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IL-10 Is Required for Regulatory T Cells to Mediate Tolerance to Alloantigens In Vivo[1]

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We present evidence that donor-reactive CD4$^+$ T cells present in mice tolerant to donor alloantigens are phenotypically and functionally heterogeneous. CD4$^+$ T cells contained within the CD45RB^{high} fraction remained capable of mediating graft rejection when transferred to donor alloantigen-grafted T cell-depleted mice. In contrast, the CD45RB^{low} CD4$^+$ and CD25$^+$CD4$^+$ populations failed to induce rejection, but rather, were able to inhibit rejection initiated by naive CD45RB^{high} CD4$^+$ T cells. Analysis of the mechanism of immunoregulation transferred by CD45RB^{low} CD4$^+$ T cells in vivo revealed that it was donor Ag specific and could be inhibited by neutralizing Abs reactive with IL-10, but not IL-4. CD45RB^{low} CD4$^+$ T cells from tolerant mice were also immune suppressive in vitro, as coculture of these cells with naive CD45RB^{high} CD4$^+$ T cells inhibited proliferation and Th1 cytokine production in response to donor alloantigens presented via the indirect pathway. These results demonstrate that alloantigen-specific regulatory T cells contained within the CD45RB^{low} CD4$^+$ T cell population are responsible for the maintenance of tolerance to donor alloantigens in vivo and require IL-10 for functional activity. ***The Journal of Immunology*****, 2001, 166: 3789–3796.**

A number of immune manipulations have been shown to induce operational tolerance to alloantigens in vivo, including blockade of accessory, adhesion, or costimulatory molecules at the time of alloantigen recognition (reviewed in Refs. 1 and 2). Despite this, the mechanisms involved in both the induction and maintenance of tolerance have been harder to characterize. Although there is evidence that clonal deletion and anergy operate under some circumstances (e.g., Refs. 3 and 4), accumulating data support a role for immune suppressive CD4$^+$ T cells (5–11). Precisely how CD4$^+$ T cells prevent allograft rejection is not known (1). It has been suggested that immune deviation toward a Th2 response may be involved, as Th2 cells have been found to be present in recipients with long-term surviving allografts (12, 13). However, the presence of Th2 cells in tolerant recipients is not a uniform finding (14), and direct evidence that Th2 cells are responsible for maintaining survival of allografts has not been reported. Indeed, in some situations, Th2 cells have been implicated in the development of chronic rejection.

In addition to data from experimental studies, there is also evidence that T cells with immune regulatory activities exist in patients with long-term surviving allografts (15, 16). However, reliable strategies for identifying such cells using functional assays in vitro have been difficult to develop (17). For the most part, when T cells from tolerant recipients are cultured in vitro, they exhibit a Th1 phenotype and proliferate normally in response to donor alloantigens, responses that are clearly not an accurate reflection of their functional activity in vivo (18, 19). The majority of these studies analyzed the response of unseparated T cells; thus, the effects of regulatory T cells may have been masked by the presence of naive T cells responding to alloantigens. Further characterization of putative regulatory T cells may be facilitated by using markers that distinguish naive from Ag-experienced CD4$^+$ T cells.

Abs reactive with different isoforms of the CD45 molecule have revealed phenotypic and functional heterogeneity in CD4$^+$ T cells (20–22). In mice, the majority of naive CD4$^+$ T cells express high levels of the CD45RB molecule (CD45RB^{high} CD4$^+$), whereas CD4$^+$ T cells that have previously encountered Ag express lower levels of CD45RB (CD45RB^{low} CD4$^+$) (23). Polyclonal activation of the CD45RB^{high} and CD45RB^{low} subsets in vitro showed that the CD45RB^{high} CD4$^+$ T cells synthesized predominantly IL-2, whereas CD45RB^{low} CD4$^+$ T cells synthesized IL-4 (24).

Functional analysis of CD45RB^{high} and CD45RB^{low} CD4$^+$ T cells responding to self and alloantigens has demonstrated that important regulatory interactions occur between these subsets in vivo (25, 26). Transfer of CD45RB^{high} CD4$^+$ T cells to scid mice led to the development of a Th1-mediated colitis and wasting disease that could be inhibited by cotransfer of the reciprocal CD45RB^{low} CD4$^+$ population (27, 28). Immune suppression in this system by the CD45RB^{low} subset was shown to be dependent on IL-10 and TGF-β, but independent of IL-4 (29, 30). Further subdivision of the CD45RB^{low} subset revealed that the regulatory T cells were enriched within the CD25$^+$ population (31). CD25 has also been shown to be a marker of regulatory T cells in other models of autoimmune disease (32, 33) and neonatal tolerance (34).

Previous studies from this laboratory have shown that donor-specific blood transfusion combined with depleting or nondepleting anti-CD4 Ab pretreatment can induce operational tolerance to B10 cardiac allografts in H-2^k recipients (35). CD4$^+$ T cells were shown to be responsible for both the induction (10) and maintenance of tolerance (M.N. and K.J.W., unpublished data). In this study, we have used Abs reactive with CD45RB and CD25 to

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subdivide the $CD4^+$ T cell subset from tolerant mice to further investigate the phenotype and function of these cells. Our results indicate that $CD4^+$ T cells capable of inhibiting allogeneic responses in vitro and in vivo were contained within the $CD45RB^{low}$ and $CD25^+$ populations. Importantly, neutralization of IL-10, but not IL-4, abrogated the adoptive transfer of tolerance to alloantigens by $CD45RB^{low}$ $CD4^+$ T cells, indicating that IL-10 plays a key role in this mechanism of immune suppression.

Materials and Methods

Mice

CBA.Ca (CBA, H-2^k), C57BL/10 (B10, H-2^b), BALB/c (H-2^d), (CBA × B10)F_1, and (CBA × BALB/c)F_1 were obtained from Harlan (Bicester, U.K.) and bred in the specific pathogen-free facility at Biomedical Services Unit, John Radcliffe Hospital (Oxford, U.K.). Sex-matched mice between 8 and 12 wk of age at the time of first experimental procedure were used in all experiments.

Reagents and mAbs

The following mAbs were used for flow cytometry and cell isolation: CD4 (anti-CD4)-Tricolor (Caltag, South San Francisco, CA); TIB120 (anti-class II; American Type Culture Collection (ATCC), Manassas, VA). YTA3.1.2 (anti-CD4), YTS169.4.2 (anti-CD8) (36) hybridomas were kindly provided by H. Waldmann (Oxford, U.K.). RM4-5 (anti-CD4) PerCP or PE, 53-6.7 (anti-CD8a) APC, 16A (anti-CD45RB) FITC or PE, IM7 (anti-CD44) PE, MEL-14 (anti-CD62L) PE, H1.2F3 (anti-CD69) PE, 7D4 (anti-CD25) biotin, and mouse rIL-2 were purchased from PharMingen (San Diego, CA). The following mAbs were used for in vivo assays: 11B11 (rat IgG1), a neutralizing anti-mouse IL-4 mAb (ATCC HB188); JES5-2A5 (rat IgG1), a neutralizing anti-mouse IL-10 mAb; and GL113 (rat IgG1), an isotype control mAb reactive with β-galactosidase (37).

Tolerance induction protocol

The protocol for the induction of operational tolerance was as described previously (10). Briefly, adult CBA mice were treated with 50 μg of the depleting anti-CD4 mAb, YTA3.1.2, 28 and 27 days before transplantation of a vascularized B10 heart. Two hundred fifty microliters of donor blood or 1×10^7 donor splenocytes (B10) were also injected on the second day of mAb treatment. The spleen and lymph nodes were harvested from mice with functioning heart grafts, confirmed by ECG and histology, over 100 days after heart transplantation, and used for cell sorting.

Skin transplantation

Full thickness tail skin grafts were transplanted to beds prepared on the flanks of recipient mice. Graft survival was defined by complete destruction of the skin graft, as assessed by visual inspection and confirmed by a third independent blinded observer. Allograft survival between two groups was compared using the log-rank test.

T cell-depleted mice

CBA mice were thymectomized and rested for 2 wk before being treated with depleting anti-CD4 (YTA3.1.2; 200 μg) and anti-CD8 (YTS169.4.2; 200 μg) mAbs. To allow time for the depletion of the majority of T cells and for clearance of the mAbs, the mice were rested for an additional 10 days. The mice were then either reconstituted i.v. with fractionated T cells or not treated. The day after reconstitution, mice received either a B10 or BALB/c skin graft.

Flow cytometry analysis

Spleen cells were resuspended in PBS supplemented with 0.1% BSA and 0.02% sodium azide (Sigma, St. Louis, MO) and incubated with CD4 PerCP, CD8 APC, and an Ab specific for an activation marker directly conjugated to PE for 30 min at 4°C. After washing the cells twice, they were resuspended in PBS containing 2% v/v formaldehyde and stored in the dark at 4°C until acquisition. The data were acquired by FACS and analyzed using the CellQuest software package (Becton Dickinson, Oxford, U.K.).

Cell purification

Lymphocyte cell suspensions were prepared from pooled spleen and lymph nodes (cervical, axillary, inguinal, mesenteric, and paraaortic) harvested from tolerant or naive mice in PBS containing 0.1% BSA (PBS/BSA). Erythrocytes were removed by hypotonic lysis. Cells were then incubated with PBS/BSA containing mAbs (anti-class II, anti-CD8) for 30 min at 4°C. After washing, the mAb-labeled cell suspension was incubated on a rotating wheel for 30 min with sheep anti-rat-coated Dynabeads at a ratio of one bead/cell (Dynabeads; Dynal, Wirral, U.K.). Negative cells were isolated by magnetic separation. The enriched population of $CD4^+$ T cells (80% pure) was then labeled with mAbs specific for CD45RB or CD25 and CD4 for 30 min and fractionated into $CD45RB^{high}$ $CD4^+$ and $CD45RB^{low}$ $CD4^+$ or $CD25^+CD4^+$ and $CD25^-CD4^+$ fractions by cell sorting using a FACSVantage (Becton Dickinson). The $CD45RB^{high}$ and $CD45RB^{low}$ populations were defined as the brightest staining 40% and the dullest staining 15% of $CD4^+$ T cells, respectively. All populations were >98% pure on reanalysis.

Proliferation assay

Various doses of fractionated $CD45RB^{high}$ $CD4^+$ or $CD45RB^{low}$ $CD4^+$ T cells were cultured with irradiated T cell-depleted spleen cells (5×10^5/well, 2000 rad) in 200 μl of RPMI 1640 containing 10% FCS (Myoclone; Life Technologies, Paisley, U.K.), 2 mM L-glutamine, 0.05 mM 2-ME, and 100 U/ml each of penicillin and streptomycin. Supernatants were harvested after 72 h for analysis of IL-2 production and 96 h for analysis of IFN-γ, IL-4, and IL-10 production. The cultures were pulsed with 0.5 μCi [^{3}H]thymidine (Amersham International, Amersham, U.K.) at 96 h and harvested onto glass fiber filters 18 h later. Proliferation was measured as [^{3}H]thymidine incorporation using liquid scintillation counting.

Detection of cytokines

Cytokine levels in supernatants were detected by two-site sandwich ELISA essentially as described for IL-2, IFN-γ, IL-4, and IL-10 (37). The lower limits of detection were IL-2, 125 pg/ml; IFN-γ, 188 pg/ml; IL-4, 7.8 pg/ml; and IL-10, 31.2 pg/ml.

Results

$CD45RB^{high}$ but not $CD45RB^{low}$ $CD4^+$ T cells from tolerant mice proliferate and produce Th1 cytokines in vitro in response to the tolerizing alloantigens

CBA mice treated with depleting anti-CD4 mAb in combination with donor alloantigen (B10) 28 days before transplantation accept donor (B10), but not third-party (BALB) heart grafts indefinitely (10). These mice are operationally tolerant to donor alloantigens, as assessed by their ability to accept second heart or skin grafts from the same, but not third-party donors 100 days after transplantation of the first graft. Moreover, the adoptive transfer of leukocytes from the tolerant mice to naive syngeneic recipients has been shown to result in the prolonged survival of heart grafts from the original donor, but not third-party strains (38). Despite the clear evidence for the existence of the tolerant state in vivo, unfractionated peripheral lymphocytes from tolerant mice proliferate and produce Th1 cytokines normally when they are cultured with donor APC in vitro (data not shown).

To address the conflicting behavior of leukocytes from tolerant mice in vivo and in vitro, $CD4^+$ T cells were isolated from CBA mice operationally tolerant of a B10 cardiac allograft (>100 days after transplantation) and further subdivided into two fractions based on the level of expression of the CD45RB Ag. FACS-sorted $CD45RB^{high}$ and $CD45RB^{low}$ $CD4^+$ T cells were then stimulated in vitro with T cell-depleted allogeneic spleen APCs. B10 and (CBA × B10)F_1 APCs were used to evaluate the contributions of the direct pathway and the direct plus indirect pathway of alloantigen presentation, respectively. T cell proliferation was evaluated by [^{3}H]thymidine incorporation; supernatants were harvested from the cultures after 72 h for determination of IL-2 content, or 96 h for analysis of IFN-γ, IL-4, and IL-10 production. The results from a representative experiment are shown in Fig. 1.

$CD45RB^{high}$ $CD4^+$ T cells from naive CBA mice proliferated and synthesized IL-2 and IFN-γ in response to B10, (CBA × B10)F_1, and (CBA × BALB/c)F_1 APCs (Fig. 1, *A* and *C*; cytokine production in response to B10 and (CBA × BALB/c)F_1 stimulators not shown). IL-4 and IL-10 production were below the limit of

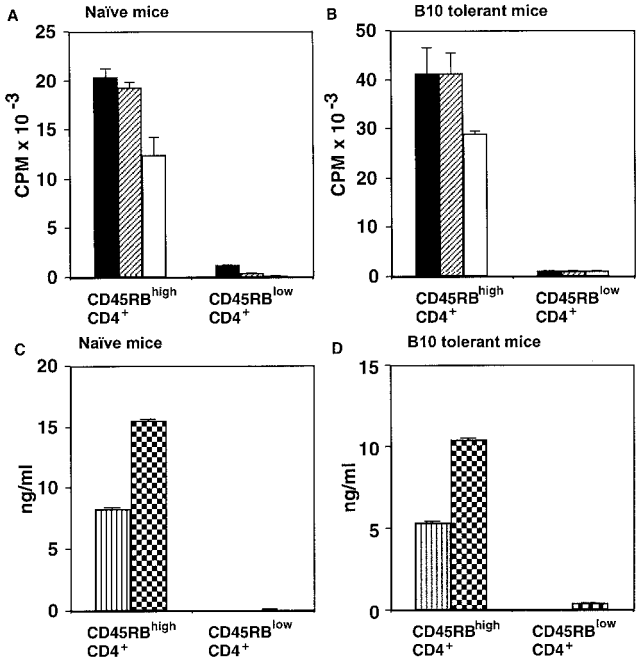

FIGURE 1. CD45RB^{high} CD4$^+$, but not CD45RB^{low} CD4$^+$ T cells from both naive and tolerant mice proliferate and produce IL-2 and IFN-γ in response to the tolerizing alloantigens. CD45RB^{high} CD4$^+$ or CD45RB^{low} CD4$^+$ T cells (2×10^5) isolated from naive mice (*A* and *C*) or B10 tolerant mice (*B* and *D*) were cultured in 96-well plates with 5×10^5 irradiated T cell-depleted spleen cells. *A* (naive) and *B* (B10 tolerant), [^{3}H]thymidine was added for 18 h after 96 h of culture. Data are expressed as mean cpm ± SEM of triplicate cultures responding to B10 (■), (CBA × B10)F_1 (▨), or (CBA × BALB/c)F_1 (□). *C* (naive) and *D* (B10 tolerant), Cytokine production in response to (CBA × B10)F_1 stimulators was assessed in the supernatant after 72 h (IL-2, ▥) or 96 h (IFN-γ, ▩) of culture. IL-4 and IL-10 levels were below the limit of sensitivity of the assay. Data are expressed as mean ± SEM of triplicate wells. Two additional experiments gave similar results.

detection of the ELISA. Like the naive population, CD45RB^{high} CD4$^+$ T cells isolated from CBA mice tolerant of B10 cardiac allografts also mounted a proliferative response and produced IL-2 and IFN-γ, but not IL-4 or IL-10, in response to each of the APC populations (Fig. 1, *B* and *D*; cytokine production in response to B10 and (CBA × BALB/c)F_1 stimulators not shown). Cytokine production was Ag specific, as cytokine levels were below the limit of detection in cultures that did not contain APC (data not shown). In contrast, CD45RB^{low} CD4$^+$ T cells isolated from either naive or tolerant mice failed to proliferate or produce Th1 (IL-2, IFN-γ) or Th2 (IL-4, IL-10) cytokines in response to B10, (CBA × B10)F_1, or (CBA × BALB/c)F_1 (Fig. 1 and data not shown). The lack of responsiveness of the CD45RB^{low} CD4$^+$ population to stimulation with alloantigens was not due to differences in the kinetics of the response, as similar results were obtained at 24, 48, and 72 h after stimulation (data not shown). The finding that the CD45RB^{low} CD4$^+$ population from naive mice failed to mount a significant response against alloantigens in vitro is in accord with previous studies (39).

Adoptive transfer of fractionated CD4$^+$ cells to T cell-depleted CBA mice

Phenotypic and functional characterization of T cell-depleted mice. To identify and characterize putative regulatory T cells present in long-term tolerant mice, an adoptive transfer system was established using T cell-depleted mice. These T cell-depleted hosts were restored with T cell subsets from naive or tolerant mice and transplanted with B10 or third-party allografts.

Initially, the phenotype of T cell-depleted mice was characterized (Fig. 2). After thymectomy and depletion of peripheral CD4$^+$ and CD8$^+$ T cells by administration of anti-CD4 and anti-CD8 mAbs, the number of T cells present in the spleen was significantly reduced (Fig. 2*B*: CD4$^+$, 37.5 ± 9.9% vs 1.6 ± 1.4%; CD8$^+$, 11.7% vs 0.5 ± 0.4% in naive vs T cell-depleted mice, respectively; $n = 4$). The small number of CD4$^+$ T cells remaining exhibited an activated phenotype (Fig. 2*C*); CD45RB$^{int\sim low}$, 34.5 ± 1.4% vs 72.3 ± 7.0%; CD44$^+$, 16.5 ± 4.5% vs 89.3 ± 3.5%; CD62L$^-$, 17.7 ± 4.5% vs 67.1 ± 4.9% in naive and T cell-depleted mice, respectively. Most of the remaining CD8$^+$ cells were also CD44$^+$ (17.7 ± 2.2% naive vs 49.3 ± 14.3% T cell depleted; data not shown). Despite the presence of these residual T cells, T cell-depleted mice were unable to reject allogeneic skin grafts (Table I).

CD45RB^{high} CD4$^+$ T cells from naive mice reconstitute rejection

To determine whether CD45RB^{high} CD4$^+$ T cells from naive CBA mice could induce rejection of B10 skin grafts, T cell-depleted CBA mice were reconstituted with varying numbers of purified CD45RB^{high} CD4$^+$ T cells, and their effect on graft survival was monitored (Table I). Mice reconstituted with less than 1×10^5 CD45RB^{high} CD4$^+$ T cells were unable to reject B10 skin grafts consistently. When 1×10^5 of CD45RB^{high} CD4$^+$ T cells were used for reconstitution, 88% of mice rejected allogeneic skin grafts. Reconstitution with 1×10^5 CD45RB^{high} CD4$^+$ T cells did not induce wasting disease, and all mice remained healthy throughout the study (data not shown). Mice reconstituted with 1×10^5

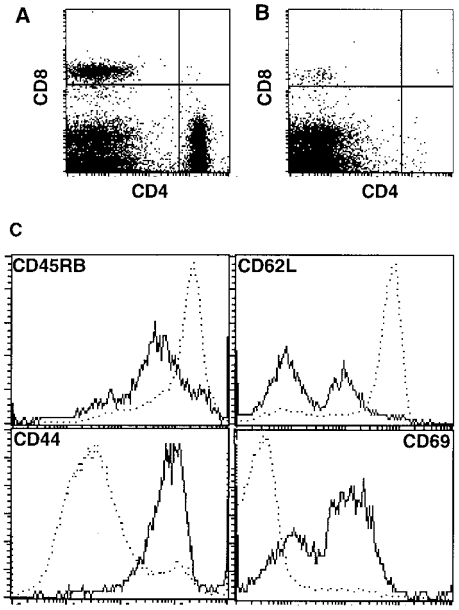

FIGURE 2. Cell surface phenotype of residual T cells in T cell-depleted mice. CD4 and CD8 profiles in the splenocytes from naive mice (*A*); T cell-depleted mice (*B*). Cell surface phenotype of residual CD4$^+$ T cells in naive (dotted line) and T cell-depleted mice (solid line) (*C*). CD4$^+$ T cells were gated for the analysis of expression of CD45RB, CD62L, CD44, and CD69. Data are presented from one of four representative experiments.

Table I. *Reconstitution of skin graft rejection by naive CD45RB^{high} CD4$^+$ T cells in T cell depleted mice*[a]

CD45RB^{high}CD4$^+$ Cell Number ($\times 10^3$)	Incidence of Rejection[b]	Median Graft Survival (days)
0	0/20	>100
3	4/7	40
10	5/7	>100
30	3/7	25
100	31/35	19
300	4/4	17.5
1000	4/4	17.5

[a] Variable doses of CD45RB^{high}CD4$^+$ T cells from naive CBA mice, purified by positive cell sorting (>98% pure) were adoptively transferred i.v. into T cell-depleted CBA recipients. Mice were transplanted with a B10 skin graft the day after cell reconstitution, and graft survival was monitored by two independent observers.

[b] Data represent the incidence of rejection (number of rejected skin grafts out of the total transplanted 60 days after transplantation).

CD45RB^{high} CD4$^+$ naive T cells will be referred to as minimally reconstituted (MR)[3] mice.

CD45RB^{low} CD4$^+$ and CD25$^+$ CD4 T cells from tolerant mice contain cells with regulatory activity in vivo

The adoptive transfer of spleen cells or purified CD4$^+$ T cells from CBA mice operationally tolerant to B10 alloantigens to naive secondary CBA recipients results in the acceptance of B10 heart grafts, indicating that tolerance in this system is an active process mediated by a subset of CD4$^+$ T cells (38) (M.N. and K.J.W., unpublished data). To address which subpopulation of CD4$^+$ T cells was responsible for immunoregulation in vivo, CD4$^+$ T cells were fractionated based upon expression of CD45RB or CD25.

FACS-sorted CD45RB^{high} or CD45RB^{low} CD4$^+$ T cells from tolerant mice were transferred into MR mice (i.e., T cell-depleted mice reconstituted with 1×10^5 naive CD45RB^{high} CD4$^+$ T cells—MR mice). The following day, mice were transplanted with B10 skin grafts, and graft survival was monitored. When 5×10^5 CD45RB^{low} CD4$^+$ T cells from tolerant mice were cotransferred, B10 skin graft rejection was abrogated and indefinite survival of the skin grafts was observed in five of six MR recipients (Fig. 3*A*; $p < 0.001$ vs MR mice; $p < 0.05$ vs MR mice treated with 5×10^5 tolerant CD45RB^{high} CD4$^+$ T cells). In contrast, cotransfer of 5×10^5 CD45RB^{high} CD4$^+$ T cells from tolerant mice resulted in the rejection of B10 skin grafts that was not statistically different from that observed in T cell-depleted mice reconstituted with 1×10^5 naive CD45RB^{high} CD4$^+$ cells alone (Fig. 3*A*; $p =$ NS MR mice treated with 5×10^5 tolerant CD45RB^{high} CD4$^+$ T cells vs MR mice).

The ability of tolerant CD45RB^{low} CD4$^+$ T cells to prevent rejection in MR mice was alloantigen specific and dose dependent. A total of 5×10^5 CD45RB^{low} CD4$^+$ T cells from CBA mice tolerant of B10 alloantigens was unable to prevent the rejection of third-party (BALB/c) skin grafts in MR mice (Fig. 3; $p < 0.01$ vs B10 graft). Immunoregulation by CD45RB^{low} CD4$^+$ T cells from tolerant mice was less effective at lower cell doses (data not shown). Moreover, cotransfer of 5×10^5 CD45RB^{low} CD4$^+$ cells from naive mice into MR mice did not prevent the rejection of B10 skin grafts (Fig. 3). These latter findings contrast with those reported by Davies and colleagues, in which CD45RB^{low} CD4$^+$ cells from naive mice were found to be capable of preventing CD45RB^{high} CD4$^+$ cells from causing rejection of neonatal islet grafts, as assessed by histology (40). This may reflect differences between the mechanisms of rejection of islet and skin allografts or the level of alloantigen expression between neonatal and adult tissue. Our results indicate that the CD45RB^{low} CD4$^+$ population in tolerant mice contains a population of regulatory T cells capable of adoptively transferring tolerance, and that this functional activity is dependent on previous exposure to donor alloantigens on the primary graft.

As CD25$^+$CD4$^+$ T cells have been shown to contain the regulatory population in a number of systems, we investigated whether CD25$^+$CD4$^+$ T cells from tolerant mice could prevent rejection in MR mice (31–34). When 5×10^5 CD25$^+$CD4$^+$ T cells from tolerant mice were cotransferred, B10 skin graft rejection was abrogated and indefinite survival of the skin grafts was observed in four of six MR recipients (Fig. 3*B*; $p < 0.001$ vs MR mice). The activity of the CD25$^+$CD4$^+$ T cells from tolerant mice was donor alloantigen specific; BALB/c skin grafts were rejected (data not shown). Cotransfer of 5×10^5 CD25$^-$CD4$^+$ T cells from tolerant mice did not prevent rejection of B10 skin grafts (Fig. 3*B*). Taken together, these findings indicate that CD45RB^{low} and CD25 can be used to enrich a subpopulation of CD4$^+$ T cells in tolerant mice that are capable of transferring specific unresponsiveness.

Immunoregulation by CD45RB^{low} CD4$^+$ T cells from tolerant mice can be demonstrated in vitro and is mediated via the indirect pathway of allorecognition

To evaluate the regulatory activity of CD45RB^{low} CD4$^+$ cells in vitro, 2×10^5 CD45RB^{high} CD4$^+$ cells from naive mice were cultured with T cell-depleted APC from the spleen of either (CBA $\times$ B10)F$_1$ (direct and indirect pathway) or B10 (direct pathway) mice in the presence or absence of 1×10^5 CD45RB^{low} CD4$^+$ cells (2:1 CD45RB^{high}:CD45RB^{low} is the physiological ratio). Data from a representative experiment are shown in Fig. 4. CD45RB^{low} CD4$^+$ T cells from tolerant mice inhibited the proliferation of naive CD45RB^{high} CD4$^+$ cells significantly in response to stimulation by (CBA $\times$ B10)F$_1$ APCs (Fig. 4*A*). Moreover, IL-2 and IFN-γ production were also significantly reduced (11.3% and 44.0%, respectively) (Fig. 4, *B* and *C*). Increasing the number of CD45RB^{low} CD4$^+$ cells from tolerant mice such that the ratio of CD45RB^{low} CD4$^+$ cells from tolerant mice to naive CD45RB^{high} CD4$^+$ cells was 1:1 resulted in more marked suppression of proliferation as well as IL-2 and IFN-γ production (12%, 0.1%, 4%, respectively; data not shown). In contrast, when B10 APCs were used as stimulators, CD45RB^{low} CD4$^+$ cells from tolerant mice were unable to inhibit proliferation or cytokine production by naive CD45RB^{high} CD4$^+$ T cells. These data suggest that indirect presentation of peptides processed from H-2^b molecules and B10 minor Ags and presented by H-2^k class II molecules expressed by (CBA $\times$ B10)F$_1$ APC is important for immunoregulation by CD45RB^{low} CD4$^+$ T cells from the tolerant mice.

CD45RB^{low} CD4$^+$ T cells from naive mice were unable to inhibit the proliferation or IFN-γ production by naive CD45RB^{high} CD4$^+$ T cells when stimulated by either (CBA $\times$ B10)F$_1$ or B10 APCs (Fig. 4, *A* and *C*; open bars). Indeed, proliferation was enhanced in some experiments (Fig. 4*A*), but this was not a consistent finding. In contrast, IL-2 production was inhibited when CD45RB^{low} CD4$^+$ cells from either naive or tolerant mice were added to the cultures (Fig. 4*B*; closed bars), indicating that inhibition of IL-2 production or consumption of IL-2 by CD45RB^{low} CD4$^+$ T cells was not dependent on previous exposure to alloantigen.

[3] Abbreviation used in this paper: MR, minimally reconstituted; MST, median survival time.

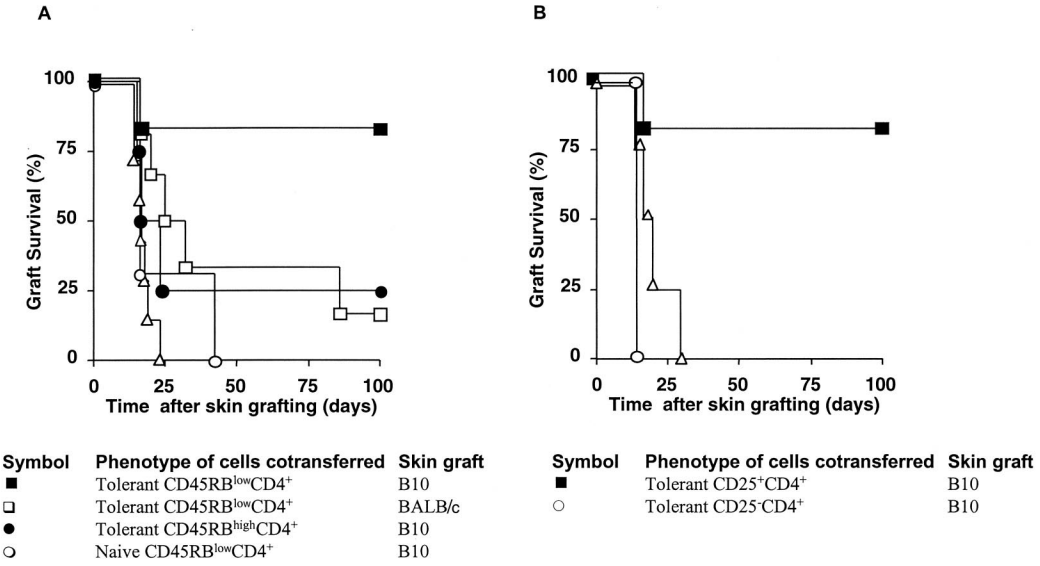

FIGURE 3. *A*, CD45RB^{low} CD4$^+$ but not CD45RB^{high} CD4$^+$ T cells from tolerant mice prevent skin graft rejection by naive CD45RB^{high} CD4$^+$ T cells. All T cell-depleted CBA mice were reconstituted with 1×10^5 naive CD45RB^{high} CD4$^+$ T cells. B10 skin grafts transplanted onto T cell-depleted CBA mice reconstituted with 1×10^5 naive CD45RB^{high} CD4$^+$ T cells alone were rejected (△; $n = 7$; median survival time (MST) 19 days). Cotransfer of 5×10^5 CD45RB^{low} CD4$^+$ T cells purified from CBA mice with long-term surviving B10 cardiac allografts prevented rejection of B10 (■; $n = 6$; MST >100 days), but not BALB/c skin grafts (□; $n = 6$; MST 26 days). Cotransfer of either 5×10^5 CD45RB^{high} CD4$^+$ T cells purified from CBA mice with long-term surviving B10 cardiac allografts (●; $n = 4$; MST 19 days) or 5×10^5 CD45RB^{low} CD4$^+$ T cells from naive CBA mice (○; $n = 7$; MST 19 days) did not prevent rejection. Repeat experiments gave similar results. *B*, CD25$^+$ CD4$^+$ T cells from tolerant mice prevent skin graft rejection by naive CD45RB^{high} CD4$^+$ T cells. All T cell-depleted CBA mice were reconstituted with 1×10^5 naive CD45RB^{high} CD4$^+$ T cells. B10 skin grafts transplanted onto T cell-depleted CBA mice reconstituted with 1×10^5 naive CD45RB^{high} CD4$^+$ T cells alone were rejected (△; $n = 4$; MST 19 days). Cotransfer of 5×10^5 CD25$^+$ CD4$^+$ T cells purified from CBA mice with long-term surviving B10 cardiac allografts prevented rejection of B10 skin grafts (■; $n = 4$; MST >100 days), whereas cotransfer of 5×10^5 CD25$^-$ CD4$^+$ T cells purified from CBA mice with long-term surviving B10 cardiac allografts did not (○; $n = 4$; MST 17 days). The data shown are representative of three independent experiments that each gave similar results.

Immune suppression transferred by tolerant CD45RB^{low} CD4$^+$ T cells is dependent on IL-10, but independent of IL-4 in vivo

Strategies that induce tolerance to alloantigens have in some circumstances been shown to be dependent on IL-4 and to involve induction of Th2 cells. To analyze the role of both IL-4 and IL-10 in the mechanism of immune suppression transferred by tolerant CD45RB^{low} CD4$^+$ T cells, these cells were transferred to MR mice and the recipients were treated weekly with either isotype control mAb or a combination of anti-IL-4 and anti-IL-10 mAbs. Neutralization of IL-4 and IL-10 completely abrogated the transfer of tolerance toward B10 alloantigens; B10 skin grafts were rejected by six of seven mice in this group ($p < 0.05$; vs GL113), with a tempo similar to MR mice. In contrast, grafts remained healthy for up to 100 days in the majority of MR mice restored with tolerant CD45RB^{low} CD4$^+$ T cells and treated with isotype control mAb (Fig. 5*A*).

To determine whether IL-4, IL-10, or both were involved in the transfer of tolerance, anti-IL-4 or anti-IL-10 was administered separately to MR mice restored with tolerant CD45RB^{low} CD4$^+$ T

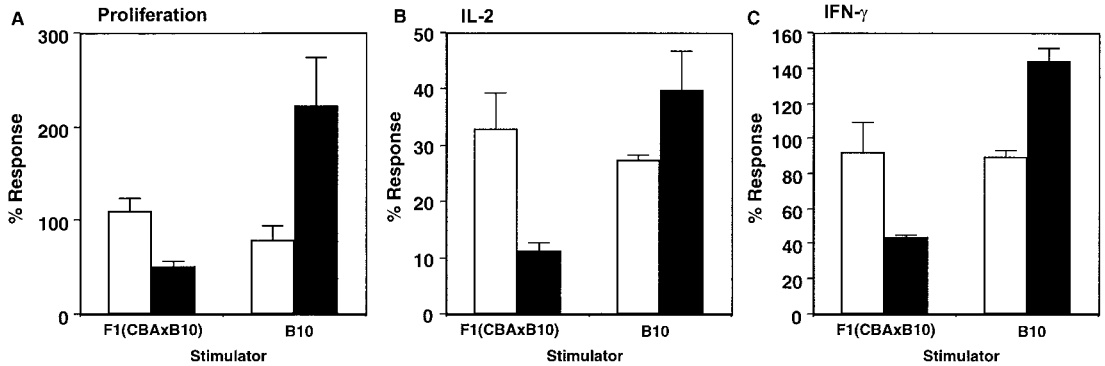

FIGURE 4. CD45RB^{low} CD4$^+$ T cells from tolerant mice suppress responses to alloantigens by naive CD45RB^{high} CD4$^+$ T cells in vitro. CD45RB^{high} CD4$^+$ T cells (2×10^5) isolated from naive mice were cocultured in 96-well plates with 1×10^5 of CD45RB^{low} CD4$^+$ T cells from either naive (□) or tolerant (■) mice in the presence of irradiated (2000 rad) T cell-depleted spleen cells (5×10^5) from either (CBA × B10)F_1 or B10 mice. At 96 h, cultures were pulsed with [^{3}H]thymidine, and cpm was measured 18 h later (*A*). Supernatants were harvested after 72 h for analysis of IL-2 (*B*) and 96 h for analysis of IFN-γ production (*C*). Data are expressed as percentage of the response of naive CD45RB^{high} CD4$^+$ T cells alone (MLR, 47,866 ± 1,202 cpm; IL-2, 4.1 ± 0.08 ng/ml; IFN-γ, 22.9 ± 0.39 ng/ml) and represent the mean for triplicate cultures. Data are expressed as mean ± SD of triplicate wells. The data shown are from one of two experiments. Proliferation in response to B10 stimulators in the presence of CD45RB^{low} CD4$^+$ T cells from tolerant mice was either the same as controls or enhanced (*A*).

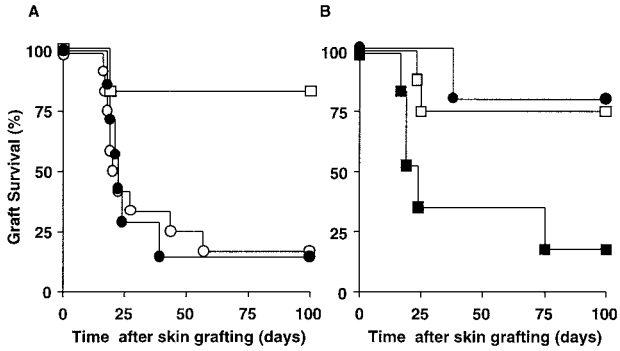

FIGURE 5. Neutralization of IL-10, but not IL-4, abrogates the regulatory functions of CD45RB^{low} CD4$^+$ T cells from tolerant mice in vivo. Survival of B10 skin grafts in T cell-depleted mice reconstituted with 1×10^5 naive CD45RB^{high} CD4$^+$ T cells in the absence ($n = 12$, ○) or presence of 5×10^5 CD4$^+$ CD45RB^{low} from tolerant mice (the remaining groups). *A*, Mice were treated at the time of T cell reconstitution with both anti-IL-4 and anti-IL-10 ($n = 7$, 5 mg/mouse of each mAb, ●), or an isotype control mAb GL113 ($n = 6$, 10 mg/mouse, □), and thereafter 1 mg/mouse anti-IL-4 Ab and 1 mg/mouse anti-IL-10 Ab or 2 mg/mouse GL113 injections every week until rejection. *B*, Mice were treated at the time of cell reconstitution with either anti-IL-4 ($n = 5$, 5 mg/mouse, ●), or anti-IL-10 ($n = 6$, 5 mg/mouse, ■), or an isotype control mAb GL113 ($n = 8$, 5 mg/mouse, □), and thereafter 1 mg/mouse every week until rejection. Data were pooled from four independent experiments.

cells. Although neutralization of IL-4 had no effect on graft prolongation (p = NS; vs GL113-treated group), treatment with anti-IL-10 inhibited the immune regulatory activity of tolerant CD45RB^{low} CD4$^+$ T cells (Fig. 5*B*). In this latter case, B10 skin grafts were rejected by five of six mice ($p < 0.01$; vs GL113-treated group). This result is not attributable to the inability of the mAb to effectively neutralize IL-4, as this same batch of Ab was shown to impair the induction of tolerance (38) and this mAb has been used to inhibit Th2 responses in vivo (41). These results indicate that IL-10, but not IL-4, is required for the function of CD4$^+$ T cells capable of transferring tolerance to alloantigens.

Discussion

In this study, we demonstrate that tolerance to alloantigen in vivo involves the induction of regulatory T cells that require IL-10 to mediate their function. Fractionation of CD4$^+$ T cells from tolerant mice with anti-CD45RB mAbs revealed functional heterogeneity in the response to alloantigen. CD45RB^{high} CD4$^+$ T cells responded normally to challenge with alloantigen in vitro and were able to reject allogeneic skin grafts when transferred alone to T cell-deficient mice (Table I). In contrast, cells contained within the CD45RB^{low} population failed to mount a proliferative response or secrete cytokines in response to alloantigen in vitro and failed to induce allograft rejection in vivo (Fig. 1). However, this population was far from inactive, as addition of these cells to cultures of naive CD45RB^{high} CD4$^+$ T cells and allogeneic APCs led to inhibition of the mixed leukocyte reaction (Fig. 4). Significantly, cotransfer of CD45RB^{low} CD4$^+$ T cells from tolerant mice to T cell-deficient mice prevented skin graft rejection initiated by naive CD45RB^{high} CD4$^+$ T cells (Fig. 3*A*). Further fractionation of CD4$^+$ T cells revealed that the regulatory cells were contained within the CD25$^+$ subset (Fig. 3*B*). The regulatory mechanism involved IL-10, but not IL-4, as administration of anti-IL-10 but not anti-IL-4 mAbs abrogated immune suppression transferred by alloreactive CD45RB^{low} CD4$^+$ T cells in vivo (Fig. 5). These data demonstrate that in mice rendered long-term tolerant to alloantigens, regulatory T cell populations capable of inhibiting graft rejection in vivo are contained within the CD25$^+$ and CD45RB^{low} CD4$^+$ subset. It seems likely that this T cell-dependent mechanism of active suppression, as opposed to clonal deletion, plays a dominant role in the maintenance of tolerance to alloantigen in this model.

In vitro analysis showed that alloreactive T cells, capable of responding to donor alloantigens presented via the direct or indirect pathways, are present at similar levels within the CD45RB^{high} CD4$^+$ population in naive and tolerant mice with long-term surviving B10 cardiac allografts (Fig. 1). Interestingly, despite the continuous presence of donor alloantigens, i.e., for 100 days since transplantation, these cells were contained within the CD45RB^{high} CD4$^+$ T cell subset. This is in contrast to findings in the rat in which after blood transfusion alone the CD45RC$^+$ population was depleted of cells reactive with blood donor alloantigens (42). The prolonged presence, 100 vs 14 days, as well as the source of donor alloantigen, a cardiac allograft vs blood transfusion, may account for this difference, but it may also reflect functional differences between T cell subsets identified by expression of different isoforms of CD45.

Despite clear evidence that immune suppressive CD4$^+$ T cells play a role in tolerance to alloantigens (6, 8, 13, 43), little is known about their mechanism of action and allorecognition properties. The findings presented in this work identify IL-10 as an essential component of the mechanism of immune suppression in mice with long-term surviving allografts. The alloreactive regulatory CD4$^+$ T cells identified in this study resemble regulatory T cells present in normal mice that control inflammatory responses to intestinal Ags. These latter cells are also contained within the CD25$^+$ CD45RB^{low} CD4$^+$ T cell population and are dependent on IL-10 (29) as well as TGF-β for their function (30). Interestingly, neutralization of TGF-β has recently been shown to abrogate tolerance to alloantigens in the rat (44) and in the mouse (45). Alloantigen-specific regulatory T cells dependent on IL-10 have also been found in patients (16). Similarly, regulatory T cells that can control pathogenic responses toward self Ags have been identified in the T cell repertoire of normal individuals and are thought to be responsible for preventing autoimmune disease (32, 33, 46, 47). These populations may be related to regulatory T cell clones that have been generated by chronic activation of both mouse and human CD4$^+$ T cells in the presence of IL-10 in vitro (48). The fact that regulatory T cells with similar properties control pathologic responses against Ags as diverse as alloantigens, autoantigens, and intestinal Ags highlights the importance of this mechanism in the regulation of the immune response.

The finding that IL-10 is required for the function of this population of regulatory T cells that inhibit graft rejection in vivo is in accord with the known anti-inflammatory properties of this cytokine. IL-10 has been shown to inhibit Ag-induced proliferation and cytokine synthesis by T cells most probably through its effects on APCs, particularly down-regulation of molecules involved in T cell costimulation (49). However, IL-10 is pleiotropic, and contradictory results concerning its role in the regulation of immune responses to transplantation Ags have been reported. In vitro, IL-10 was shown to inhibit alloantigen-induced proliferation in a dose-dependent manner (50). Somewhat surprisingly, systemic injection or local high concentrations of IL-10 was found to accelerate graft rejection (51). In contrast, expression of viral IL-10 in nonvascularized cardiac allografts by retroviral transduction of the graft before transplantation resulted in graft prolongation (52), and neutralization of IL-10 by anti-IL-10 therapy abrogated skin graft prolongation induced by portal venous injection of allogeneic splenocytes (53). Moreover, high levels of IL-10 have been described as associated with tolerance to HLA-mismatched bone

marrow stem cells (54), and in renal transplant patients with EBV-induced posttransplant lymphoproliferative disorder increased levels of IL-10 correlated with operational tolerance to the graft in aciclovir-treated patients (55). These conflicting data may be explained by the observation that there are dose-dependent differences in the effect of IL-10 in vivo (56).

Neutralizing IL-4 failed to affect the transfer of tolerance to alloantigens by CD45RB^{low} CD4$^+$ T cells in vivo in this study. Consistent with this, CD45RB^{low} CD4$^+$ T cells from tolerant mice failed to produce detectable levels of IL-4 in response to stimulation with the tolerogen in vitro. There are conflicting reports on the role of Th2 cells and IL-4 in tolerance to alloantigens. Induction of tolerance in a number of model systems has been shown to correlate with increases in Th2 cytokines (12, 57), and IL-4 has been shown to be absolutely required for neonatal tolerance induction (58, 59). However, in adult animals there is no direct evidence that Th2 cells are involved in the induction or maintenance of tolerance (14). Indeed, Th2 cells have been shown to trigger, not prevent graft rejection (60). Although Th2 cells per se may not be responsible for tolerance induction, the cytokines they produce or stimulate may be critical. Thus, although the majority of experiments using IL-4 knockout mice have suggested that IL-4 does not play a role in long-term graft acceptance (61), other studies reveal that IL-4 does play a role when conditions are limiting (11, 38). Taken together, these data suggest that IL-4 may be involved in the induction, but not the maintenance of tolerance, playing a role in the growth or expansion of regulatory T cells, but not their effector function (38). The precise role that particular immune suppressive cytokines play in the mechanism of action of regulatory T cells responsive to either self or alloantigens remains controversial and may in part be dependent on the phase of the tolerance process (induction vs maintenance) examined.

CD45RB^{low} CD4$^+$ T cells from long-term tolerant mice were able to regulate responses to alloantigen in vitro only when donor alloantigens were presented via the indirect pathway of allorecognition by the APCs present in the culture. This suggests that tolerant CD45RB^{low} CD4$^+$ T cells function predominantly by recognizing alloantigen via the indirect pathway of allorecognition. Teleologically, this makes a great deal of sense; in tolerant recipients with long-term surviving allografts, the main route of alloantigen presentation under normal circumstances will be via the indirect pathway. After transplantation, passenger leukocytes migrate out of the graft into the draining lymphoid tissue and initiate the rejection response (62). Administration of immunosuppressive agents or the induction of tolerance to donor alloantigens before transplantation will inhibit rejection, but in most cases does not inhibit leukocyte migration. Thus, long-term surviving grafts have few passenger leukocytes, and the transplanted tissue itself may not possess the costimulatory capacity to stimulate naive T cells to respond (63). In contrast, T cells capable of responding to alloantigens via the indirect pathway will be stimulated continuously after transplantation (64). In support of this conclusion, it has been shown that regulatory T cells require constant stimulation by donor alloantigens to maintain their function both following infusion of alloantigen (65, 66) and after transplantation (67). Thus, presentation of donor-derived allopeptides by recipient APC is sufficient to maintain the functional activity of CD45RB^{low} CD4$^+$ T cells in tolerant recipients and is required for the detection of the functional activity of these cells in vitro.

In summary, we have demonstrated that tolerance to alloantigens in vivo is maintained by a subpopulation of CD4$^+$ T cells that is characterized by expression of CD45RB^{low} or CD25$^+$, requires IL-10 to function in vivo, and responds to donor alloantigen via the indirect pathway of allorecognition. The characterization of both the phenotype and function of the regulatory T cells responsible for both the induction and maintenance of tolerance to alloantigens is key for the development and implementation of new strategies for inducing tolerance in transplantation and autoimmune disease as well as for identifying markers of tolerance that can be used to assess transplant patients with long-term surviving grafts. In many experimental and clinical situations in which operational tolerance to donor alloantigens can be demonstrated, in vivo antidonor reactivity remains detectable in vitro. It is possible that the presence of regulatory cells in these situations may be masked by naive, CD45RB^{high} cells responding to donor Ag. These observations may therefore allow transplant patients with long-term surviving to be assessed more effectively for the level of tolerance to donor Ags they exhibit.

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