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CD25$^+$CD4$^+$ Regulatory T Cells Prevent Graft Rejection: CTLA-4- and IL-10-Dependent Immunoregulation of Alloresponses[1]

Cherry I. Kingsley, Mahzuz Karim, Andrew R. Bushell,[2] and Kathryn J. Wood

Specific and selective immunological unresponsiveness to donor alloantigens can be induced in vivo. We have shown previously that CD25$^+$CD4$^+$ T cells from mice exhibiting long-term operational tolerance to donor alloantigens can regulate rejection of allogeneic skin grafts mediated by CD45RB^{high}CD4$^+$ T cells. In this study, we wished to determine whether donor-specific regulatory cells can be generated during the induction phase of unresponsiveness, i.e., before transplantation. We provide evidence that pretreatment with anti-CD4 Ab plus a donor-specific transfusion generates donor-specific regulatory CD25$^+$CD4$^+$ T cells that can suppress rejection of skin grafts mediated by naive CD45RB^{high}CD4$^+$ T cells. Regulatory cells were contained only in the CD25$^+$ fraction, as equivalent numbers of CD25$^-$CD4$^+$ T cells were unable to regulate rejection. This pretreatment strategy led to increased expression of CD122 by the CD25$^+$CD4$^+$ T cells. Blockade of both the IL-10 and CTLA-4 pathways abrogated immunoregulation mediated by CD25$^+$ T cells, suggesting that IL-10 and CTLA-4 are required for the functional activity of this population of immunoregulatory T cells. In clinical transplantation, the generation of regulatory T cells that could provide dynamic control of rejection responses is a possible route to permanent graft survival without the need for long-term immunosuppression. *The Journal of Immunology,* **2002, 168: 1080–1086.**

E vidence that immune regulation plays an important role in tolerance induction to peripheral self Ags and alloantigens has been clearly demonstrated in experimental models of autoimmune disease (1–5) and transplantation (6–14). The discovery that CD4$^+$ T cells are pivotal in mediating immune regulation (1, 2, 4, 12) has focused attention on the identification of cell surface molecules that allow further enrichment of regulatory populations and elucidation of their mechanisms of action.

Selection of CD4$^+$ T cells expressing CD25 (IL-2Rα chain) has been shown to enrich for immunoregulatory activity (15). Although CD25$^+$ cells represent a minor subset (5–10%) of CD4$^+$ T cells in naive mice and CD25 is generally regarded as an activation marker, depletion of CD25$^+$ cells in normal mice leads to autoimmune disease (15). Moreover, the adoptive transfer of CD25$^+$CD4$^+$ T cells from normal mice can prevent autoimmune disease caused by pathogenic effector cells (16–20). Although the mechanisms of immune regulation mediated by CD25$^+$CD4$^+$ T cells are not fully characterized, in vivo studies using neutralizing and blocking Abs have highlighted the role of the cytokines IL-10 (21, 22) and TGF-β (19) and the CD28 homolog CTLA-4 (CD152; Refs. 19 and 23).

We have recently shown that CD45RB^{low}CD4$^+$ and CD25$^+$CD4$^+$ T cell subpopulations isolated from animals with long-term surviving allografts can regulate rejection of skin allografts mediated by CD45RB^{high}CD4$^+$ T cells from naive mice (14), indicating that long-term operational tolerance in this system is dependent on active immune regulation. Similar results have recently been reported by Gregori et al. (24) using CD25$^+$CD4$^+$ T cells from mice with long-term islet allografts. The possibility that specific regulatory T cells could be generated before transplantation and afford the graft protection from the outset is appealing. Furthermore, if such cells can expand by an "infective" process (25), it might be possible to achieve the tantalizing goal of lifelong operational tolerance without the need for prolonged immunosuppression. An understanding of the mechanism of such regulation will play a vital role in the development of such tolerogenic strategies.

Preliminary evidence that regulatory cells develop as a consequence of pretreatment with donor alloantigen in combination with anti-CD4 therapy was initially provided by the finding that adoptive transfer of cells from pretreated but untransplanted animals into naive recipients resulted in prolongation of cardiac graft survival (26). The hypothesis that regulation in this system is CD4 dependent was demonstrated by the fact that naive leukocytes are unable to break the unresponsive state unless putative regulatory cells are targeted by the addition of depleting anti-CD4 Ab (10).

In this study, we investigated the hypothesis that infusion of donor alloantigen under the cover of an anti-CD4 Ab leads to the development of CD4$^+$ regulatory T cells that can be enriched on the basis of CD25 expression. We demonstrate that this pretreatment strategy generates alloantigen-specific CD25$^+$CD4$^+$ T cells with the ability to regulate rejection of skin allografts. Prior exposure to anti-CD4 Ab and donor alloantigen increases the proportion of CD4$^+$CD25$^+$ T cells that express CD122 (IL-2Rβ). Furthermore we demonstrate that blockade of both CTLA-4 and IL-10 pathways abrogates the immune regulation of alloresponses mediated by CD25$^+$CD4$^+$ T cells, thus providing evidence that both CTLA-4 and IL-10 play an important role in this mechanism of immune regulation in vivo.

Materials and Methods

Mice

CBA.Ca (CBA, H-2^k), C57BL/10 (B10, H-2^b), BALB/c (H-2^d), and CBA Rag $1^{-/-}$ (H-2^k) (kindly provided by Dr. D. Kioussis, Division of Molecular Immunology, National Institute for Medical Research, Mill Hill, London, U.K.) mice were obtained from and housed in the Biomedical Services Unit of the John Radcliffe Hospital (Oxford, U.K.). Sex-matched mice between 6 and 12 wk of age at the time of first experimental procedure were used in all experiments.

Reagents and mAbs

The following reagents were used for flow cytometry and cell isolation: TIB120 (anti-class II; American Type Culture Collection, Manassas, VA), RM4-5 (anti-CD4) CyChrome, RM4-5 (anti-CD4) PerCP, TM-β1 (anti-CD122) FITC, 16A (anti-CD45RB) PE, 7D4 (anti-CD25) biotin, streptavidin-PE, and streptavidin-allophycocyanin were purchased from BD PharMingen (San Diego, CA). The hybridomas YTA3.1.2, (anti-CD4; Ref. 27), YTS177.9 (anti-CD4; Ref. 25), and YTS169 (anti-CD8; Ref. 25) were kindly provided by Prof. H. Waldmann (Sir William Dunn School of Pathology, Oxford, U.K.).

The following mAbs were used for in vivo experiments: anti-mouse CTLA-4 (clone UC10-4F10-11; 0.8 mg/week; Ref. 28; hybridoma kindly provided by Dr. J. Bluestone, Diabetes Center, University of California, San Francisco, CA), purified hamster IgG (0.8 mg/week; Jackson ImmunoResearch Laboratories, West Grove, PA), 1B1.2 (rat IgG1; 1 mg at time of cell transfer, 0.5 mg/week thereafter), a blocking mAb reactive with mouse IL-10R (29), and GL113 (rat IgG1; 1 mg at time of cell transfer, 0.5 mg/week thereafter; Ref. 30), an isotype control mAb reactive with β-galactosidase.

Pretreatment protocol

Adult CBA mice received 200 μg of the anti-CD4 mAb YTS177 i.v. on days -28 and -27. On day -27, they also received 250 μl of B10 (donor-specific transfusion (DST)[3]) or BALB/c (third-party) blood i.v. Spleens were harvested on day 0 for cell isolation (Fig. 1).

Skin transplantation

T cell-deficient (T cell-depleted (Ref. 14) or Rag$1^{-/-}$) mice either were reconstituted i.v. with fractionated T cells or remained untreated. The day after reconstitution, all mice received a B10 skin graft. Full-thickness tail skin was transplanted to graft beds prepared on the flanks of recipient mice. Grafts were monitored two to three times per week, and graft rejection was defined by complete destruction of the skin. Allograft survival between any two groups was compared by the log-rank sum test (31) using software developed and kindly provided by Dr. S. Cobbold (Sir William Dunn School of Pathology).

Cell purification and adoptive transfer

CD45RB^{high}CD4$^+$ T cells were isolated from lymph nodes and spleens of naive CBA mice, and CD25$^+$CD4$^+$ T cells were obtained from spleens of animals pretreated with the YTS177/DST-tolerizing protocol or the relevant control protocol.

Lymph nodes and spleens were harvested and single cell suspensions were prepared. After red cell lysis, the cells were resuspended in PBS/1% BSA at 2×10^8/ml and were then incubated with YTS169 (200 μg/ml) and TIB120 (100 μg/ml) for 20 min at 4°C to deplete CD8$^+$ and class II$^+$ cells, respectively. The cell suspension was washed and added to sheep anti-rat-coated Dynabeads (Dynal Biotech, Oslo, Norway) at a ratio of one bead per cell and this was then incubated on a rotating wheel at 4°C for 15 min. Negative cells were isolated by magnetic separation, counted, resuspended to 2×10^8 cells/ml, and stained with mAbs specific for CD4, CD45RB, or CD25 for 20 min. Cells were fractionated into CD45RB^{high}CD4$^+$ or CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ fractions by cell sorting using a FACSVantage (BD Biosciences, Mountain View, CA). The CD45RB^{high} population was defined as the brightest staining 40% of CD4$^+$ T cells. On reanalysis, all populations were >95% pure. Each experiment contained minimally reconstituted (MR)-only mice to validate the efficacy of the CD45RB^{high} effector population.
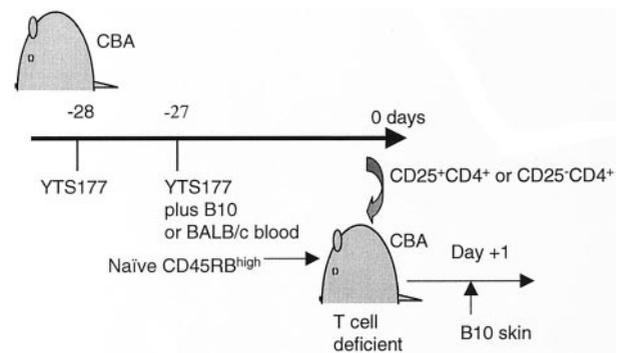
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[3] Abbreviations used in this paper: DST, donor-specific transfusion; MST, median survival time; MR, minimally reconstituted.

FIGURE 1. Protocol for pretreatment and adoptive transfer. CBA mice were pretreated with YTS177 on days -28 and -27. On the second day of pretreatment, they received either a transfusion of donor-specific B10 blood or third-party BALB/c blood. On day 0, spleens were harvested, cells were sorted into CD25$^+$CD4$^+$ or CD25$^-$CD4$^+$ T populations, and they were then reconstituted together with CD45RB^{high}CD4$^+$ T cells (sorted from naive animals) into T cell-deficient recipient mice. These mice were grafted 1 day later with a B10 skin graft.

Flow cytometric analysis

All incubation steps were conducted for 30 min at 4°C. Single cell splenocyte suspensions were prepared, erythrocytes removed by hypotonic lysis, and the cells were resuspended in FACS buffer consisting of PBS supplemented with 2% FCS (PAA Laboratories, Linz, Austria) and 0.02% sodium azide (Sigma-Aldrich, St. Louis, MO). The cells were then incubated with Abs for cell surface staining (CD122-FITC, CD4-PerCP, and CD25-biotin), washed, incubated with streptavidin-allophycocyanin, washed again, and fixed by incubating in PBS containing 4% (v/v) formaldehyde (Sigma-Aldrich). For CTLA-4 staining, cells were incubated in permeabilization buffer consisting of FACS buffer supplemented with 0.5% saponin (Sigma-Aldrich) for 30 min and were then washed and incubated with CTLA-4-PE Ab for intracellular staining. The cells were washed, fixed in PBS containing 2% (v/v) formaldehyde, and stored at 4°C until acquisition. Data were acquired using a FACSort and were analyzed using the CellQuest software package (BD Biosciences, Oxford, U.K.). Statistical analysis was performed using the two-tailed paired t test.

Results

Pretreatment with YTS177/DST generates CD25$^+$CD4$^+$ T cells that prevent skin graft rejection

Previous work from our laboratory has demonstrated that mice exhibiting operational tolerance to donor alloantigens in vivo contain CD4$^+$ regulatory T cells that can be enriched on the basis of low expression of CD45RB or expression of CD25 (14). In the present study, we examined the induction phase of tolerance to provide an insight into the development of these regulatory populations and explore their mechanisms of action.

CD45RB^{high}CD4$^+$ T cells (1×10^5) from naive animals reconstitute acute skin graft rejection when infused into T cell-deficient mice (median survival time (MST) = 22.5 days, $n = 8$; Fig. 2). These animals are referred to as MR mice. Unreconstituted but grafted T cell-deficient mice all accepted their skin grafts over 100 days (data not shown). To investigate the ability of putative regulatory T cells to prevent skin graft rejection, fractionated populations were cotransferred with 1×10^5 CD45RB^{high}CD4$^+$ T cells. Cotransfer of 5×10^5 CD25$^+$CD4$^+$ T cells isolated from mice 28 days after pretreatment with YTS177/DST prevented rejection mediated by the CD45RB^{high} cells with five of six mice accepting their skin grafts over 100 days (MST > 100 days, $n = 6$, $p < 0.05$ vs MR mice; Fig. 2). These surviving grafts were between 90 and 100% of their original size, had no signs of tissue necrosis, and showed dense hair growth indicating the efficiency of regulation in this system. To determine whether the regulatory
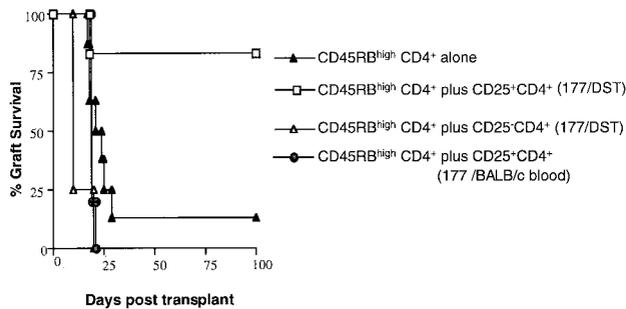
FIGURE 2. CD25⁺CD4⁺ T cells from YTS177/DST-pretreated mice prevent skin graft rejection mediated by CD45RB� ʰⁱᵍʰCD4⁺ T cells. All T cell-deficient CBA mice were reconstituted with 1×10^5 naive CD45RBᵍʰCD4⁺ T cells (MR mice). Cells were isolated from mice 28 days after pretreatment. Mice reconstituted with 1×10^5 CD45RBʰⁱᵍʰCD4⁺ T cells alone acutely rejected B10 skin grafts (▲; $n = 8$, MST = 22.5). Cotransfer of 5×10^5 CD25⁺CD4⁺ T cells isolated from YTS177/DST-pretreated CBA mice prevented rejection of B10 skin grafts (□; $n = 8$, MST > 100 days), whereas 5×10^5 CD25⁻CD4⁺ T cells from YTS177/DST-pretreated CBA mice were unable to prevent rejection (△; $n = 4$, MST = 10 days). Cotransfer of CD25⁺CD4⁺ T cells isolated from CBA mice pretreated with BALB/c blood under the cover of YTS177 (third-party control; ●; $n = 5$, MST = 19 days) did not prevent rejection of B10 skin grafts.
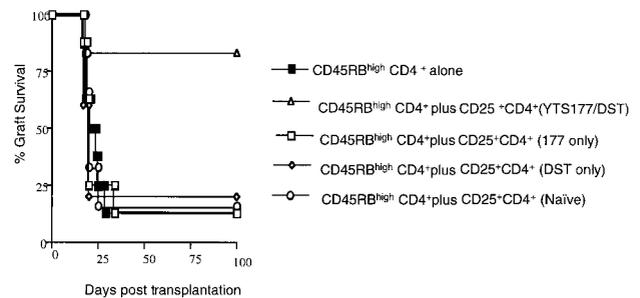

FIGURE 3. Generation of CD25⁺CD4⁺ regulatory T cells requires the simultaneous presence of donor alloantigen and anti-CD4 Ab. All T cell-deficient CBA mice were reconstituted with 1×10^5 naive CD45RBʰⁱᵍʰCD4⁺ T cells. Mice reconstituted with 1×10^5 CD45RBʰⁱᵍʰCD4⁺ T cells alone acutely rejected B10 skin grafts (■; $n = 8$, MST = 22.5). Cotransfer of 5×10^5 CD25⁺CD4⁺ T cells isolated from YTS177/DST-pretreated CBA mice prevented rejection of B10 skin grafts (△; $n = 8$, MST > 100 days), whereas cotransfer of 5×10^5 CD25⁺CD4⁺ T cells from mice pretreated with either YTS177 only (□; $n = 8$, MST = 20) or DST only (◇; $n = 5$, MST = 20 days) or isolated from naive CBA mice (○; $n = 6$, MST = 20 days) did not prevent rejection of skin grafts.

cells were only contained in the CD25⁺ fraction, MR mice were infused with 5×10^5 CD25⁻CD4⁺ T cells from YTS177/DST-pretreated mice. This resulted in the rapid rejection of B10 skin grafts (MST = 10 days, $p < 0.01$ vs MR mice reconstituted with 5×10^5 CD25⁺CD4⁺ T cells from YTS177/DST pretreated animals; Fig. 2), demonstrating that at these cell numbers, CD25⁻CD4⁺ T cells from pretreated mice were unable to regulate rejection.

Regulation of skin graft rejection is alloantigen specific

To investigate whether the regulation in this system was alloantigen specific, we isolated CD25⁺CD4⁺ T cells from mice pretreated with third-party (BALB/c) blood under the cover of YTS177. Cotransfer of 5×10^5 CD25⁺CD4⁺ T cells isolated from these mice with 1×10^5 CD45RBʰⁱᵍʰCD4⁺ T cells (from naive animals) resulted in acute rejection of B10 skin grafts (MST = 19 days, $p < 0.05$ vs 177/DST (B10 blood) pretreated mice; Fig. 2), demonstrating that regulation in this system is an alloantigen-specific phenomenon.

Generation of regulatory CD25⁺CD4⁺ T cells requires the simultaneous presence of donor alloantigen and anti-CD4 Ab

To determine whether the presence of both donor alloantigen and anti-CD4 Ab is required for the generation of a population of CD25⁺CD4⁺ regulatory T cells, we pretreated mice with either YTS177 only (days −28 and −27) or DST (day −27) only and transferred CD25⁺CD4⁺ T cells into MR mice.

CD25⁺CD4⁺ T cells (5×10^5) isolated after pretreatment with either 177 (only) or DST (only) were unable to regulate rejection mediated by 1×10^5 CD45RBʰⁱᵍʰCD4⁺ T cells (MST = 20 and 20 days, respectively, $p < 0.05$, 177 alone or DST alone vs YTS177/DST-pretreated mice; Fig. 3).

In models of autoimmunity, CD25⁺CD4⁺ T cells with regulatory capacity in vivo have been isolated from naive unmanipulated cell donors (1–5). Therefore, we wished to determine whether CD25⁺CD4⁺ isolated from naive animals could regulate allograft rejection in our system. Cotransfer of 5×10^5 CD25⁺CD4⁺ T cells isolated from naive animals into MR mice resulted in acute

rejection of B10 skins in five of six animals (MST = 20 days, $p < 0.01$ vs YTS177/DST-pretreated mice; Fig. 3), demonstrating that in this system, equivalent numbers of CD25⁺CD4⁺ T cells from naive animals are unable to regulate skin allograft rejection mediated by CD45RBʰⁱᵍʰ effector cells.

Taken together, the data clearly demonstrate that the generation of CD25⁺ cells with the capacity to regulate skin allograft rejection in this model depends on coexposure of recipient cells to alloantigen in the form of a DST and anti-CD4 Ab.

Pretreatment with the tolerizing YTS177/DST protocol: effect on expression of CD25 and CTLA-4

Having established that infusion of alloantigen in combination with the anti-CD4 mAb YTS177 generates CD25⁺CD4⁺ T cells that can regulate skin allograft rejection in a donor alloantigen-specific manner in vivo, we wished to determine whether CD4⁺ T cells from pretreated animals had particular phenotypic changes compared with CD4⁺ T cells from naive or control pretreated mice. Our initial attention focused on CD25, and we asked whether pretreated mice contained a higher proportion of CD25⁺CD4⁺ T cells or expressed CD25 at a higher level than control mice. Preliminary analysis showed only a very modest increase in the number of CD25⁺CD4⁺ T cells following pretreatment with the YTS177/DST tolerogenic protocol, but we speculated that significant changes might be more readily detected following re-exposure to alloantigen. Therefore, mice given the YTS177/DST pretreatment were rechallenged with the original tolerizing Ag in the form of a second DST at day 0 (normally the day of cell isolation and transfer in the adoptive transfer protocol) and were analyzed 2 days later by flow cytometry. When compared with CD4⁺ T cells from naive mice or control mice given DST only at day −27 and DST rechallenge at day 0, a higher proportion of CD4⁺ T cells from pretreated and rechallenged mice were positive for CD25 (15.8% compared with 10.01 and 11.1% in naive and and DST (only) controls, respectively). However, mice pretreated with YTS177 (only) at days −28 and −27 then rechallenged with donor Ag at day 0 also displayed an increase in the proportion of cells positive for CD25 (13.5%).

CTLA-4 is a CD28 homolog that has a negative regulatory role in T cell activation (32–34) and has recently been shown to be constitutively expressed on CD4⁺CD25⁺ immunoregulatory cells

(19, 23, 35). Therefore, we examined the expression of CTLA-4 by $CD25^+CD4^+$ T cells in our system. Intracellular staining revealed that a greater proportion of $CD25^+CD4^+$ T cells from mice pretreated with the YTS177/DST protocol and re-exposed to donor Ag were positive for CTLA-4 than were cells from either naive or DST (only) control mice (45% compared with 32 and 36%, respectively). However, control animals pretreated with YTS177 (only) then re-exposed to alloantigen also showed a similar increase in the proportion of $CD25^+CD4^+$ T cells that were positive for CTLA-4 (42%). Therefore, at present we are unable to conclude that the YTS177/DST-tolerizing protocol uniquely leads to an increase in the proportion of cells that express either CD25 or CTLA-4. However, we are aware that a limiting factor in this system at present is that our phenotypic analysis is unable to differentiate between alloantigen-specific $CD25^+CD4^+$ T cells generated by the YTS177/DST pretreatment and those "background" $CD25^+CD4^+$ T cells that occur spontaneously and, as shown in Fig. 3, are unable to regulate rejection responses in this model. Experiments in progress, which attempt to enrich for these Ag-specific cells, may shed further light on their CD25 and CTLA-4 expression.

Induction of unresponsiveness leads to increased expression of CD122 by $CD25^+CD4^+$ T cells

In an attempt to characterize the regulatory population further, we investigated the phenotype of cells isolated from YTS177/DST-pretreated animals. CD25 is up-regulated as a consequence of T cell activation, but, in view of the fact that in our system $CD25^+CD4^+$ T cells clearly regulate responses to alloantigens, we looked for other phenotypic changes that might distinguish between activated and regulatory populations. We chose to examine the expression of CD122 (the IL-2Rβ chain), and we speculated that reduced expression of this molecule might allow $CD25^+CD4^+$ regulatory cells to compete for alloantigen or costimulatory molecules without the ability to bind IL-2 with high affinity. As described above, cells were isolated from YTS177/DST-pretreated animals rechallenged with a second DST, and from naive mice and relevant control animals. However, contrary to our hypothesis, flow cytometric analysis revealed that CD122 was expressed by a greater proportion of $CD25^+CD4^+$ T cells following the induction of specific unresponsiveness and Ag rechallenge in vivo ($p \leq 0.002$, YTS177/DST plus DST day 0 vs all other groups, $n = 4$ each group; Fig. 4).
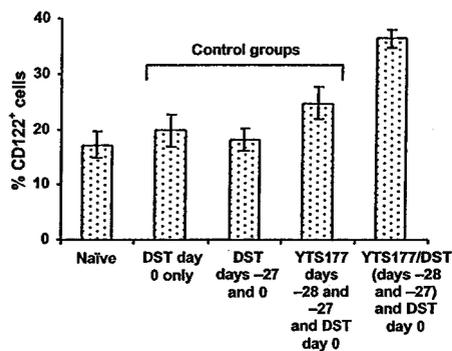

FIGURE 4. Pretreatment and Ag rechallenge increase the proportion of $CD25^+CD4^+$ T cells expressing CD122. CBA mice were pretreated with the tolerizing 177/DST protocol, followed by an additional DST at day 0. Spleen cells were isolated 48 h later and were analyzed for CD4, CD25, and CD122 expression by FACS. Data represent the percentage of $CD25^+CD4^+$ T cells expressing CD122. Control pretreatments were as follows: DST at day 0; DST at days −27 and 0; YTS177 at days −28 and −27 plus DST at day 0. Results are shown as mean ± SD.

Immune regulation by $CD25^+CD4^+$ T cells from YTS177/DST-pretreated mice is dependent on the CTLA-4 pathway

CTLA-4 has been implicated in the mechanism of action of $CD25^+CD4^+$ regulatory T cells in autoimmune models (19, 23, 35). Therefore, we sought to establish whether CTLA-4 was involved in the regulatory activity of the $CD25^+CD4^+$ T cells in our system. MR mice were reconstituted with 5×10^5 $CD25^+CD4^+$ T cells from YTS177/DST-pretreated animals the day before receiving a skin graft. Recipients received either anti-CTLA-4 mAb or control hamster IgG at the time of cell transfer and weekly thereafter. Treatment continued for 6 wk or until rejection was observed. Treatment with the control Ig did not inhibit the regulatory activity of $CD25^+CD4^+$ T cells from YTS177/DST-pretreated mice, as animals receiving this treatment accepted their skin grafts long term ($n = 4$, MST > 100 days; Fig. 5). In contrast, administration of anti-CTLA-4 Ab abrogated regulation, with six of six mice rejecting their skin grafts acutely (MST = 17 days, $p < 0.01$, CTLA-4 vs hamster Ig-treated mice; Fig. 5). As an important control for this experiment, MR mice were given anti-CTLA-4 Ab without coadministration of $CD25^+CD4^+$ T cells. These mice rejected their skins with the same tempo as MR mice without anti-CTLA-4 Ab (MST = 17 days both groups; Fig. 5), showing that blockade of CTLA-4 does not in itself enhance the ability of the $CD45RB^{high}$ effector cells to mediate graft rejection.

Immune regulation by $CD25^+CD4^+$ T cells from YTS177/DST-pretreated mice is dependent on IL-10

In a number of experimental models in which immunoregulation is mediated by either $CD45RB^{low}$ or $CD25^+CD4^+$ T cells, regulation has been shown to be dependent upon or at least involve IL-10 (14, 22, 36). Indeed, previous work from this laboratory using $CD4^+$ T cells isolated from long-term tolerant mice that had accepted allografts for over 100 days demonstrated that regulation of skin graft rejection could be abrogated in 80% of mice by administration of a neutralizing IL-10 Ab (14). To assess the role of IL-10 in regulation in our system, MR mice were reconstituted with 5×10^5 $CD25^+CD4^+$ from YTS177/DST-preteated animals
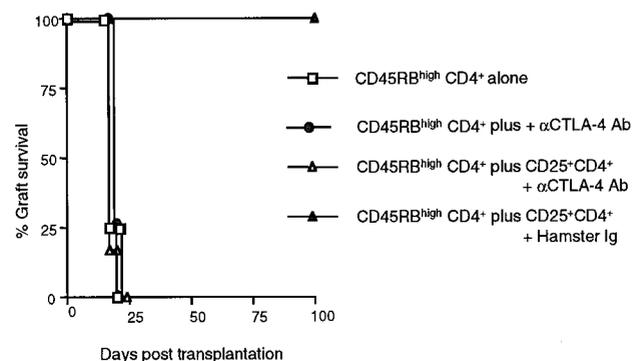

FIGURE 5. Blocking CTLA-4 abrogates regulation. All T cell-deficient CBA mice were reconstituted with 1×10^5 naive $CD45RB^{high}CD4^+$ T cells. T cell-deficient mice reconstituted with 1×10^5 $CD45RB^{high}CD4^+$ T cells alone acutely rejected B10 skin grafts. (□; $n = 4$, MST = 17 days). Mice reconstituted with 1×10^5 $CD45RB^{high}CD4^+$ T cells and treated with an anti-CTLA-4 Ab acutely rejected B10 skin grafts with the same tempo (●; $n = 4$, MST = 17 days). Mice reconstituted with both 1×10^5 naive $CD45RB^{high}CD4^+$ T cells and 5×10^5 $CD25^+CD4^+$ T cells isolated from YTS177/DST-pretreated CBA mice and treated with an anti-CTLA-4 Ab acutely rejected B10 skin grafts (△; $n = 6$, MST = 17 days), whereas administration of a control Ab had no effect on the ability of the $CD25^+CD4^+$ T cells to regulate rejection (▲; $n = 4$, MST > 100 days).
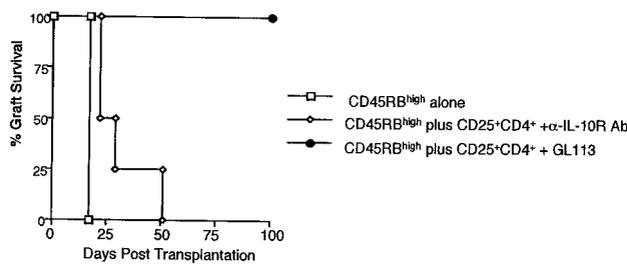
FIGURE 6. Blockade of the IL-10R abrogates regulation mediated by CD25$^+$CD4$^+$ T cells. All T cell-deficient CBA mice were reconstituted with 1×10^5 naive CD45RB^{high}CD4$^+$ T cells. T cell-deficient mice reconstituted with 1×10^5 CD45RB^{high}CD4$^+$ T cells alone acutely rejected B10 skin grafts (□; $n = 8$, MST = 17 days). Mice reconstituted with both 1×10^5 naive CD45RB^{high}CD4$^+$ T cells and 5×10^5 CD25$^+$CD4$^+$ T cells isolated from YTS177/DST-pretreated CBA mice and treated with an anti-IL-10R blocking Ab rejected B10 skin grafts acutely (◇; $n = 4$, MST = 22.5 days), whereas administration of a control Ab had no effect on the ability of the CD25$^+$CD4$^+$ T cells to regulate rejection (●; $n = 4$, MST > 100 days).

1 day before skin grafting and at the time of cell transfer received either the IL-10R Ab 1B1.2 or isotype control Ab GL113.

Treatment with control Ab did not affect the regulatory activity of CD25$^+$CD4$^+$ T cells from pretreated mice, as all animals receiving this treatment accepted their skin grafts long term (MST = 100, $n = 4$; Fig. 6). In clear contrast, blockade of the IL-10 pathway abolished regulation with the majority of grafts rejected by day 25 (MST = 22.5 days, $n = 4$; Fig. 6).

Taken together the results of the anti-CTLA-4 and IL-10R Ab experiments clearly suggest common mechanisms of regulation in naturally occurring CD25$^+$CD4$^+$ regulatory T cells and those generated through pretreatment with alloantigen challenge under the cover of anti-CD4 Ab.

Discussion

Previous work from our laboratory has demonstrated that the maintenance of operational tolerance to donor alloantigens in vivo is mediated by a population of regulatory T cells whose activity is contained within the CD45RB^{low} or CD25$^+$CD4$^+$ T cell subset (14). In the current study, we wished to examine the induction phase of specific immunological unresponsiveness to alloantigens to provide a better understanding of the generation of these cells and their mechanism of action. We have demonstrated that pretreatment with donor alloantigen under the cover of an anti-CD4 Ab induces donor-specific CD25$^+$CD4$^+$ regulatory T cells that are present before transplantation, express elevated levels of CD122, and require CTLA-4 and IL-10 to mediate their function.

CD25$^+$CD4$^+$ T cells purified from YTS177/DST-pretreated animals were able to suppress skin graft rejection mediated by CD45RB^{high}CD4$^+$ T cells in five of six reconstituted recipients. Conversely, at equivalent cell numbers, CD25$^-$CD4$^+$ T cells from pretreated animals were unable to prevent rejection, demonstrating that in this system the regulatory activity of the CD4$^+$ population was contained within the CD25$^+$ fraction (Fig. 2). This observation is in accordance with our previous data demonstrating that CD25$^-$CD4$^+$ T cells from long-term tolerant animals do not possess regulatory activity (14). In contrast, two recent studies have shown that peripheral CD4$^+$CD25$^-$ T cells contain regulatory cells that prevent autoimmune disease (36, 37). Analysis of CD4$^+$ subpopulations has demonstrated that almost all CD25$^+$ cells are contained within the CD45RB^{low} population (15), whereas ~60% of CD45RB^{low} cells are CD25$^-$ (36). In a variety of models, CD45RB^{low} cells have been shown to regulate immune pathology

induced by CD45RB^{high} cells. Thus, the transfer of CD25$^-$CD4$^+$ cells results almost inevitably in the transfer of CD45RB^{low} cells. Although we cannot rule out the possibility that at higher cell numbers CD25$^-$ cells might mediate regulation in our system, it is clear that at equivalent cell numbers CD25$^-$ cells are ineffective in comparison with their CD25$^+$ counterparts.

In the current study, the regulatory populations were obtained from mice pretreated with the YTS177/DST pretreatment protocol. Given the fact that in autoimmune models (2, 15) and at least one transplantation model (38) regulatory cells could be obtained from naive animals, it was important to determine whether in our system alloantigen-specific regulatory cells could be found in naive animals. CD25$^+$CD4$^+$ T cells (5×10^5) isolated from naive mice were unable to regulate the rejection of skin allografts. This differs from models of autoimmune disease, such as the colitis model described by Read et al. (19) where CD25$^+$CD4$^+$ T cells from naive animals could effectively regulate the pathogenic effects of CD45RB^{high}CD4$^+$ T cells and prevent disease. However, in such colitis models, T cells from naive animals would have had prior exposure to the relevant gut Ags, potentially driving the development or expansion of regulatory populations. The generation of regulatory cells from naive animals has also been demonstrated in a pancreas allograft model, where CD45RB^{low}CD4$^+$ T cells from naive animals could regulate rejection mediated by CD45RB^{high}CD4$^+$ T cells (38). The explanation for this difference seen between these data and ours is not apparent at present; however, in a previous study (14) we have also shown that CD45RB^{low}CD4$^+$ T cells from naive animals were unable to regulate rejection. On balance, the data suggest that the generation of CD25$^+$CD4$^+$ regulatory T cells requires prior exposure to specific Ags. In the transplantation setting this may, for example, be in the form of a DST or cardiac allograft, whereas in autoimmune models such Ag would be naturally present in the body. It is almost certain that the precursor frequency of alloantigen-specific regulatory CD25$^+$CD4$^+$ T cells from naive animals is too low to allow regulation at the cell doses used and that our pretreatment regimen serves to increase this precursor frequency.

To determine whether the generation of alloantigen-specific regulatory cells required the presence of both alloantigen and anti-CD4 Ab, we pretreated with either Ab or DST alone and investigated whether CD25$^+$CD4$^+$ T cells isolated from these animals could display regulatory activity. Reconstitution of MR mice with 5×10^5 CD25$^+$CD4$^+$ T cells isolated from either DST alone- or YTS177 alone-pretreated mice resulted in acute skin graft rejection (Fig. 3), supporting the view (10) that for effective generation or expansion of these regulatory cells the recipient immune system must encounter donor alloantigen at the time of immunomodulation by anti-CD4 Ab.

To understand the effects of pretreatment on the CD4$^+$ populations isolated, we investigated the phenotype of cells obtained from pretreated animals. Compared with naive animals, YTS177/DST pretreatment and Ag rechallenge only modestly increased the proportion of CD4$^+$ T cells that expressed CD25. This result is perhaps not unexpected, as T cell activation as a consequence of exposure to Ag induces CD25 expression (39). However, in our system, pretreatment with DST alone at days -27 and 0 did not lead to a rise in the proportion of CD4$^+$ cells expressing CD25, suggesting that the modest increase in the proportion of CD25$^+$ cells seen in the tolerizing protocol indicates more than simply "Ag exposure." Administration of YTS177 (days -28 and -27) plus DST at day 0 did lead to an increase in the proportion of CD4$^+$ T cells expressing CD25, but to a lesser degree than after the combined YTS177/DST therapy. One potential explanation for the slight increase in the proportion of CD4 cells expressing CD25

in what were regarded as control animals in this study is that when given alone at days −28 and −27, YTS177 does lead to some prolongation of primary cardiac allograft survival in this strain combination (MST = 20 days; Ref. 40 and A. R. Bushell, unpublished data). However, as shown in Fig. 3, pretreatment with YTS177 alone does not lead to regulation in this sensitive adoptive transfer system, suggesting either that increase in CD25 expression is not an absolute indicator of regulation or that only the combined pretreatment (YTS177/DST) sufficiently increases the precursor frequency of alloantigen-specific regulatory cells. Experiments are in progress that attempt to enumerate and isolate donor-specific cells within the CD25$^+$ population. From these experiments, it might be possible to determine whether Ag-specific regulatory cells require a certain threshold expression of CD25 to exert their regulatory function.

In our system, as in many others, regulation is mediated by the CD25-positive but not CD25-negative subset of CD4$^+$ T cells. CD25 is a key component of the high-affinity IL-2R and, given that IL-2 is a critical cytokine in most immune responses, we speculated that if CD25$^+$ regulatory T cells were deficient in other components of the IL-2R they might bind IL-2 with only low affinity and thereby act as abortive competitors for alloantigen or for co-stimulatory or adhesion molecules on APC. Therefore, our attention turned to CD122 (the IL-2Rβ chain) because this chain is largely responsible for IL-2 signal transduction (41). In our system, we were surprised to find that a greater proportion of CD25$^+$CD4$^+$ cells obtained from tolerized animals expressed CD122 compared with cells obtained from control animals (Fig. 4). CD122 is used by the receptors of both IL-2 and IL-15 (42), and it is possible that increased expression of CD122 by regulatory cells could allow higher affinity binding of IL-2, IL-15, or both. Preferential binding of IL-2 may reduce the availability of this cytokine to effector cells, thus reducing their expansion. IL-15 has been shown to reduce the susceptibility of T cells to activation-induced cell death (43). Therefore, increased expression of CD122 may provide regulatory cells with a survival advantage, allowing immune regulation to persist. Given our data regarding CD122 expression, it is interesting to note that CD122 has recently been shown to be constitutively expressed on ex vivo isolated human regulatory CD25$^+$CD4$^+$ T cells (44, 45).

CTLA-4, a CD28 homolog, is expressed on T cells after activation and has been shown to down-regulate T cell responses (32, 34, 46, 47). Evidence for a critical role of CTLA-4 is provided by the observation that, in vivo, CTLA-4-deficient mice develop a lymphoproliferative disorder resulting from uncontrolled expansion of CD4$^+$ T cells and die within 3–4 wk (47). As recent studies have shown that immunoregulatory CD25$^+$CD4$^+$ T cells express CTLA-4 (19, 23, 35), we wished to investigate its expression in our system. Although when compared with naive mice CD25$^+$ cells from YTS177/DST-pretreated animals showed an increased intracellular content of CTLA-4, a similar increase was also seen in control animals pretreated with YTS177 alone. Thus, in this model, where probably only a small proportion of CD25$^+$ T cells are specific for donor alloantigens, we are unable to conclude that an increased CTLA-4 content/expression correlates with the ability of CD25$^+$CD4$^+$ T cells to mediate donor alloantigen-specific regulation. However, when CTLA-4 was targeted in vivo, the results were unequivocal. As shown in Fig. 5, administration of anti-CTLA-4 Ab completely abolished regulation mediated by CD25$^+$CD4$^+$ T cells isolated from YTS177/DST-pretreated animals, providing clear evidence for a role for CTLA-4 in this as in other systems. Thus, although we were unable to detect a specific increase in CTLA-4 expression following pretreatment with the tolerizing protocol using phenotypic analysis, the in vivo data

clearly implicate CTLA-4 as an important factor in the regulation mediated by CD25$^+$CD4$^+$ T cells in this model.

Various outcomes have been identified following cross-linking of CTLA-4 in vitro. These include recruitment of the phosphatase SHP-2 into the immunological synapse (48, 49) and the secretion of the immunoregulatory cytokine TGF-β (50). TGF-β blockade has been shown to abrogate regulation by CD25$^+$CD4$^+$ T cells in an autoimmune model (19).

IL-10 has been shown to display a range of immune suppressive effects, including inhibition of APC function (51), induction of anergy (52), differentiation of regulatory (Tr1) T cells in vitro (4), and control of the expansion of other T cell populations (53). We had previously identified IL-10 as a key factor in the regulation of skin graft rejection mediated by CD45RB^{low}CD4$^+$ T cells obtained from long-term tolerant mice (14); thus, we asked whether IL-10 also played a critical regulatory role in the adoptive transfer system using CD25$^+$CD4$^+$ T cells from pretreated-only mice. Blockade of the IL-10 pathway using an anti-IL-10R Ab abolished the ability of CD25$^+$CD4$^+$ T cells to regulate skin graft rejection mediated by naive CD45RB^{high}CD4$^+$ T cells.

How IL-10 exerts its immune suppressive effects in our system is at present unknown. However, recent data from Cottrez (54) has demonstrated a link between IL-10 and TGF-β, with IL-10 enhancing the expression of TGF-βR expression on activated and resting T cells. As cross-linking of CTLA-4 has been shown to induce the production TGF-β (50), we can speculate that there may be a common mechanism of action linking CTLA-4 and IL-10.

To our knowledge, this is the first demonstration that alloantigen-specific CD25$^+$ regulatory cells can be generated following manipulation of the adult immune system. We believe that an understanding of the way in which these cells develop could have implications for the treatment of autoimmune disease as well as in the field of tolerance induction in transplantation. The fact that in this system regulation is dependent on CTLA-4 and IL-10 provides evidence for common mechanisms between recently generated and naturally occurring regulatory T cells.

Acknowledgments

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