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CUTTING EDGE

Cutting Edge: CD28 Controls Peripheral Homeostasis of $CD4^+CD25^+$ Regulatory T Cells[1]

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CD28/B7 blockade leads to exacerbated autoimmune disease in the nonobese diabetic mouse strain as a result of a marked reduction in the number of $CD4^+CD25^+$ regulatory T cells (Tregs). Herein, we demonstrate that CD28 controls both thymic development and peripheral homeostasis of Tregs. CD28 maintains a stable pool of peripheral Tregs by both supporting their survival and promoting their self-renewal. CD28 engagement promotes survival by regulating IL-2 production by conventional T cells and CD25 expression on Tregs. The Journal of Immunology,* 2003, 171: 3348–3352.

In both mice and humans, $CD4^+CD25^+$ regulatory T cells (Tregs)[3] constitute 5–15% of peripheral $CD4^+$ T cells and are immunosuppressive in vivo and in vitro (1, 2). Thymic-derived Tregs have been shown to regulate autoimmune disease via active suppression of self-reactive T cells in various models of autoimmunity (3). Mice engineered to express both a transgenic TCR and its cognate Ag in the thymus have an increased percentage of Tregs in the thymus and the periphery (4, 5), suggesting that Tregs develop from self-reactive T cells that have escaped negative selection. Moreover, there is evidence to indicate that autoantigens and IL-2-dependent events are essential for the maintenance and/or induction of Ag-specific Tregs in the periphery (6, 7).

Previously, we showed that Tregs control development of diabetes in the nonobese diabetic (NOD) mouse model of spontaneous autoimmune diabetes. In addition, in the absence of CD28-mediated costimulatory signals, NOD mice developed exacerbated diabetes associated with a profound decrease in the number of peripheral Tregs (8). Prevention of disease could be achieved by the reconstitution of Tregs from wild-type (WT) NOD mice, implicating CD28/B7 interactions in promoting regulatory function and peripheral homeostasis of Tregs. The role of CD28 costimulation in classical T cell activation has been investigated extensively. Because Tregs are naturally anergic and do not produce IL-2 (a major CD28-dependent event), it is unclear how CD28 regulates Treg development and homeostasis.

Materials and Methods

Mice

Six- to 10-wk-old C57BL/6 and BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA), NOD mice (Taconic Farms, Germantown, NY), CD28-deficient mice on BALB/c background, and Bcl-x_L transgenic mice on a C57BL/6 background were housed under specific pathogen-free conditions at the Animal Barrier Facility (University of California, San Francisco, CA).

Abs and other reagents

mAbs: 145-2C11 (anti-CD3ε), AT83a (rat anti-Thy1.2), 16-10A1 (anti-B7-1), and GL-1 (anti-B7-2) were prepared in our laboratory. Murine CTLA4 Ig was a generous gift from Genetics Institute (Cambridge, MA). Recombinant human (rh) IL-2 was a gift from Chiron (Emeryville, CA). Fluorochrome-labeled mAbs against CD25 (7D4), CD62L (Mel-14), and CD4 (GK1.5) were purchased from Southern Biotechnology Associates (Birmingham, AL). Allophycocyanin and PerCP-labeled mAbs against CD4 (RM4-5) were purchased from BD PharMingen (San Diego, CA). Biotin-labeled anti-glucocorticoid-induced TNFR family related (GITR) Abs were purchased from R&D Systems (Minneapolis, MN). CFSE was purchased from Molecular Probes (Eugene, OR).

Cell sorting and flow cytometry

Tregs were sorted from lymph node (LN) and spleen cells on the Mo-Flo cytometer (Cytomation, Fort Collins, CO) based on the expression of CD4, CD25, and CD62 ligand (CD62L) to >95% purity. For some experiments, $CD4^+$ T cells were enriched from pooled LN and spleens by negative selection on autoMACS (Miltenyi Biotec, Auburn, CA), and cultured overnight at 5×10^6 cell/ml in 20 U/ml rhIL-2 in complete medium as previously described (8). Tregs were sorted the next day as described above. Flow cytometric analyses were performed on a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, San Jose, CA).

Adoptive transfer and Ab/cytokine administration

Sorted T cells were labeled with 1.5 μM CFSE, and $1–3 \times 10^6$ cells were transferred via retro-orbital injection. The recipient mice were treated by i.p. injection with a mixture of anti-B7-1 and anti-B7-2 Abs (100 μg each) in PBS, 200 μg of control mAb (rat anti-human Bw6), CTLA4 Ig, or PBS as specified in figure legends. In some experiments, a rat anti-human HLA-Bw6 mAb was administered as control for the anti-B7 mAbs, whereas PBS was used in other

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[3] Abbreviations used in this paper: Treg, regulatory T cells; NOD, nonobese diabetic; WT, wild type; GITR, glucocorticoid-induced TNFR family related; LN, lymph node; CD62L, CD62 ligand; rh, recombinant human; HPRT, hypoxanthine phosphoribosyltransferase; Ct, threshold cycle; SP, single positive.

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experiments. No observable differences were noted among control mAb-, PBS-treated, or untreated mice.

Real-time PCR analysis of steady state IL-2 mRNA in spleens

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) from spleens of WT and $CD28^{-/-}$ BALB/c mice immediately after sacrifice. cDNA was synthesized from 2 μg of each RNA sample using SuperScript II RNase H- reverse transcriptase and oligo dT as primer (Invitrogen), and 25 ng of the cDNA was used in each real-time PCR for IL-2 or hypoxanthine phosphoribosyltransferase (HPRT). Primers and probes for both genes were purchased as reagent kits from Applied Biosystems (Foster City, CA). The real-time PCR was performed on ABI Prizm 7700 using TaqMan Universal PCR master mix (Applied Biosystems) in duplicates and the average threshold cycles (Ct) of the duplicate were used to calculate the fold change between WT and CD28KO mice. Ct for HPRT were used to normalize the samples, and fold change was calculated using the following formula: fold change = 2^{-n}, n = $((CD28^{-/-}\ Ct_{IL\text{-}2} - CD28^{-/-}\ Ct_{HPRT}) - (WT\ Ct_{IL\text{-}2} - WT\ Ct_{HPRT}))$.

Results and Discussion

CD28 controls thymic development of $CD4^+$ $CD25^+$ regulatory T cells

A comparison of the thymic CD4 single-positive (SP) $CD25^+$ T cells in WT and CD28-deficient mice demonstrated that disruption of the CD28/B7 pathway resulted in a dramatic (80%) reduction in both the percentage and the number of CD4 SP $CD25^+$ T cells in the thymus (Fig. 1*A*). Similarly, mice treated with a combination of anti-B7-1 and anti-B7-2 Abs every other day for 10 days resulted in a 66% reduction in the number of $CD4^+$SP $CD25^+$ T cells in the thymus compared with PBS-treated littermate controls (Fig. 1*B*). Thus, our data suggest that Tregs develop in a CD28/B7-dependent manner in adult mice.

CD28 controls the peripheral homeostasis of Tregs

The treatment of NOD mice with CTLA4 Ig fusion protein results in a rapid reduction in the number of Tregs in the LN and spleen (8). Similar results have been obtained using anti-B7 Abs in all mouse strains tested (BALB/c, B6, and NOD, data not shown). However, it was possible that the depletion of Tregs in the periphery following CD28/B7 blockade was a consequence of the decreased generation of these cells in the thymus as opposed to a direct effect on the peripheral Treg population. The Treg population in adult thymectomized mice was significantly reduced following anti-B7 treatment (Fig. 1*C*), comparable to that observed in sham-thymectomized animals. Moreover, unlike $CD28^{-/-}$ mice, young (10–14 days) $CTLA\text{-}4^{-/-}$ mice have normal levels of $CD62L^{high}$ Tregs in the periphery (data not shown). These results suggest that CD28 is important both for the development of Tregs in the thymus and for their maintenance in the periphery.

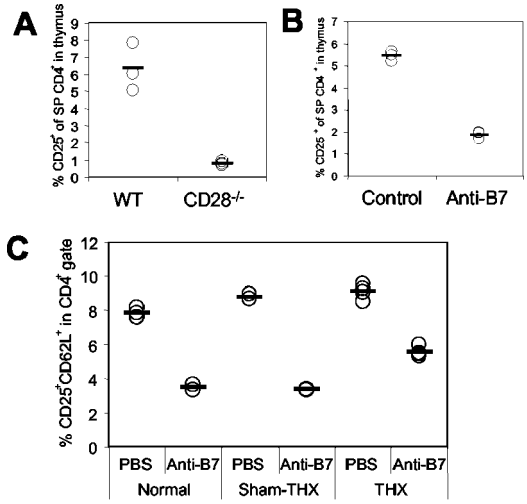

FIGURE 1. CD28 is essential for both thymic development of Tregs and their peripheral homeostasis. *A*, Percentages of $CD25^+$ cells among CD4 SP thymocytes in WT ($n = 3$) or $CD28^{-/-}$ BALB/c mice ($n = 3$) were determined by flow cytometry. *B*, WT NOD mice were injected with anti-B7-1 and anti-B7-2 (100 μg each, $n = 3$) or PBS ($n = 2$) every other day for 10 days. On day 10, the percentages of $CD25^+$ cells among $CD4^+$ SP thymocytes were determined. *C,* BALB/c mice were thymectomized ($n = 8$), sham-thymectomized ($n = 4$), or not treated ($n = 5$) on day 0 and injected with PBS or anti-B7-1 and anti-B7-2 (100 μg each) on days 5, 7, 9, 11, and 13. The percentages of Tregs in CD4 gate of lymph node cells were determined on day 14. Each circle represents one animal and the black bar represents the mean of the group. Results are representative of two independent experiments.

Treg proliferation and survival depends on CD28 costimulation

Purified labeled $CD4^+CD62L^{high}CD25^+$ (Tregs) or $CD4^+$ $CD62L^{high}CD25^-$ cells were transferred into secondary hosts and the recipients were treated with a combination of anti-B7-1 and anti-B7-2 mAbs or control Abs with irrelevant specificity. The numbers of labeled Tregs in the LN and spleens of recipient mice were determined by flow cytometry based on the co-expression of CD4 and CFSE on days 15 and 30 after cell transfer. The total number of cells recovered at various time points after transfer was estimated to be ~10% of the initial input. This relatively low rate of recovery is most likely due to non-specific trapping of transferred cell in tissues immediately after injection. It has been documented that despite the low recovery, the remaining transferred cells exhibited homing and activation patterns predicted for their endogenous counterparts (9). Therefore, we believe that the cells recovered from the LN and spleens in our experiments are representative of the transferred cells and are likely to reflect the behavior of the corresponding endogenous cell population. In contrast to the purported "anergic" phenotype of Tregs observed in vitro (10, 11), the Tregs underwent brisk proliferation in vivo, with 10–15% diluting the CFSE label within 2 wk after transfer (data not shown) and up to 40% 30 days after transfer (18% in the experiment shown in Fig. 2*A*). Moreover, because we could not detect transferred cells once their CFSE label is at background level, the actual proportion of transferred Tregs that have undergone proliferation is likely to be higher than our estimation. In contrast, only 6.5% of the $CD25^-$ cells transferred in the same manner showed CFSE dilution by day 30 (Fig. 2*A*). The recipient mice were not lymphopenic, therefore the proliferation observed represented the true steady state homeostatic activity of these cells. Moreover, the proliferation was CD28-dependent, as it was almost completely blocked by anti-B7 mAb treatment (Fig. 2, *B* and *C*). Because this block in proliferation was observed at all time points examined (days 1, 7, 15, and 30), we think it is very unlikely that a subpopulation of cells have lost all CFSE and escaped detection. In addition, this is not due to killing of the Tregs by direct binding of the anti-B7 mAb because a similar reduction in Treg proliferation was observed when WT Tregs were transferred to mice that were deficient in both B7-1 and B7-2 (data not shown), demonstrating that B7 expression on the host cells was essential for Treg proliferation. These results support the notion that Tregs express TCRs that react with self Ags (4–6), and continuously respond to autoantigens in a costimulation-dependent manner to maintain their homeostasis in vivo.

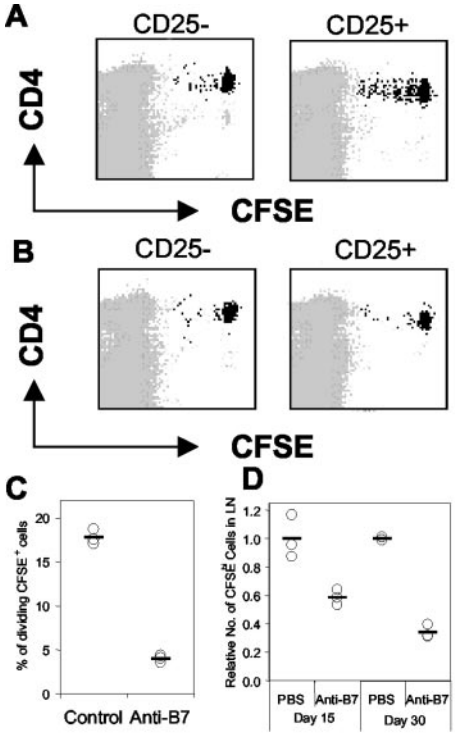

FIGURE 2. CD28 regulates both proliferation and survival of Tregs in the periphery. Sorted CFSE-labeled Tregs or CD4$^+$CD62L$^+$CD25$^-$ cells were transferred to syngeneic mice, proliferation and cell survival were analyzed on days 15 and 30 after transfer. Representative dot plots of LN cells harvested on day 30 in control-treated animals (*A*) and anti-B7-treated animals (*B*) are shown. Proliferation of CD25$^+$ cells on day 15 (*C*, $n = 3$ in each group) and the number of nondividing CFSE^{high} cells on indicated days after transfer (*D*, $n = 3$ in each group) were determined by flow cytometry. Symbols are as described for Fig. 1. Results are representative of five independent experiments.

In addition to its role in Treg proliferation, CD28/B7 interactions also regulate the survival of Tregs. The number of CFSE^{high} (undivided) Tregs decreased by 20–40% on day 15 and by 50–70% on day 30 in anti-B7-treated mice compared with control mice (Fig. 2*D*). The number of CFSE^{high} CD4$^+$CD25$^-$ cells was unaffected by anti-B7 treatment at either time point (data not shown), suggesting that the survival of conventional T cells was largely CD28-independent. Proliferation of Tregs was almost completely blocked 15 days after anti-B7 treatment initiation, whereas the survival of the cells was partially affected, suggesting that the two processes are differentially regulated by CD28.

Treg survival depends on both IL-2 and B7

IL-2 has been shown to be essential for Treg homeostasis, and mice deficient in IL-2 or IL-2R have very low numbers of Tregs in the periphery (7, 12–14) and develop rampant systemic autoimmune diseases (15–17). Moreover, CD28 plays a critical role in IL-2 production by activated T cells (18), and the steady state IL-2 mRNA was 2- to 5-fold less in normal unperturbed CD28$^{-/-}$ mice compared with that in normal WT mice (Fig 3*A*). To determine whether the diminished steady state IL-2 production in CD28$^{-/-}$ mice affected normal Treg homeostasis, the survival of purified WT Tregs transferred into WT and CD28$^{-/-}$ hosts was compared. One month after transfer into CD28$^{-/-}$ recipients, WT Tregs were barely detectable in the LNs and spleens (Fig. 3, *B* and *C*). These results demonstrated that CD28 expression on Tregs was insufficient to support Treg homeostasis. Thus, CD28 functioned on conventional T cells to regulate Treg survival through the induction of Treg-extrinsic survival factor(s), such as IL-2. To determine whether exogenous IL-2 could overcome the Treg survival defect in CD28$^{-/-}$ mice, we precultured Tregs in 20 U/ml IL-2 overnight and examined their survival in WT or CD28$^{-/-}$ hosts. The short-term culture in IL-2 did not significantly up-regulate CD25 expression on the Tregs (data not shown). In contrast, the IL-2 treatment restored Treg homeostasis in IL-2$^{-/-}$ mice (data not shown) and completely protected the Tregs in the CD28$^{-/-}$ hosts for the observation period (Fig. 3, *D* and *E*). Thus, the IL-2 level is likely to be one of the limiting factors in sustaining Treg homeostasis in CD28$^{-/-}$ mice.

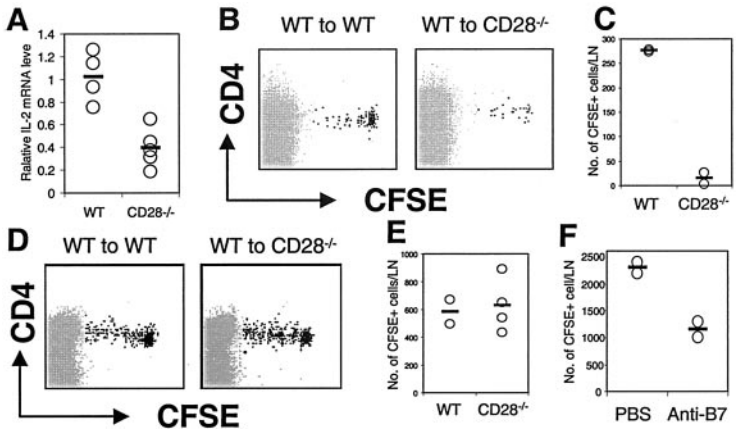

FIGURE 3. Both IL-2 and B7 are necessary for Treg survival. *A*, Steady state IL-2 mRNA level was determined in the spleens of WT ($n = 4$) and CD28$^{-/-}$ ($n = 5$) mice by reverse transcription of RNA followed by real-time PCR analysis. *B* and *C*, Tregs cells were purified from WT BALB/c mice and transferred to either WT or CD28$^{-/-}$ BALB/c mice after CFSE labeling. Representative dot plots (*B*) and total number of CFSE$^+$ cells (C, $n = 3$ in WT and 2 in CD28$^{-/-}$) in the LN harvested on day 30 after transfer are shown. *D* and *E*, WT BALB/c Tregs were purified from LN and spleens after overnight culture in 20 U/ml IL-2 and transferred to WT ($n = 2$) or CD28$^{-/-}$ ($n = 4$) BALB/c hosts after CSFE labeling. Representative dot plots (*D*) and total number of CFSE$^+$ cells (*E*) in the LN harvested on day 30 after transfer are shown. *F*, WT BALB/c Tregs were purified from LN and spleens after overnight culture in 20 U/ml IL-2 and transferred to WT BALB/c host after CFSE labeling. The mice were then treated with anti-B7 mAbs ($n = 2$) or PBS ($n = 2$) every third day for 30 days. Numbers of CFSE^{high} cells in LN were determined by flow cytometry on day 30 after cell transfer. Symbols are as described for Fig. 1. Results are representative of two (*A*–*C*), three (*D* and *E*), and five (*F*) independent experiments.

It is possible that $CD28^{-/-}$ mice have additional defects in supporting Treg homeostasis besides IL-2. Therefore, we examined whether exogenous IL-2 could replace the need for CD28 costimulation in the periphery using the adoptive transfer system. Tregs were precultured with 20 U/ml IL-2 overnight before adoptive transfer into WT hosts. The mice were then treated with anti-B7 or control mAbs, and the survival of transferred Tregs was determined 30 days post-transfer. This treatment led to over 60% reduction in the number of Tregs (Fig. 3*F*), similar to that observed without IL-2 preculture (Fig. 2*D*). Thus, IL-2 alone was not sufficient for supporting Treg survival in the absence of CD28 signaling.

CD28 maintains a high level of CD25 expression on Tregs

Costimulation through CD28 has been shown to be necessary for inducing several cell intrinsic survival factors, such as Bcl-x_L (19). Therefore, we tested whether these molecules were involved in CD28 regulation of Treg survival. Transgenic mice expressing Bcl-x_L under the control of the Lck proximal promoter were treated with CTLA4 Ig. Although $CD4^+CD25^+$ in these transgenic mice overexpress Bcl-x_L, Tregs were not protected from depletion after CD28/B7 blockade (Fig. 4*A*). CD28-dependent OX40 induction on $CD4^+$ T cells has been implicated in promoting T cell survival and the generation of memory T cells (20, 21). In addition, OX40 was expressed on resting WT, but not on $CD28^{-/-}$ Tregs (data not shown). However, unlike $CD28^{-/-}$ mice, $OX40^{-/-}$ mice have normal level of Tregs in the periphery (Fig. 4*B*) and thymus (data not shown). Taken together, these results suggest that CD28 regulation of Treg survival was independent of Bcl-x_L and OX40.

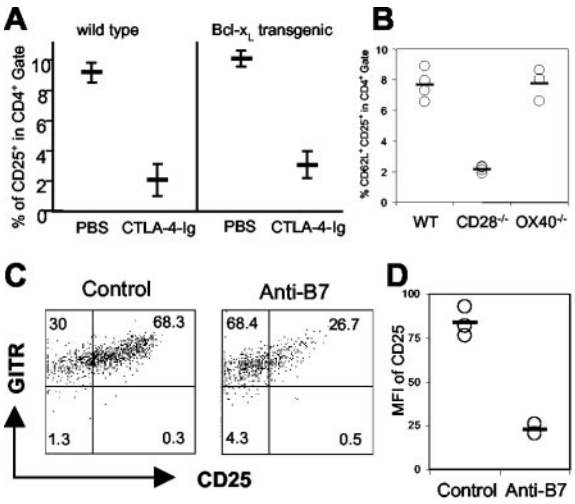

FIGURE 4. CD28 regulation of Treg survival is independent of Bcl-x_L and OX40. *A*, Bcl-x_L transgenic and WT littermates were treated with CTLA4 Ig or PBS as control every other day for 10 days. Percentages of $CD25^+$ cells in the CD4 gate in the LN were determined by flow cytometry. *B*, Percentages of Tregs in the CD4 gate in $OX40^{-/-}$ mice were compared with those in WT and $CD28^{-/-}$ mice ($n = 4$ in each group). *C* and *D*, Tregs were purified from WT BALB/c mice and transferred to syngeneic hosts after CFSE labeling. The recipient mice were treated with either anti-B7 mAbs or control mAb every third day for 15 days ($n = 3$ in each group). Levels of CD25 and GITR expression on $CD4^+$ $CFSE^{high}$ cells were determined by flow cytometry on day 15 after cell transfer. Representative dot plots (*D*) and charts of MFI of CD25 staining on $CD4^+$ $CFSE^+$ cells are shown. The position of the quadrants was set such that all the endogenous Tregs fall in the *upper right quadrant* and all the $CD4^+CD25^-$ cells fall in the *left two quadrants*. The numbers in dot plots indicate the percentages of transferred cells in respective quadrants. Symbols are as described for Fig. 1. Results are representative of two (*A* and *B*) and five (*C* and *D*) independent experiments.

Because IL-2 was essential for normal Treg homeostasis, we speculated that the intrinsic function of CD28 may be to regulate Treg survival indirectly through the up-regulation of CD25 expression on Tregs. Therefore, we determined the level of CD25 expression on adoptively transferred CFSE-labeled Tregs after anti-B7 treatment. Two weeks after the initiation of anti-B7 treatment the mean fluorescent intensity of CD25 staining on the transferred cells was reduced significantly (Fig. 4, *C* and *D*). The loss of CD25 expression was not due to the general loss of cell viability, because expression of GITR, another molecule abundantly expressed on Tregs (22, 23), remained high after anti-B7 treatment (Fig. 4*C*). The selective loss of CD25 expression after CD28 signal blockade raised the possibility that CD28 might only be required to induce and maintain high level of CD25 expression, but not necessary for Treg development and homeostasis. Therefore, we examined GITR and CD25 expression in the $CD28^{-/-}$ mice, and found that the two markers coexpressed on a subpopulation of $CD4^+$ cells, identical to the pattern observed in WT mice, although the cells were present at much lower percentage in the $CD28^{-/-}$ mice. Thus, CD28 is necessary for both CD25 expression and Treg homeostasis. Moreover, it is unlikely that CD28 maintains CD25 expression indirectly through IL-2 induction; rather, evidence suggests that CD28 acts directly on Tregs to sustain their CD25 expression. Tregs precultured in IL-2 before transfer into $CD28^{-/-}$ host (with diminished IL-2 expression) maintained normal level of CD25 (data not shown) and survived (Fig. 3*D*), whereas similarly treated Tregs transferred into WT mice treated with anti-B7 rapidly lost CD25 as seen in Fig. 4*D*. Notably, the decreased expression of CD25 on Tregs preceded their disappearance in the periphery, consistent with the idea that CD28 indirectly regulates Treg survival through CD25. Thus, even under conditions of adequate IL-2 production (or exogenous IL-2 treatment, data not shown), the absence of CD28 signal, thus CD25 expression, would lead to the loss of Tregs. These results extend a previous report that showed an essential role of IL-2 receptor in Treg homeostasis (14) suggesting further that costimulation through CD28 is critical in maintaining a high level of CD25 expression on Tregs.

In conclusion, we have demonstrated that CD28 provides a unique costimulatory signal to promote thymic development and peripheral homeostasis of Tregs. In addition, CD28 maintains a stable pool of peripheral Tregs by supporting both their self-renewal and their survival. These functions of CD28 were independent of Bcl-x_L or OX40 and were mediated through IL-2 and CD25. We propose that CD28 directly regulates Treg proliferation possibly by increasing TCR signaling strength, and indirectly regulates Treg survival by promoting IL-2 production by conventional T cells and by up-regulation of CD25 on Tregs.

Understanding the mechanisms of CD28/B7 costimulation in the maintenance of Tregs has important implications for the treatment of autoimmunity and transplant rejection via CD28 blockade. Although treatments such as CTLA4 Ig block T cell activation, they also deplete tolerance-promoting Tregs. Therefore, it will be important to determine the signals provided by CD28/B7 interactions that maintain the development and survival of regulatory T cells. Ultimately, we may be able to harness

these signals to generate Tregs that promote tolerance in the setting of autoimmunity and transplantation.

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