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 (Treg cells) are largely autoreactive yet escape clonal deletion in the thymus. We demonstrate here that CD27–CD70 co-stimulation in the thymus rescues developing Treg cells from apoptosis and thereby promotes Treg cell generation. Genetic ablation of CD27 or its ligand CD70 reduced Treg 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-Treg cell generation, Foxp3 induction, or mature Treg cell function. Rather, CD27 signaling enhanced positive selection of Treg cells within the thymus in a cell-intrinsically manner. CD27 signals promoted the survival of thymic Treg 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 Treg 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 Treg cell development. Our data suggest that mTECs and DCs form dedicated niches in the thymic medulla, in which CD27–CD70 co-stimulation rescues developing Treg cells from apoptosis, subsequent to Foxp3 induction by TCR and CD28 signals.

To achieve immunological tolerance, self-reactive T cells are either eliminated by clonal deletion in the thymus or actively suppressed by regulatory T cells (Treg cells) in the periphery. The best characterized Treg cells are CD4⁺ cells that express Foxp3 and CD25 (Sakaguchi et al., 2008). These Treg 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 Treg 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 Treg cells for immunological tolerance (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001).

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.

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The discovery of T<sup>reg</sup> cells was based on the observation that neonatal thymectomy in mice led to severe autoimmunity, which could be prevented by transfer of CD4<sup>+</sup>CD25<sup>+</sup> T cells (Sakaguchi et al., 1995). T<sub>reg</sub> cells develop in the thymus in the first weeks after birth, after the peripheral lymphoid organs have been populated with conventional CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fontenot et al., 2005a). T<sub>reg</sub> 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<sub>reg</sub> cells arise from CD4<sup>+</sup> thymocytes (Fontenot et al., 2003). Foxp3 expression can also be induced in mature, conventional CD4<sup>+</sup> 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<sup>+</sup>CD8<sup>+</sup> stage on MHC class I- and MHC class II-expressing epithelial cells in the thymic cortex. The resulting CD4<sup>+</sup> and CD8<sup>+</sup> (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 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<sup>+</sup> T cells, T<sub>reg</sub> 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<sub>reg</sub> cells can somehow escape negative selection in the thymus. Indeed, it has been observed that certain CD4<sup>+</sup> thymocytes acquire Foxp3 expression upon contact with Aire-expressing mTECs, survive selection against autoreactivity, and exit to peripheral lymphoid organs as CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells (Aschenbrenner et al., 2007).

Foxp3 induction relies on TCRαβ signaling that results from interaction with MHC class II<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> 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<sup>+</sup>CD8<sup>+</sup> conventional T cell precursors (van Santen et al., 2004; Taylor et al., 2007). Moreover, Foxp3 induction and thymic T<sub>reg</sub> cell development are highly dependent on CD28 co-stimulation (Tai et al., 2005), whereas CD28 signaling promotes the deletion of autoreactive CD4<sup>+</sup> thymocytes (McKean et al., 2001). The question has been raised therefore which signals enable T<sub>reg</sub> 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; Tessleraa 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<sup>+</sup>CD8<sup>+</sup> thymocytes (Martorell et al., 1990). In mature peripheral T cells, CD27–CD70 co-stimulation promotes CD8<sup>+</sup> 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<sup>+</sup>CD8<sup>+</sup> thymocyte compartment (Gravestein et al., 1996). However, development of CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>β</sub> T cells proved normal in Cd27<sup>-/-</sup> 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<sub>reg</sub> 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<sub>reg</sub> cell development. CD27–CD70 interactions in the thymus enhanced positive selection of T<sub>reg</sub> cells and rescued developing T<sub>reg</sub> 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<sub>reg</sub> cells.

**RESULTS**

The CD27–CD70 pathway is important for the development of T<sub>reg</sub> 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<sup>−/−</sup>) 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<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells in these mice (Fig. 1, A and B). In contrast, numbers of conventional, Foxp3<sup>−</sup>CD4<sup>+</sup> 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<sup>+</sup>CD8<sup>+</sup> populations in thymus and spleen were comparable between all
genotypes (Fig. 1, B and C). Thus, in mice lacking CD27 or CD70, generation of T<sub>reg</sub> cells was specifically impaired, whereas the development of conventional T cells appeared normal.

During T<sub>reg</sub> cell development in the thymus, T<sub>reg</sub> 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<sub>reg</sub> cell development (Burchill et al., 2008; Lio and Hsieh, 2008). Thymic CD4<sup>+</sup>Foxp3<sup>+</sup>CD25<sup>+</sup> cells are referred to as pre-T<sub>reg</sub> cells and include a large proportion of cells destined to become mature T<sub>reg</sub> cells (Lio and Hsieh, 2008). CD27 signals were not required to generate this cell population, as the frequency of pre-T<sub>reg</sub> cells was normal in the thymus of Cd27<sup>−/−</sup> mice (Fig. 1 D and Fig. S1 A). Generation of pre-T<sub>reg</sub> cells as well as mature T<sub>reg</sub> 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<sub>reg</sub> cell development in the absence of CD27. For this purpose, Cd27<sup>−/−</sup> mice were administered neutralizing antibodies to CD80 and CD86 over a period of 10 d, after which T<sub>reg</sub> cells...
Figure 2. CD27–CD70 co-stimulation promotes Treg 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, Treg 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+ Treg 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 sum 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+ Treg 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 sum 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 Treg cells among total CD4+ cells and the absolute number of conventional CD4+Foxp3− T cells and CD4+Foxp3+ Treg 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).
(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 Treg 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 Treg cell development but not required for development of conventional CD4+ T cells.

CD27 signaling prevents apoptosis of Treg cells but does not affect their functional differentiation

To determine whether CD27 co-stimulation affected the functional programming of Treg cells, their suppressive activity was assessed. Treg 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−/− Treg 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\textsubscript{reg} cells, as assessed in this in vitro assay. Furthermore, Foxp3 was expressed at a similar level in T\textsubscript{reg} cells from WT, C\textsubscript{d27}^+/−, and C\textsubscript{d70}^+/− 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\textsubscript{reg} cells.

We next determined whether CD27–CD70 co-stimulation affected the development and survival of T\textsubscript{reg} cells. For this purpose, pre-T\textsubscript{reg} cells were purified from the thymus of WT and C\textsubscript{d27}^+/− mice on basis of a CD69\textsuperscript{+}HSA\textsuperscript{−}CD25\textsuperscript{+}CD4\textsuperscript{−}CD8\textsuperscript{−} 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\textsubscript{reg} cells hardly gave rise to Foxp3\textsuperscript{+} cells (Fig. 3 B) and most cells died (Fig. 3 C). In the presence of IL-2, pre-T\textsubscript{reg} cells developed into Foxp3\textsuperscript{+} T\textsubscript{reg} cells, with C\textsubscript{d27}^−/− precursors giving rise to significantly less Foxp3\textsuperscript{+} cells than WT precursors (Fig. 3 B). Moreover, significantly more Annexin-V\textsuperscript{+} apoptotic cells were observed in the C\textsubscript{d27}^+/− cultures as compared with the WT cultures (Fig. 3 C), suggesting that CD27 provided important survival signals to developing T\textsubscript{reg} cells. Direct ex vivo analysis of Annexin-V binding also indicated that CD4\textsuperscript{+}CD25\textsuperscript{+} cells from C\textsubscript{d27}^−/− mice underwent more apoptosis than their WT counterparts (Fig. 3 D and Fig. S1 D). This effect was selective for T\textsubscript{reg} cells because there was no difference in the proportion of apoptotic cells within the conventional CD4\textsuperscript{+}CD8\textsuperscript{−}CD25\textsuperscript{−} or CD8\textsuperscript{+}CD4\textsuperscript{−} populations of WT and C\textsubscript{d27}^+/− 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 C\textsubscript{d27}^+/− cells. 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 C\textsubscript{d27}^−/− CD25\textsuperscript{+}CD4\textsuperscript{+} thymocytes, CD70 showed a similar trend (Fig. 3 E). These proapoptotic molecules were not differentially expressed in CD25\textsuperscript{−}CD4\textsuperscript{+} thymocytes from WT or C\textsubscript{d27}^+/− mice (Fig. 3 E), suggesting that CD27 specifically counteracts the mitochondrial apoptosis pathway in developing T\textsubscript{reg} cells. To further assess whether CD27 signaling promoted the survival of developing T\textsubscript{reg} cells by inhibiting the mitochondrial apoptosis pathway, we examined whether deliberate expression of the antiapoptotic Bcl-2 protein could rescue the survival of C\textsubscript{d27}^−/− T\textsubscript{reg} cells. CD45.2\textsuperscript{+} C\textsubscript{d27}^−/− 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\textsuperscript{+} 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 C\textsubscript{d27}^−/− cells (CD45.2\textsuperscript{−}) among gated GFP\textsuperscript{+}CD25\textsuperscript{+}CD4\textsuperscript{+} T\textsubscript{reg} cells (Fig. 3 F). C\textsubscript{d27}^−/− BM cells contributed only ~10% of the total GFP\textsuperscript{+} T\textsubscript{reg} cell population when they had been transduced with empty GFP vector, confirming the superiority of WT precursors over C\textsubscript{d27}^+/− precursors to develop into T\textsubscript{reg} cells. However, the proportion of C\textsubscript{d27}^+/− cells contributing to the total GFP\textsuperscript{+} T\textsubscript{reg} cell pool was significantly enhanced to almost 50% when C\textsubscript{d27}^+/− BM cells had been transduced with the Bcl-2\textsubscript{ires}GFP vector (Fig. 3 G). The contribution of Bcl-2 to T\textsubscript{reg} cell development from C\textsubscript{d27}^+/− BM cells was also reflected in absolute numbers of T\textsubscript{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\textsuperscript{+}CD4\textsuperscript{+} cells from C\textsubscript{d27}^+/− BM cells (Fig. 3 H). The fact that thymic C\textsubscript{d27}^+/− T\textsubscript{reg} cell numbers but not conventional CD4\textsuperscript{+} T cell numbers increased upon apoptosis inhibition by Bcl-2 lends further support to our findings that CD27 deficiency enhances apoptosis of developing T\textsubscript{reg} cells but not conventional CD4\textsuperscript{+} T cells.

**CD70 is expressed on Aire\textsuperscript{+} and Aire\textsuperscript{−} mTECs**

In the mouse, CD70 is expressed on DEC205\textsuperscript{+} cells in the thymic medulla (Tessler 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 C\textsubscript{d70}^+/− 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; Komada 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\textsuperscript{+} and ER-TR5\textsuperscript{+} 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\textsuperscript{−} mTECs but also on the great majority of (if not all) Aire\textsuperscript{+} 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\textsubscript{reg} cell selection (Aschenbrenner et al., 2007; Lei et al., 2011).

**CD70 on TECs promotes positive selection of T\textsubscript{reg} cells**

To address whether CD27–CD70–dependent survival signals were required for positive selection of thymic T\textsubscript{reg} cells, we used a model of superantigen (sAg)-driven T\textsubscript{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\textsuperscript{a} MHC class II molecules and form high-affinity ligands for V\textsubscript{B}5 TCRs. In B6 mice, however, the sAgs bind to I-A\textsuperscript{b} MHC class II molecules and form low-affinity ligands for V\textsubscript{B}5 TCRs (Luther and Acha-Orbea, 1997).
To assess whether CD27 promoted positive selection of Ag-specific T<sub>reg</sub> cells, Vβ5 expression was analyzed in the T<sub>reg</sub> cell compartment (Fig. 5 B). In DBA/2 recipient mice, the frequency of WT Vβ5<sup>+</sup> T<sub>reg</sub> cells was significantly higher than that of Cd27<sup>−/−</sup> Vβ5<sup>+</sup> T<sub>reg</sub> cells (Fig. 5 C). In B6 mice, the frequencies were not significantly different. The frequencies of control Vβ4<sup>+</sup> T<sub>reg</sub> cells, which do not recognize these sAgs (Luther and Acha-Orbea, 1997), were similar among WT and Cd27<sup>−/−</sup> T<sub>reg</sub> cells (Fig. 5 C). Furthermore, the frequencies of Vβ5<sup>+</sup>and Vβ4<sup>+</sup> conventional CD4<sup>+</sup> T cells were comparable between WT and Cd27<sup>−/−</sup> cells in B6 and DBA/2 mice (not depicted). These results suggest that CD27 co-stimulation promotes the positive selection of Vβ5<sup>+</sup> T<sub>reg</sub> cells.

To determine whether CD70 expression by epithelial cells alone sufficed to promote positive selection of T<sub>reg</sub> cells, irradiated DBA/2 mice were reconstituted either with a 1:1 mixture of WT (Cd27<sup>+/−</sup>) and Cd27<sup>−/−</sup> BM cells or with a 1:1 mixture of Cd27<sup>+/−</sup>; Cd70<sup>Cre/Cre</sup> and Cd27<sup>−/−</sup>; Cd70<sup>Cre/Cre</sup> 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 chimeras, the positive selection of Vβ5<sup>+</sup> T<sub>reg</sub> cells was significantly impaired by CD27 deficiency, both when Cd27 was present or absent on hematopoietic cells (Fig. 5 D). These data suggest a scenario in which T<sub>reg</sub> 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<sub>reg</sub> 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<sub>reg</sub> cell selection.

CD70 is present on DCs in the thymic medulla, and CD8<sup>+</sup> conventional DCs (cDCs) support T<sub>reg</sub> 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<sub>reg</sub> cell selection. Previously, it has been shown that DCs play a role in thymic T<sub>reg</sub> 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<sup>+</sup>B220<sup>lo</sup> DC purified from WT and Cd70<sup>Cre/Cre</sup> mice on the basis of CD8α and SIRPα expression (Fig. 6 B). Cd70 mRNA was
detected in both CD8α+ and SIRPα+ WT DCs but not in Cd70Cre/Cr Cre BM cells, resulting in expression of CD70 on hematopoietic cells or not, as indicated. After 8 wk, the development of Treg cells was analyzed as outlined for A–C. Bar diagrams indicate the percentage of Vβ5+ Treg cells (D) and the absolute number of Treg cells (E) within WT and Cd27−/− or Cd70Cre/Cr Cre BM 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.

Figure 5. CD27–CD70 co-stimulation promotes positive selection of sAg-specific Treg 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

CD70 on DCs as well as CD70 on epithelial cells supports Treg cell development in vivo

To test whether CD70 on hematopoietic cells supports Treg cell development in vivo, we created BM chimeras. Cd70Cre/Cr 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 Treg cell precursors and CD70 on a BM-derived cell type (most likely the DCs identified above) contributes to Treg cell development.

To test the relative contributions of CD70 on BM–derived versus epithelial cells in Treg 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, Cd70Cre/Cr Cre BM was transferred into WT mice. To exclude a role for CD70 on epithelial cells, WT BM was transferred into Cd70Cre/Cr Cre mice. The fourth setting, in which Cd70Cre/Cr Cre BM was introduced into Cd70Cre/Cr 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 Treg cell output but not to a significant extent (Fig. 7 B). When CD70 was lacking on both cell types, however, Treg cell development was significantly impaired. These data indicate that CD70 on both BM DCs and epithelial cells contributes to Treg cell development.

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 Treg 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 Treg cell development is quantitatively impaired by lack of co-stimulatory
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 Treg cell development has been described (Liston et al., 2008), it is generally accepted that Treg cell precursors find the optimal signals to differentiate into mature Treg cells when they are present at a low frequency (Bautista et al., 2009). Thus, even within the thymic medulla, the possibility for a Treg cell precursor to become a mature Treg 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 Treg cells. Aire-expressing mTECs that have been implicated in Treg 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 Treg 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 Treg cell numbers in the thymus, strongly suggesting that the clusters of mTECs and DCs are important for Treg 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 Treg cell development and implicate CD70 on both of these cell types in Treg cell development. CD70 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 Treg cell numbers in the thymus, strongly suggesting that the clusters of mTECs and DCs are important for Treg cell development (Lei et al., 2011).
promote T\textsubscript{reg} cell development, in which CD80/86 and CD70 play an important role. However, SIRP\textalpha\textsuperscript{+} cDCs could also induce T\textsubscript{reg} cell development in vitro, in a CD70-independent manner. SIRP\textalpha\textsuperscript{+} cDCs are a circulatory population and express higher levels of CD80, CD86, and MHCI\textsuperscript{II} than thymus-resident CD8\textalpha\textsuperscript{+} cDCs (Proietto et al., 2008; Li et al., 2009; Lei et al., 2011). The ability of SIRP\textalpha\textsuperscript{+} cDCs to promote T\textsubscript{reg} cell development independently of CD70 may therefore be caused by their high expression of other co-stimulatory molecules. Alternatively, SIRP\textalpha\textsuperscript{+} cDCs may present different autoantigens in MHC class II than CD8\textalpha\textsuperscript{+} cDCs and positively select a different pool of T\textsubscript{reg} cell precursors.

Interestingly, it was previously demonstrated that like CD70, CD80 and/or CD86 on both TECs and DCs contribute to T\textsubscript{reg} cell development (Proietto et al., 2008). Furthermore, CD40 on epithelial cells or hematopoietic cells can also contribute to T\textsubscript{reg} cell development (Spence and Green, 2008). It is likely that CD40 engagement promotes T\textsubscript{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\textsubscript{reg} cell development in the absence of CD27. During the priming of naïve 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\textsubscript{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\textsubscript{reg} cell development to the next stage. In contrast, CD27 deficiency did not impact pre-T\textsubscript{reg} cell numbers, Foxp3 induction, or programming of suppressive function in T\textsubscript{reg} cells. Rather, CD27 signaling counteracted the mitochondrial apoptosis pathway in developing T\textsubscript{reg} cells, as demonstrated by the increased exposure of phosphatidylserine and increased expression of proapoptotic Bcl-2 molecules in CD27-deficient T\textsubscript{reg} cells, as well as the capacity of ectopic Bcl-2 to rescue the development of CD27-deficient T\textsubscript{reg} cells. Several lines of evidence argue that T\textsubscript{reg} cells rely on survival signals for their thymic development. First, CD4\textsuperscript{+}CD25\textsuperscript{+} thymocytes are more resistant to negative selection than CD4\textsuperscript{+}CD25\textsuperscript{−} thymocytes (van Santen et al., 2004; Taylor et al., 2007). Furthermore, deficiency in the proapoptotic Bcl-2 protein Bim leads to a large increase specifically in T\textsubscript{reg} cell numbers (Ouyang et al., 2010). Our data lend further support to the idea that T\textsubscript{reg} cell development relies on signals that inhibit the intrinsic apoptosis pathway and impact 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\textsubscript{reg} cells without affecting that of
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 Treg cell development. TCR and CD28 signals kick start the developmental process by induction of Foxp3 expression. Thereafter, IL-2/IL-2 receptor signaling stabilizes Treg cell differentiation, whereas CD27–CD70 co-stimulation rescues developing Treg cells from apoptosis and thereby allows them to take part in the peripheral Treg cell population.

MATERIALS AND METHODS

Mice. B6 WT, Cd70+/−, Cd70Cre/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). Cd70−/− mice were made on a 129/J/Ola background and backcrossed to B6 for eight generations (Hendriks et al., 2000). Cd70Cre/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 Cd70Cre/Cre mice. Cd70−/− mice are deficient for CD70 expression because exon 1 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 Treg cell generation from Cd70Cre/Cre donor cells because both Cd70Cre/Cre and Cd70−/−;Cd70Cre/Cre donor cells were of CD45.2+ origin. In the Bcl2 transduction experiments, c-Kir+ 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-Kir GFP+ cells were sorted by flow cytometry, and appropriate mixes of a total 10^6 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 (Scharfe).

Antibodies and flow cytometry. Cells were stained with the following fluorescein-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 (MB.9-4) from BD; and anti-CD45.2 (104), anti-CD27 (LG.3A10), anti-SIRPα (P84), and anti-Foxp3 (FJK-166) 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 MoFlo (Dako). Sorted cells were always at least 98% pure. Data were analyzed using Flowjo software (Tree Star).

conventional αβ T cell. 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 Treg 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 Treg cells may be explained by the higher-affinity TCR signals they receive in the thymus (Ciofani and Zúñiga-Pflücker, 2010).

Another critical factor in Treg 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 Treg 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 Treg cells (Fontenot et al., 2005b; Tai et al., 2005). Importantly, CD27 as well as CD28 contribute to Treg 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 Treg cell generation (Tai et al., 2005; this study).

Treg cell numbers in Cd27−/− or Cd70Cre/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 Treg 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 Treg 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 Treg cells (Claus et al., 2012). Therefore, the regulation of Treg cell numbers via the CD27–CD70 pathway can play an important physiological role. Furthermore, we have recently shown that Cd27−/− and Cd70Cre/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 Treg cells also contributed to enhanced EAE in Cd27−/− and Cd70Cre/Cre mice. The use of MHC II tetramers or TCR sequencing technology to detect antigen–specific Treg cells in cancer or autoimmune models may help to shed light on the role of the CD27–CD70 pathway in peripheral Treg cell responses.

In conclusion, although Treg cell development abides by many of the rules applicable to conventional αβ T cell development, Treg 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 mechanisms thereof are reduced to maximally about half of control numbers in the periphery. In Fig. 5 (D and E), CD27 was used as a marker for Treg cell generation from Cd70Cre/Cre donor cells because both Cd70Cre/Cre and Cd27−/−;Cd70Cre/Cre donor cells were of CD45.2+ origin. In the Bcl2 transduction experiments, c-Kir+ 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-Kir GFP+ cells were sorted by flow cytometry, and appropriate mixes of a total 10^6 cells were injected intravenously in irradiated hosts.

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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 SIRPa 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 (Teselar 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^- (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., 2001b) or human IgG1 control antibody was added to the cultures. To disrupt CD70 interactions, the anti-CD70 mAb FR70 (provided by H. Yagita, Juntendo University School of Medicine, Tokyo, Japan; Oshima et al., 2010b) or human IgG1 control antibody was added to the cultures. For CD27 stimulation, a fusion protein of the extracellular domain of mouse CD70 and the Fc portion of human IgG1 (FcCD70; Peperzak et al., 2001b) or a control rat IgG2b isotype control (eBioscience) was added to culture. Controls 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, 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–mice 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 SH12 (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 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-GELO 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 DNA was isolated from pellets cells using the RNeasy mini kit (QIAGEN). 5 μg of RNA was used for MLPA (MRC-Holland) using the RM040-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.

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