appropriate mixes of a total 10^5 cells were injected intravenously in irradiated hosts.

CD80/CD86 in vivo neutralization. *Cd27*$^{-/-}$ mice were injected i.p. every 2 d, for 2 wk, with 100 µg of each anti-CD80 (16-10A1) and anti-CD86 (GL1) mAb or corresponding isotype controls.

Single cell isolation. To isolate lymphocytes from thymus and spleen, organs were passed through 100-µM nylon mesh (BD), and red blood cells were lysed in 0.14 M NaCl and 0.017 M Tris-HCl, pH 7.2, for 1 min at room temperature (rT). To isolate BM, femurs were removed from mice and flushed with PBS using a 25-G needle. Red blood cell lysis was subsequently performed for 1 min at rT. Cells from spleen, thymus, or BM were resuspended in PBS containing 2% bovine serum albumin and counted on a CASY cell counter (Schärfe).

Antibodies and flow cytometry. Cells were stained with the following fluorochrome-conjugated mAbs: anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD25 (PC-61), anti-CD44 (IM7), anti-CD11c (N418), anti-CD45RA (RA3-6B2), anti-Vβ4 (KT4), and anti-Vβ5 (MR9-4) from BD; and anti-CD45.2 (104), anti-CD27 (LG.3A10), anti-SIRPα (P84), and anti-Foxp3 (FJK-16s) from eBioscience. Intracellular staining for Foxp3 was performed using the staining buffer set from eBioscience. Cells were analyzed on Cyan (Dako) or FACSCanto (BD) flow cytometers. Cells were purified by flow cytometric sorting on either a FACSAria (BD) or MoFlow (Dako). Sorted cells were always at least 98% pure. Data were analyzed using FlowJo software (Tree Star).

Thymic DC isolation. Mouse thymus was harvested and roughly diced into small pieces using scissors in 25 ml IMDM containing 1 mg/ml collagenase type IV (Worthington) and 25 µg/ml DNase (Sigma-Aldrich) and incubated at 37°C for 1 h. Liberated cells were filtered through 100-µM nylon mesh and incubated with CD11c microbeads (Miltenyi Biotech) for 30 min before being run through a MACS column (Miltenyi Biotech). Enriched thymic DCs were then stained for expression of CD11c, CD45RA, CD8α, and SIRPα and sorted by flow cytometry. RNA was isolated from purified thymic DCs using the RNeasy kit (QIAGEN), and cDNA was transcribed using random hexamers. Primers to detect CD70 and HPRT message were previously described (Tesselaar et al., 2003).

Cell culture. Cells were cultured in either IMDM or RPMI containing 8% FCS, penicillin, streptomycin, and β-mercaptoethanol (tissue culture medium). For co-cultures of thymic DCs and T cells, 10^4 sorted thymic DCs were cultured with 3×10^4 sorted thymic $CD4^+CD8^-CD25^-$ cells in 96-well U-bottom plates for 5 d. Thereafter, cells were harvested and stained for the surface markers CD4 and CD25 before being stained intracellularly for Foxp3 and analyzed by flow cytometry. For T_{reg} cell suppressor assays, $CD4^+CD25^+$ cells (T_{reg} cells) were sorted from WT or *Cd27*$^{-/-}$ mice and $CD4^+CD25^-$ responder T cells were sorted from WT mouse spleen. U-bottom 96-well plates were coated with 2 µg/ml anti-CD3 mAb 145.2C11 for 2 h at 37°C. Responder T cells were labeled with 5 µM CFSE in PBS supplemented with 2% FCS for 5 min at 37°C. T_{reg} and responder T cells were cultured in tissue culture medium at various ratios as indicated in coated U-bottom plates for 3 d in the presence of irradiated splenocytes. After 3 d, cells were harvested and analyzed for dilution of CFSE by flow cytometry.

FTOCs. FTOCs were maintained at 37°C, 5% CO_2 in FTOC medium, which is RPMI-1640 with 10% FCS (STEMCELL Technologies), 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U penicillin, and 100 µg/ml streptomycin. B6 fetal thymic lobes at E15 were cultured for 14–16 d in FTOC medium on Nuclepore filters (Whatman) and then analyzed by flow cytometry. Cultures were provided with fresh FTOC medium at day 7. For CD27 stimulation, a fusion protein of the extracellular domain of mouse CD70 and the Fc portion of human IgG1 (FcCD70; Peperzak et al., 2010b) or human IgG1 control antibody was added to the cultures. To disrupt CD27–CD70 interactions, the anti-CD70 mAb FR70 (provided by H. Yagita, Juntendo University School of Medicine, Tokyo, Japan; Oshima et al., 1998) or a control rat IgG2b isotype control (eBioscience) was added to culture. Cultures containing antibodies were rested for 24 h in fresh medium before analysis.

CLSM. Mouse thymus was embedded in Cryo-Block medium (LTI) and frozen at −80°C, after which sections were made, fixed in acetone, air dried, and stored at −20°C. Sections were rehydrated in PBS for 30 min at rT, immersed in PBS, 5% BSA for 30 min at rT, and stained with antibodies in PBS, 1% BSA for 1 h at rT. Primary antibodies used were rat anti–mouse CD70 mAb FR70 (homemade purified Ig), rat anti–mouse mAb ER-TR5 (provided by P.J. Leenen, Erasmus University, Rotterdam, Netherlands; Van Vliet et al., 1984), rabbit anti–mouse Keratin-5 polyclonal PRB-160P (Covance), Alexa Fluor 488–conjugated rat anti–mouse Aire mAb 5H12 (eBioscience), FITC-conjugated Armenian hamster anti–mouse CD11c mAb N418 (eBioscience), and Allophycocyanin-conjugated rat anti–mouse CD8α mAb 53-6.7 (BD). Second step antibodies were Alexa Fluor 488– or Alexa Fluor 568–conjugated anti–rat IgG or anti–rabbit IgG (Invitrogen). In case two rat mAbs were used sequentially, sections were incubated with normal rat serum as a blocking step in between. After antibody staining, sections were incubated as indicated with DAPI (Sigma-Aldrich) at 0.15 µg/ml in PBS for 20 min at rT. Stained sections were mounted using Vectashield (Vector Laboratories) or FLUORO-GEL with TES buffer (Electron Microscopy Sciences) and observed under a TCS NT CLSM (Leica), using an HCX PL APO CS objective lens with 40× magnification and 1.3 aperture. Signals were acquired with LAS AF Lite software (Leica), and Fiji software was used for image processing.

Multiplex ligation-dependent probe amplification (MLPA). Thymic $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were sorted, and RNA was isolated from pelleted cells using the RNeasy mini kit (QIAGEN). 5 ng of RNA was used for MLPA (MRC-Holland) using the RM002-B1 mouse apoptosis probe mix as previously described (Eldering et al., 2003). Three separate experiments were performed, and data for *Cd27*$^{-/-}$ cells were normalized to WT levels in each experiment.

Online supplemental material. Fig. S1 shows flow cytometry gating schemes for pre-T_{reg} cells, CD80/CD86 blockade, CD69 and HSA staining on pre-T_{reg} cells, and Annexin-V staining. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20112061/DC1.

We thank H. Veiga-Fernandes, R.G. Domingues, I. Barbosa, and S. Madeira (IMM) for help with the *Bcl2* transduction experiments, H. Yagita for FR70 antibody, P.J. Leenen for the ER-TR5 antibody, E.A.M. Veraar for FcCD70 production, and the staff of the animal and flow cytometry facilities of our institutes for expert technical assistance.

This work was supported by grants NKI 2004-3087 and NKI 2008-2023 from the Dutch Cancer Society to J. Borst, European Molecular Biology Organization long-term fellowships to J.M. Coquet and J.C. Ribot, a Rubicon (The Netherlands Organisation for Scientific Research [NWO]) fellowship to J.M. Coquet, and a Fundação para a Ciência e Tecnologia project grant (PTDC/SAU-MII/104158/2008) and a European Research Council starting grant (StG260352) to B. Silva-Santos. J.F. Neves is funded by the Fundação para a Ciência e Technologia of Portugal; D.J. Pennington is funded by the Wellcome Trust.

The authors declare no commercial affiliations or competing financial interests.

Submitted: 27 September 2011
Accepted: 15 February 2013

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