CTLA-4 up-regulation plays a role in tolerance mediated by CD45

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Cytolytic T lymphocyte–associated antigen 4 (CTLA-4) is a critical down-regulatory molecule in T cells that plays a major role in peripheral tolerance. Although the CD45 protein tyrosine phosphatase is a potent immunomodulatory target, the mechanisms by which antibody against CD45RB isoforms (anti-CD45RB) induces allograft tolerance remain unclear. We show here that anti-CD45RB treatment alters CD45 isoform expression on T cells, which is associated with rapid up-regulation of CTLA-4 expression. These effects appear specific and occur without up-regulation of other activation markers. Administration of a blocking monoclonal antibody to CTLA-4 at the time of transplantation prevents anti-CD45RB therapy from prolonging islet allograft survival. In addition, treatment with cyclosporin A blocks anti-CD45RB–induced CTLA-4 expression and promotes acute rejection. These data suggest that anti-CD45RB acts through mechanisms that include CTLA-4 up-regulation and demonstrate a link between CD45 and CTLA-4 that depends on calcineurin-mediated signaling. They demonstrate also that CTLA-4 expression may be specifically targeted to enhance allograft acceptance.

Tolerance remains the ideal for permanent engraftment of transplanted organs and treatment of autoimmune diseases. Cytolytic T lymphocyte–associated antigen 4 (CTLA-4) is an important T cell down-regulatory molecule that is required for induction of peripheral tolerance in a number of models. Mice that lack CTLA-4 develop fatal autoimmune disease, which results from unchecked T cell activation and lymphoproliferation[1,2]. In addition, monoclonal antibody (mAb)-mediated blockade of CTLA-4 prevents development of tolerance to soluble antigen[3], augments anti-tumor responses[4], exacerbates autoimmune disease[5,6] and can even induce autoimmunity in normal mice. Although crucial in regulating autoimmunity, the role of CTLA-4 in allograft rejection and tolerance is less clear. CTLA-4 signaling is essential for the induction of long-term allograft survival through CD28 blockade with CTLA-4–immunoglobulin (CTLA-4–Ig)[8]. However, CTLA-4 is not absolutely required for the induction of long-term engraftment by antibodies to CD40 ligand (anti-CD40L), which acts (at least in part) through pathways that are independent from B7-CD28[8]. The role of CTLA-4 in transplant tolerance that results from other therapeutic strategies is unknown.

CTLA-4 is primarily expressed as an intracellular molecule that cycles to the cell surface where it can then interact with B7 counterligands (CD80 and CD86) on antigen presenting cells (APCs)[9,10]. CTLA-4 expression is up-regulated by T cell activation, which normally requires signals through both the TCR and the CD28 costimulatory pathways[10,11]. However, mAbs to CTLA-4 require extensive cross-linking to trigger negative signaling[12,13]. The lack of soluble agonist ligands for CTLA-4 has frustrated attempts to exploit this down-regulatory pathway for therapeutic purposes.

The CD45 family of transmembrane protein tyrosine phosphatases (PTPases) plays a critical role in lymphocyte activation by regulating the phosphorylation of Src family protein tyrosine kinases (PTKs) and the T cell receptor (TCR) ζ chain[14,15]. Multiple CD45 isoforms are generated by alternative splicing of exons 4–6 (commonly known as exons A, B and C). The various isoforms differ in the size of their extracellular domains while sharing identical cytoplasmic PTPase domains. Individual lymphocytes simultaneously express multiple CD45 isoforms. However, the higher and lower molecular weight (M_r) isoforms are differentially distributed on subsets of CD4 cells that have distinct functions including differences in *in vitro* and *in vivo* regulatory activity and cytokine production[16–19]. In mice, these subsets are best delineated by high and low

Table 1. Induction of tolerance by anti-CD45RB requires signals through CTLA-4

Treatment	*n*	*Graft* survival in individual mice	Median
None	5	11, 12, 12, 13, 13[c]	12
Anti-CD45RB[a]	14	10, 16, 19, 19, 25, 79, >120×8	>120[d]
Anti -CTLA4[b]	5	8, 12, 13, 14, 15	13
Anti-CD45RB + anti-CTLA4[b]	9	18, 18, 19, 19, 22, 23, 24, 24, 26	22

Chemically diabetic C57BL/6 recipients of BALB/c islets were untreated (controls) or treated with anti-CD45RB, anti-CTLA-4 or a combination of both mAbs, as described in Methods. [a]100 μg of antibody injected intravenously 1 day before, the day of and 5 days after transplantation. [b]200 μg of antibody injected intraperitoneally 1 day before, the day of and 1 day after transplantation. [c]Survival times are given in days. [d]$P<0.004$ *versus* all other groups.

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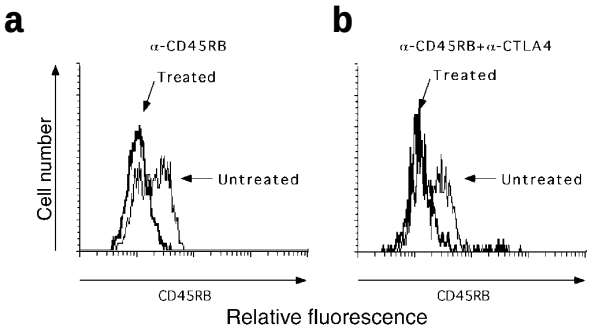

Figure 1. Anti–CTLA-4 does not alter the anti-CD45RB–mediated shift in CD45 isoforms. Representative CD45RB expression on $CD4^+$ cells from (**a**) untreated *versus* anti-CD45RB–treated animals and (**b**) untreated *versus* anti-CD45RB + anti-CTLA-4–treated animals. Splenocytes were isolated from mice on day 6 after treatment with three doses of anti-CD45RB or three doses each of anti-CD45RB and anti-CTLA4. After gating on $CD4^+$ cells, CD45RB expression was analyzed. Fluorescence of isotype controls was always within the first decade and did not differ between treatment groups (data not shown). (Similar results were obtained in four independent experiments.)

expression of CD45 exon B–containing isoforms (respectively designated $CD45RB^{hi}$ and $CD45RB^{lo}$)[16,17].

The expression of CD45 isoforms is highly regulated and dynamic. T cell activation is associated with a decrease in the higher M_r isoforms and concomitant up-regulation of the lower M_r isoforms. Such findings support the notion that $CD45RB^{hi}$ cells are naïve, whereas the $CD45RB^{lo}$ population contains antigen-experienced and memory T cells. However, the regulation of CD45 isoform expression appears to be more complicated as T cells expressing the lower M_r isoforms can re-express the larger isoforms[20–22].

The regulated expression of CD45 isoforms on distinct T cell subsets suggests biological importance. Although the exact function of individual isoforms remains unclear, expression of different isoforms does alter TCR-mediated signal transduction. For example, reconstitution of CD45-deficient T cell lines with distinct isoforms results in differential utilization of particular signaling pathways and differential secretion of IL-2[23–25].

CD45 is a potent immunomodulatory target. Several doses of anti-CD45RB can induce long-term engraftment and donor-specific tolerance to murine renal and islet allografts[26,27]. Although anti-CD45RB is thought to interfere with activation signal 1, the exact mechanisms by which this agent prevents rejection are not yet clear. The effective mAb to CD45RB, MB23G2, causes a shift towards expression of the lower M_r ($CD45RB^{lo}$) isoforms. It is not known how cells normally regulate CD45 isoform content nor do we understand how anti-CD45RB alters CD45 isoform expression. Regardless, the up-regulation of $CD45RB^{lo}$ isoforms is associated with augmented T_H2 cytokine secretion[26]. Altered isoform expression may also promote tolerance through other mechanisms. Because CTLA-4 is expressed by a subset $CD45RB^{lo}$ "memory" cells[28] and tolerogenic CD45RB mAbs induce a shift in CD45 isoform expression[26], we examined the role of CTLA-4 in the induction of long-term graft survival by anti-CD45RB.

Results

Anti-CD45RB–mediated engraftment requires CTLA-4

The role of CTLA-4 in long-term engraftment that results from anti-CD45–mediated interference with signal 1 is unknown. To address this question, we determined whether or not CTLA-4 blockade would prevent long-term anti-CD45RB–induced survival of islet allografts. Untreated control animals rapidly reject their allografts and become hyperglycemic in 11–12 days (**Table 1**). Treatment of recipients with three doses of anti-CD45RB as sole therapy, results in long-term engraftment in approximately 50% of the recipients in this high-responder strain combination. However, when recipients received a blocking mAb against CTLA-4 at the time of engraftment,

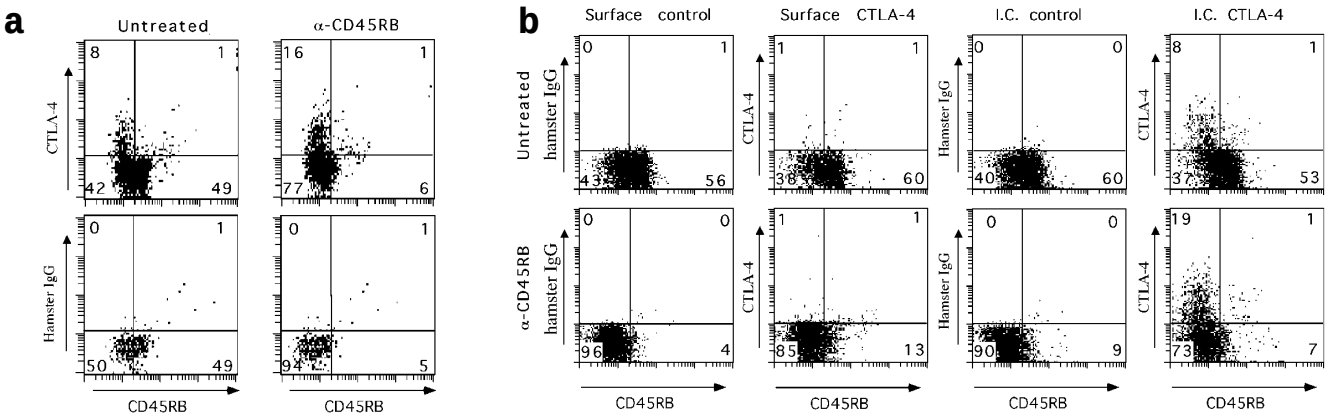

Figure 2. CTLA-4 expression is up-regulated by anti-CD45RB treatment. (**a**) Surface CD45RB and intracellular CTLA-4 expression on $CD4^+$ cells was determined by three-color immunofluorescence analysis of splenocytes from untreated animals or animals on day 6 after treatment with anti-CD45RB. After staining with CyChrome 5–conjugated anti-CD4 and FITC–anti-CD45RB, cells were permeabilized and stained with either PE-anti–CTLA-4 or control PE-conjugated hamster Ig. After gating on $CD4^+$ lymphocytes, green and red fluorescence were analyzed and are displayed on a log scale. The percentages of cells within each quadrant are shown. Surface CTLA-4 expression (nonpermeabilized cells) was essentially negative (data not shown). (Data are representative of at least five animals in each group.) (**b**) CTLA-4 and CD45RB expression on CD4 cells from untreated *versus* day 6 anti-CD45RB–treated animals after activation-induced cycling of CTLA-4 between the cytoplasm and cell surface. Purified CD4 cells were stimulated with plate-bound anti-CD3 + anti-CD28 for 4 h in the presence of PE-conjugated hamster Ig (I.C. control) or PE-anti–CTLA-4 (I.C. CTLA-4). After incubation, cells were stained with FITC–anti-CD45RB and analyzed by flow cytometry. Surface expression was determined by exposing CD4 cells to PE-conjugated hamster Ig or PE-anti–CTLA-4 (surface control and surface CTLA-4, respectively) only after removal from plate-bound stimulation. The percentages of cells within each quadrant are shown. (Data are representative of three experiments.) Cells cultured with PE-anti–CTLA-4 in wells that lacked anti-CD3 and anti-CD28, exhibited only surface CTLA-4 (data not shown).

anti-CD45RB was no longer able to promote long-term engraftment and rejection occurred promptly (median survival time 22 days). This was not due to a generalized augmentation of the rejection response by CTLA-4 blockade because animals treated with anti–CTLA-4 alone did not reject their grafts more quickly than untreated controls.

The rapidity with which anti-CD45RB–treated animals rejected grafts when the CTLA-4 pathway was blocked raised the question of whether CTLA-4 signals were directly involved in anti-CD45RB–mediated engraftment. For example, anti–CTLA-4 might prevent induction of a requisite shift in CD45 isoforms by anti-CD45RB.

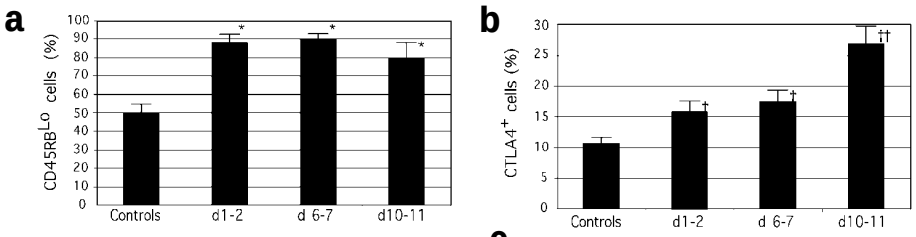

Figure 3. Time course of the shift in CD45 isoforms and CTLA-4 induction after anti-CD45RB treatment. Splenocytes were isolated from untreated and treated animals at various time points after initiation of anti-CD45RB treatment. CD4^{+} splenocytes were examined by three-color immunofluorescence as described above. CD4 cells were evaluated for (**a**) CD45RB^{lo} expression (**b**) CTLA4 expression and (**c**) CTLA4 expression on CD45RB^{lo} cells. (Results are expressed as the mean+s.d. of percentage of positive cells). Animals in each group: controls, n=9; days 1–2, n=5; days 6–7, n=6; days 10–11, n=3. *$P \leq 0.001$ *versus* control; $^{\dagger}P \leq 0.015$ *versus* control; $^{\dagger\dagger}P < 0.0001$ *versus* control and $P \leq 0.025$ *versus* other groups; **$P < 0.03$ *versus* all other groups.

Anti–CTLA-4 does not influence CD45 isoform regulation

To address this latter point, we compared CD45RB expression on CD4 cells from animals treated with either anti-CD45RB alone or the combination of anti-CD45RB + anti-CTLA-4. In both cases, an equivalent loss of CD45RB high isoforms was apparent (**Fig. 1**). There was little or no change in overall CD45 expression, which indicated a concomitant up-regulation of lower M_r isoforms[26] (and data not shown). In addition, there was no change in the total number of T cells in the spleen, which indicated that simple depletion of CD45RB^{hi} cells (~50% of the CD4 population) had not occurred. Thus, anti–CTLA-4 does not inhibit the shift in isoforms induced by anti-CD45RB.

Anti-CD45RB treatment up-regulates CTLA-4 expression

Despite being virtually undetectable in freshly isolated CD45RB^{hi} CD4 cells, CTLA-4 is constitutively expressed by a proportion of "antigen-experienced" CD45RB^{lo} cells that appear primed towards down-regulation through this pathway[28]. Given that anti-CD45RB treatment induces a shift from high to low (CD45RB^{lo}) M_r CD45 isoforms and that prolongation of allograft survival by anti-CD45RB is highly dependent on CTLA-4 signaling, it is possible that anti-CD45RB acts by up-regulating CTLA-4 expression. This was addressed by comparing CTLA-4 expression on CD4^{+} cells from mice that had received anti-CD45RB treatment with CD4^{+} cells from untreated control mice (**Fig. 2a**). As CTLA-4 expression is primarily intracellular, CTLA-4 expression was examined on permeabilized cells. In untreated control animals, CTLA-4 was constitutively expressed by a population of CD45RB^{lo} cells. As shown, ~20% of the CD45RB^{lo} population constitutively expressed CTLA-4. Because only ~50% of the resting CD4 cells were CD45RB^{lo}, just 10% of the overall CD4 population expressed CTLA-4. On day 6 after initiation of treatment with anti-CD45RB, ~95% of the CD4 cells were CD45RB^{lo} and constitutive CTLA-4 expression had essentially doubled (**Fig. 2a**).

Although CTLA-4 expression remains primarily intracellular, T cell activation results in rapid cycling of preformed CTLA-4 between endosomal compartments and the cell surface[9,10,29]. To examine regulation of this cycling after induction of CTLA-4 expression by anti-CD45RB, CD4 cells from treated (day 6 after anti-CD45RB administration) and untreated animals were incubated in wells coated with anti-CD3 and anti-CD28. After 4 h, CD4 cells from both treated and untreated animals exhibited only minimal surface expression of CTLA-4 (**Fig. 2b**). However, addition of fluorochrome-conjugated anti–CTLA-4 to the cells during short-term (4-h) activation resulted in internalization of mAb as CTLA-4 cycled to the surface and back (and see[9,28]). We found that twice as many CD4 cells exhibited CTLA-4 expression in anti-CD45RB–treated animals than in untreated animals (**Fig. 2b**). This agreed closely with the results obtained by direct (intracellular) staining of permeabilized cells (**Fig. 2a**). Thus, CTLA-4 induced by anti-CD45RB has rapid access to the cell surface and undergoes normal regulation of surface expression and endocytosis.

Examination of the time course of changes in CD45 isoform and

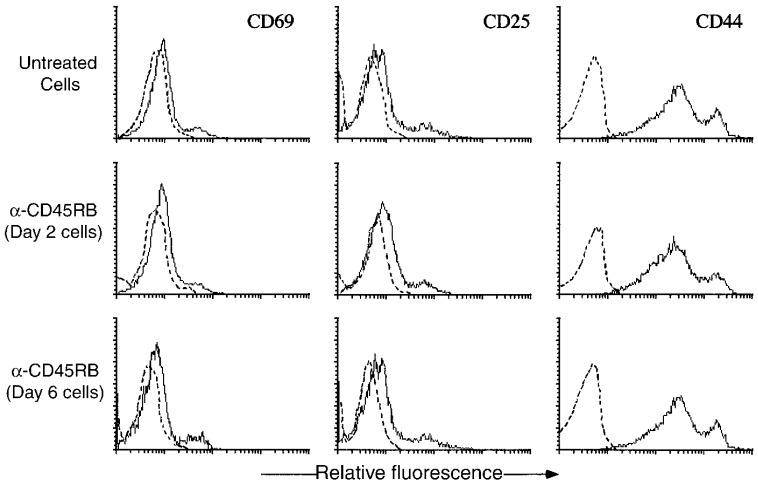

Figure 4. Anti-CD45RB treatment does not induce expression of other activation markers. CD69, CD25 and CD44 expression on CD4 cells from untreated control animals and animals on day 2 and day 6 after initiation of treatment with three doses of anti-CD45RB. (Similar results were obtained in at least three experiments in each group. The dotted lines represent immunofluorescence of isotype controls.)

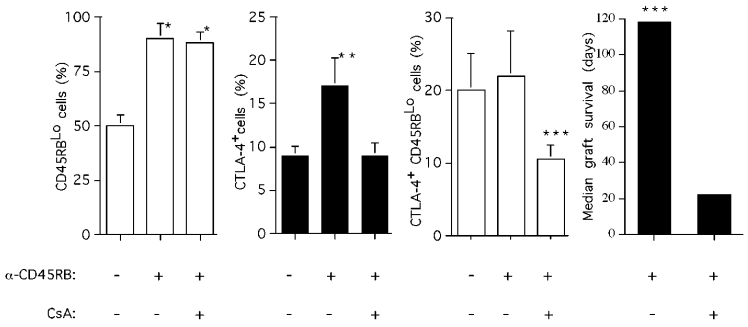

Figure 5. CsA blocks the anti-CD45RB–mediated increase in CTLA-4 expression on CD4 cells and inhibits prolongation of allograft survival. (**a–c**) Mice were untreated, received anti-CD45RB alone or received anti-CD45RB + CsA. On day 6, splenocytes were evaluated by three-color immunofluorescence as described above. CD4 cells were evaluated for (**a**) CD45RB^{lo} expression (**b**) CTLA-4 expression or (**c**) CTLA-4 expression on CD45RB^{lo} cells. (Results are expressed as mean+s.d. percentage of positive cells for four animals in each treatment group. *P<0.0001 *versus* control, **P<0.004 *versus* other groups, ***P<0.02 *versus* other groups.) (**d**) Chemically diabetic C57BL/6 recipients received islet allografts from BALB/c donors and received either anti-CD45RB alone or anti-CD45RB + CsA (n=7). (Data show median graft survival. ***P=0.014 CsA treatment *versus* no CsA.)

CTLA-4 expression revealed that within 1–2 days after anti-CD45RB treatment, a marked shift in CD45RB expression had already occurred (**Fig. 3**). This shift persisted for at least 10 days after treatment. CTLA-4 expression also underwent a rapid and statistically significant increase. By comparing the percentage of CD45RB^{lo} cells that express CTLA-4, it can be seen that after a slight lag, CTLA-4 expression on CD45RB^{lo} cells was relatively constant for the first week after treatment. However, CTLA-4 expression continued to rise so that by day 10–12, 30%, and in some animals 40%, of the CD4$^+$ CD45RB^{lo} cells expressed CTLA-4.

Anti-CD45RB does not induce T cell activation

To rule out the possibility that the shift in CD45 isoforms and rise in CTLA-4 expression were simply the result of T cell activation by anti-CD45RB, we compared CD4$^+$ splenocytes from treated and untreated animals for expression of the activation markers CD69, CD25 and CD44 on day 2 and day 6 after treatment (**Fig. 4**). As seen, anti-CD45RB treatment does not alter expression of molecules that are closely associated with T cell activation and entry into the cell cycle. In addition, CD45RB and CD44 expression are usually inversely correlated with one-another, with CD45RB^{hi} cells expressing low amounts of CD44 and CD45RB^{lo} cells expressing high amounts of CD44[30]. However, after anti-CD45RB treatment, CD44 expression remained bimodal even though over 90% of the cells were CD45RB^{lo}. This shows the relative specificity of the effects of anti-CD45RB and provides further evidence that CD45RB^{hi} cells are not simply being depleted (which would have removed the CD44^{lo} population).

Calcineurin links CD45RB, CTLA-4 and graft survival

The events that link the shift in CD45 isoforms to an up-regulation of CTLA-4 expression without overall T cell activation are unknown. To initiate identification of signaling pathways involved in these events, we examined the effects of calcineurin inhibition with the widely used immunosuppressant agent, cyclosporin A (CsA)[31]. Mice were treated with CsA alone, anti-CD45RB alone or these two agents in combination. Alone, CsA had no effect on CD45RB or on basal CTLA-4 expression (data not shown). In addition, CsA-treatment had no effect on the anti-CD45RB–induced shift in CD45 isoforms (**Fig. 5a**). In contrast, CsA completely blocked anti-CD45RB–induced up-regulation of CTLA-4 (**Fig. 5b**,**c**). Thus, only half as many CD45RB^{lo} cells expressed CTLA-4.

If up-regulation of CTLA-4 expression is critical to the induction of long-term allograft survival by anti-CD45RB, then inhibiting CTLA-4 up-regulation should have a detrimental effect on the efficacy of anti-CD45RB treatment. Animals that underwent allograft transplantation were treated with either anti-CD45RB or with both anti-CD45RB and CsA (**Fig. 5d**). The addition of CsA to anti-CD45RB resulted in acute rejection with a median graft survival of 22 days that was identical to that observed when allograft recipients treated with anti-CD45RB also received a mAb that blocked CTLA-4 signaling (**Table 1**).

Discussion

Although the biochemical mechanisms remain unresolved, CTLA-4–derived signals are crucial for the regulation of peripheral tolerance[3,32]. Unfortunately, no CTLA-4 agonist has been identified and, until now, there has been no known therapeutic means to utilize this negative regulator to promote peripheral tolerance *in vivo*. Here we demonstrate that CTLA-4 expression can be up-regulated without overt T cell activation, which allows this potent inhibitory pathway to be harnessed for the induction of long-term allograft survival. We hypothesize that anti-CD45RB acts through a newly identified mechanism: the rapid shift towards the lower M_r CD45 isoforms primes CD4 cells for CTLA-4 expression. By the time host APCs have up-regulated their CD80 and CD86 costimulatory ligands, potentially alloreactive CD4 cells either express, or have the capacity to rapidly express, CTLA-4 and are subject to down-regulation. In this regard, CD45RB^{lo} cells not only express CTLA-4 but they are more sensitive than CD45RB^{hi} cells to CTLA-4–mediated inhibition *in vitro*[28]. In addition to demonstrating an important *in vivo* role for CTLA-4 in anti-CD45RB–mediated allograft survival, we have also observed that CD4 cells from anti-CD45RB–treated animals exhibit increased sensitivity to inhibition through CTLA-4 cross-linking *in vitro* (not shown).

Recent studies suggest that a CD4$^+$CD25$^+$ population, which contains cells that constitutively express CTLA-4, can play an important role in regulating autoimmunity[7,33,34]. In one report, these regulatory cells were found to also express the lower M_r CD45 isoforms[33]. CTLA-4 ligation appears to augment inhibitory activity by these cells, which suggests another means by which CTLA-4 can down-modulate the immune system. The relationship between these regulatory cells and the CD45RB^{Lo} cells induced by anti-CD45RB treatment is unclear. However, we did not detect any increase in CD25 expression on CD4 cells in anti-CD45RB–treated animals.

The regulation of CTLA-4 expression is complex and occurs at multiple levels. Both CTLA-4 transcription and mRNA stability are up-regulated by T cell activation[11,35]. Maximal protein expression is seen 48–72 h later and appears to require entry into the cell cycle[10,11]. Although CTLA-4 expression is largely intracellular, TCR ligation induces rapid movement from endosomes to the plasma membrane, it's site of action and ligand binding[9,10]. Nonetheless, CTLA-4 is quickly endocytosed, which precludes significant accumulation on the surface at physiologic temperatures[10]. Surface expression and endocytosis are highly regulated through reversible tyrosine phosphorylation of the cytoplasmic tail, which inhibits interaction with the AP-2 clathrin adaptor complex, thereby promoting surface expression[29,36]. In addition,

intracytoplasmic CTLA-4 may also be regulated by interaction with the AP-1 adaptor complex, which targets CTLA-4 to lysosomal compartments for degradation[37].

We have demonstrated that anti-CD45RB treatment augments constitutive expression of intracellular CTLA-4 on CD4 cells. This CTLA-4 appears to undergo normal regulation of surface expression and endocytosis and exhibits ready access to the cell surface upon T cell activation. Although anti-CD45RB could raise intracellular CTLA-4 expression by reducing AP-1–mediated shuttling of CTLA-4 to the lysosomes, complete inhibition by CsA suggests primary regulation at the level of transcription.

The signaling pathways that lead from T cell activation to CTLA-4 transcription are not well defined. Basal transcription is extremely low and optimal induction requires stimulation through both the TCR and CD28[11,35]. Analysis of a 335-bp upstream regulatory region of the CTLA-4 promoter reveals potential binding sites for nuclear factor AT (NFAT), AP-1, NF-κB and a number of other transcription factors[35]. Although actual binding of these factors to the CTLA-4 promoter has not been confirmed, CsA inhibits activation-induced CTLA-4 expression[10,11], which suggests regulation by NFAT or other calcineurin-regulated transcription factors. However, an indirect effect mediated by blockade of cell cycle progression cannot be excluded[10]. In contrast to activation-induced CTLA-4 expression, essentially nothing is known about the regulation of basal expression. Additionally, the notion that CTLA-4 expression can be augmented short of full-scale T cell activation is entirely new.

CD45 isoform expression is closely regulated at the level of alternative splicing. We have shown that the lower M_r isoforms are generated by a "negative" splicing factor that recognizes and splices out the alternative exons[38]. The shift from high to low M_r CD45 isoforms that occurs after T cell activation gave rise to the widely held view that CD45RB^{hi} cells are naïve, whereas CD45RB^{lo} cells have been previously activated and contain the memory population[17,39]. Nonetheless, *in vivo* and *in vitro* studies in humans and rats demonstrate that CD45 isoforms can switch from low to high, which suggests that isoform expression is carefully regulated but does not strictly reflect maturational state[20,22,40]. We have shown that certain CD45RB mAbs can dissociate CD45 isoform expression from other markers of T cell memory or activation. How anti-CD45RB alters CD45 isoform expression is unclear. However, the rapidity of the response suggests that antibody-mediated ligation results in endocytosis of the larger CD45 isoforms and re-expression of smaller isoforms.

We have previously shown that the expression of distinct CD45 isoforms results in altered cytokine secretion and differential signaling through SLP-76 and Vav[23,24]. This is likely to result from the interactions of individual isoforms with distinct ligands. Although no definitive extracellular ligands for CD45 have been identified, co-capping experiments suggest that the distinct extracellular domains of individual isoforms may direct distinct "lateral" interactions with other surface molecules on the same cell[41]. This would place the PTPase domains of different isoforms within distinct signaling complexes and could alter basal PTK activity and/or phosphorylation of particular signaling intermediates. Our findings suggest that either ligation of the higher M_r isoforms or expression of the lower M_r isoforms up-regulates constitutive CTLA-4 expression through a calcineurin-mediated pathway.

The interaction between CD45 and CTLA-4, and indeed their connection through calcineurin, was completely unanticipated. Nonetheless, potential interactions between CD45 and the calcineurin pathway have been reported previously. It has been shown that co-cross-linking CD45 and CD3 and dimerization of epithelial growth factor receptor–CD45 chimera (EGFR-CD45 chimeras) disrupt Ca^{2+} flux[42,43]. The anti-CD45RB mAb MB23G2 used in this report has been shown to augment anti-CD3–induced tyrosine phosphorylation of phospholipase Cγ1 in a T cell hybridoma[27]. However, in preliminary studies, we were unable to demonstrate that anti-CD45RB alone, or cross-linked by anti–rat Ig, increased intracellular Ca^{+2} (data not shown).

Our findings suggest that ligation of CD45 with anti-CD45RB alters signaling through calcineurin and specifically up-regulates CTLA-4 expression. We show that CTLA-4 expression can be induced without overt T cell activation. This provides a practical means of harnessing this down-regulatory pathway for the induction of long-term allograft survival. Further identification of the specific pathways involved should allow us to optimize regulation of this potent inhibitory signal for the generation of tolerance in both autoimmune and transplant settings.

Methods

Animals. Male 7–10-week-old C57BL/6 (H-2^b) recipients and BALB/c (H-2^d) donor mice (Charles River, Boston, MA) were housed individually, with free access to food and water, after transplantation.

Antibodies. For *in vivo* use, the anti-CD45RB mAb MB23G2 (ATCC, Rockville, MD) and the anti–CTLA-4 mAb 4F10 (kindly provided by J. Bluestone, University of Chicago) were purified on protein G columns according to the manufacturer's instructions (Pharmacia, Piscataway, NJ). For *in vitro* use, anti-CD8 (TIB210) and B220 (TIB164) were both from ATCC, anti–MHC Class II (212.A1) and anti-FcR (24G2) were kindly provided by C. Janeway, Yale University. Unconjugated anti-CD3 (2C-11) and anti-CD28, fluorochrome-conjugated mAbs against CD3, CD4, CD8, CD25, CD44, CD45, CD45RB, CD69 and CTLA-4 as well as fluorochrome-conjugated hamster Ig and rat Ig control antibodies were from PharMingen (San Diego, CA).

Islet isolation and transplantation. Diabetes was induced in C57BL/6 mice with a single 200 mg per kg body weight (mg/kg) intraperitoneal (i.p.) injection of streptozotocin and confirmed by persistent hyperglycemia (blood glucose >400 mg/dl). After *in situ* digestion with collagenase P (Sigma, St. Louis, MO), islets were separated by density gradient centrifugation and 400 hand-picked islets were transplanted under the left kidney capsule, as described[26]. Glycemia of <200 mg/dl by day 3 after transplantation and >250 mg/dl (after initial engraftment) defined primary graft function and graft loss, respectively. All *in vivo* studies were done in compliance with National Institutes of Health (NIH) and Yale Animal Care and Use Committee guidelines.

Treatment protocols. Recipient mice received 100 μg of intravenously injected anti-CD45RB (MB23G2) on days -1, 0 and 5, as described[26]. Anti–CTLA-4 (200 μg) was administered by i.p. injection on days -1, 0 and 1[8]. CsA (20 mg/kg/day) was administered by i.p. injection[44] on days -3 through 7. Control allograft recipients were untreated.

Immunofluorescence. Monoclonal antibodies were used at saturating concentrations to analyze surface expression by two and three-color direct immunofluorescence. CD45RB expression was analyzed by staining with fluoresecein isothiocyanate (FITC)-conjugated anti-CD45RB (clone 16A), which does not cross-react with the epitope bound by MB23G2 (data not shown). Cell surface expression of CTLA-4 was analyzed by standard immunofluorescence on nonpermeabilized cells. Intracellular CTLA-4 expression was examined by fixing cells in 2% paraformaldehyde, permeabilization with 0.5% saponin and incubation with PE-anti–CTLA-4 or control PE-conjugated hamster Ig, as described[9]. In the case of multi-color analysis, cells were stained with other markers before incubation with saponin. Cell phenotype was analyzed by using a FACStar (Becton Dickinson, San Jose, CA) 5,000 cells per sample, as described[26]. Negative controls used rat or hamster IgG fluorochrome conjugates.

Activation-induced CTLA-4 expression. CD4 cells were purified from splenocytes from treated and untreated animals by negative selection using a cocktail of mAbs against FcR, Class II, B220 and CD8, followed by incubation with immunomagnetic beads coated with anti–rat Ig and anti–murine IgG and IgM (Biomag; PerSeptive Diagnostics, Cambridge, MA). CD4 cells (50,000 cells/well) were placed in 96-well flat-bottomed plates that had been previously coated with anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml), as described[9,28]. Cells were incubated for 4 h at 37 °C in the presence of PE-anti–CTLA-4 or control PE-conjugated hamster Ig, washed, stained with FITC–anti-CD45RB (16A) and analyzed by flow cytometry. To differentiate between activation-induced accumulation of CTLA-4 on the cell surface and the intracellular compartment (due to endocytosis), CD4 cells in parallel wells were cultured for 4 h (without anti–CTLA-4) and stained for surface expression with anti–CTLA-4 or control hamster Ig (at 4 °C), as described[9,28].

Statistical analysis. Graft survival was compared using the Mann-Whitney test. Other analyses used the Student *t*-test.

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