Both $CD4^+CD25^+$ and $CD4^+CD25^-$ Regulatory Cells Mediate Dominant Transplantation Tolerance[1]

Luis Graca,[2] Sara Thompson, Chun-Yen Lin, Elizabeth Adams, Stephen P. Cobbold, and Herman Waldmann

$CD4^+CD25^+$ T cells have been proposed as the principal regulators of both self-tolerance and transplantation tolerance. Although $CD4^+CD25^+$ T cells do have a suppressive role in transplantation tolerance, so do $CD4^+CD25^-$ T cells, although 10-fold less potent. Abs to CTLA-4, CD25, IL-10, and IL-4 were unable to abrogate suppression mediated by tolerant spleen cells so excluding any of these molecules as critical agents of suppression. $CD4^+CD25^+$ T cells from naive mice can also prevent rejection despite the lack of any previous experience of donor alloantigens. However, this requires many more naive than tolerized cells to provide the same degree of suppression. This suggests that a capacity to regulate transplant rejection pre-exists in naive mice, and may be amplified in "tolerized" mice. Serial analysis of gene expression confirmed that cells sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations were distinct in that they responded to TCR ligation with very different programs of gene expression. Further characterization of the differentially expressed genes may lead to the development of diagnostic tests to monitor the tolerant state. ***The Journal of Immunology,*** **2002, 168: 5558–5565.**

Ever since the description of classical transplantation tolerance by Medawar and colleagues (1), the attainment of clinical transplantation tolerance has been considered the Holy Grail of immunology. In rodents, the therapeutic administration of nondepleting mAbs (2), such as the combination of anti-CD4 and anti-CD8, at the time of transplantation can lead to a robust form of peripheral tolerance (2–6). The tolerant state so achieved creates an environment in the host which can disarm nontolerant naive cells from rejecting the transplant (dominant tolerance), as well as enabling the emergence of novel regulatory cells from the naive lymphocyte population (infectious tolerance). This has been observed not only with tolerance induced following coreceptor blockade of CD4 and CD8 (3), but also with tolerance resulting from costimulation blockade with nondepleting anti-CD40L (CD154) mAb (7). Thus far, the regulatory cells mediating dominant tolerance have been identified as $CD4^+$ T cells in all the models studied (3, 8–11).

In parallel studies, animal models of autoimmune disease and inflammatory bowel disease have provided compelling evidence of $CD4^+$ regulatory cells that prevent immunopathology (12–15). The phenotype of these regulatory T cells has been further refined, so that $CD4^+CD25^+CD45RB^{low}$ T cells are now considered the principle exponents (16–18). These cells have been shown capable of regulating $CD4^+CD25^-$ or $CD4^+CD45RB^{high}$ nontolerant cells both in vitro and in vivo, preventing the onset of autoimmunity and gut immunopathology (19–22). They have also been shown capable of suppressing in vitro proliferation and IFN-γ secretion by $CD8^+$ T cells (23).

To establish the relationship, if any, between the T cells that regulate transplant rejection and those that regulate self-immunopathology, we compared the suppressive ability of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from mice rendered tolerant to skin transplants, as well as from naive mice with no previous experience of those particular transplantation alloantigens. We found that both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from tolerant mice could mediate suppression, although the $CD4^+CD25^-$ cells were required in larger numbers. However, as mice have 10 times more $CD4^+CD25^-$ than $CD4^+CD25^+$ T cells, we are led to conclude that regulatory cells within both populations are involved in suppression, perhaps acting in concert. In contrast, it was only the $CD4^+CD25^+$, but not the $CD4^+CD25^-$, cells from naive mice that could prevent naive splenocyte cells from rejecting a skin graft, although at least 5-fold more cells were required than from tolerant donors. This could mean that tolerance-inducing protocols either drive an expansion of regulatory T cells (both $CD4^+CD25^+$ and $CD4^+CD25^-$), or that they bring about selective deletion of nontolerant cells; or indeed both—the outcome being a tolerance-permissive regulator to immune-effector ratio. These results appear to differ from previously published work (24, 25) where only the $CD4^+CD25^+$ cells from tolerant animals have been shown to be regulatory. These differences may be apparent rather than real, simply reflecting insufficient cell doses in the previous studies.

Transcriptional profiling by serial analysis of gene expression (SAGE)[3] (26) of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from naive mice was used to establish that the two populations have very distinct gene profiles. These may reflect the differing functions of such populations, and with further characterization, may provide diagnostics to allow monitoring of the contributions of each $CD4^+$ subpopulation in circumstances where therapeutic tolerance is desirable.

Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

Received for publication January 11, 2002. Accepted for publication March 29, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was funded by a program grant from the Medical Research Council (U.K.). L.G. was supported by the Calouste Gulbenkian Foundation's PhD Program in Biology and Medicine and the Portuguese Foundation for Science and Technology/ Praxis XXI.

[2] Address correspondence and reprint requests to Dr. Luis Graca, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K. E-mail address: luis.graca@pathology.oxford.ac.uk

[3] Abbreviations used in this paper: SAGE, serial analysis of gene expression; MST, median survival time.

0022-1767/02/$02.00

Materials and Methods

Mice

CBA/Ca (H-2^k), human-CD52 transgenic CP1-CBA/Ca (H-2^k) (27), and B10.BR (H-2^k) mice were bred and maintained in the specific pathogen-free facilities of the Sir William Dunn School of Pathology (Oxford, U.K.). All groups were age- and sex-matched. All procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986.

Thymectomy and skin grafting

Mice were anesthetized with a mixture of 10 mg/ml Hypnodil and 2 μg/ml Sublimaze (Janssen Pharmaceutica, Tilburg, The Netherlands). A total of 0.12 ml/20 g of body weight was injected i.p. Thymectomy was conducted as described by Monaco et al. (28). In short, a longitudinal incision was made on the anterior surface of the neck, and the thymus was removed as two intact lobes by the application of negative pressure through a glass tip inserted in the anterior mediastinum. Skin grafting was conducted according to a modified technique of Billingham et al. (1). Briefly, full thickness tail skin (1 $\times$ 1 cm) was grafted on the lateral flank. Grafts were observed on alternate days after the removal of the bandage at day 8 and considered rejected when no viable donor skin was present. Statistical analysis of graft survival was made by the log rank method (29).

Cell separation and adoptive cell transfer

Cells were obtained from spleens of adult CBA/Ca mice. A single-cell suspension was obtained by passing the splenocytes through a 70-μm cell strainer (BD Biosciences, Oxford, U.K) and the erythrocytes were depleted by water lysis. Cells were counted, diluted in PBS, and injected i.v. into the tail vein. Cell separation was performed by negative selection of CD8$^+$ cells, MHC class II$^+$ cells, and B cells by incubation with the mAbs M5/114, 187.1, and YTS156.7, and subsequent incubation with goat-anti-rat IgG Dynabeads (Dynal Biotech, Oslo, Norway) and magnetic removal. For CD4$^+$ separation, cells were incubated with anti-CD4 microbeads (Miltenyi-Biotech, Bergisch Gladbach, Germany) according to manufacturer's instructions, and positively selected over two magnetic columns using the "posseld" program of AutoMACS (Miltenyi-Biotech). For separation of CD4$^+$CD25$^+$ T cells, the CD8, class II, and B cell-depleted single-cell suspension was incubated 45 min at 4°C with 7D4-biotin (BD PharMingen, San Diego, CA) 1:100 in PBS, 1% w/v BSA, 5% v/v heat-inactivated normal rabbit serum, and 0.1% w/v sodium azide. Following washing, the cells were incubated 15 min at 4°C with 2 μl/10^7 cells streptavidin-microbeads (Miltenyi Biotech), and positively selected over two columns using AutoMACS posseld program. CD4$^+$CD25$^-$ cells were sorted from the negative fraction obtained following CD4$^+$CD25$^+$ separation, by incubation with 100 μl/10^7 cells with streptavidin-microbeads, and subsequent negative selection of any remaining CD25$^+$ cells using AutoMACS "possels", and finally, a positive selection step with anti-CD4 microbeads as described above. All sorted fractions were labeled with CD8(YTS156.7)-FITC, CD25(PC61)-PE (BD PharMingen), and CD4(H129.19)-CyCr (BD PharMingen); and assessed in a flow cytometer. Typical purity of CD4$^+$ cells and CD4$^+$CD25$^-$ cells was >98%, and purity of CD4$^+$CD25$^+$ >90%. Cells were counted, diluted in PBS, and injected i.v. into the tail vein.

Cell depletion, tolerance induction, and mAbs

For depletion of CP1-CBA T cells, 0.2 mg of CAMPATH-1H (30) was injected i.p. Tolerance was induced in CBA/Ca mice by treatment with 1 mg YTS177.9 (2) and 1 mg YTS105.18 (2) at days 0, 2, and 4 after B10.BR skin transplantation. These mAbs, as well as M5/114 (31), 187.1 (32), YTS156.7 (33), PC61 (34), 4F10 (35), JES5 (36), 11B11 (37), and YCATE55.9 (3) were produced in our laboratory by culture in hollow fiber bioreactors, purified from culture supernatants by 50% ammonium sulfate precipitation, dialysed against PBS, and purity checked by native and SDS gel electrophoresis (PhastGel; Pharmacia, St. Albans, U.K.).

Flow cytometry analysis

Peripheral blood samples were depleted of erythrocytes by water lysis, washed, and resuspended in PBS, 1% w/v BSA, 5% v/v heat-inactivated normal rabbit serum, and 0.1% w/v sodium azide. Cells were incubated for 45 min at 4°C with directly conjugated CAMPATH-1H-FITC, CD8α (53-6.7)-PE (BD PharMingen), and CD4-CyCr. The cells were washed, resuspended in PBS, 1% w/v BSA, 0.1% w/v sodium azide, and fixed in 2% v/v formaldehyde solution. Three-color FACSCaliber analysis (BD Biosciences) was performed using CellQuest (BD Biosciences) software.

SAGE libraries and differential analysis of gene expression

CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells were sorted from naive CBA/Ca mice and activated with overnight incubation with solid-phase anti-CD3 mAb (KT3) at 37°C. T cell populations were pelleted and resuspended in Promega SV total RNA isolation system (Promega Z3100; Promega, Madison, WI) lysis buffer (175 μl/2 $\times$ 10^6 cells), and total RNA was isolated according to the manufacturer's instructions. First strand cDNAs were prepared from 1 μg of total RNA from each cell fraction using Superscript II (Life Technologies, Gaithersburg, MD). Reverse transcription was initiated using the anchoring primer 5′-GACTCGAGTTGACATCGAGG(T)$_{20}$V-3′ with incorporation of the SMARTII oligonucleotide (Clontech Laboratories, Palo Alto, CA) 5′-AAGCAGTGGTAACAACGCAGAGTACGCG GG-3′at the 5′ end. The cDNAs were preamplified with the forward 5′-AGTGGTAACAACGCAGAGTAC-3′ and reverse 5′-GACTCGAGTTGA CATCGAG-3′ primers using the Advantage-GC cDNA PCR kit (Clontech Laboratories) with 1 M of the GC-Melt, following the manufacturer's protocol. cDNAs were subjected to 16 cycles of preamplification, 94°C for 30 s, 68°C for 7 min. The preamplification steps were monitored by PCR using various housekeeping and cytokine primers as tests. SAGE was performed using *Nla*III as the anchoring enzyme, BsmF1 as the tagging enzyme, and *Sph*I as the cloning enzyme, as described (26). DNA sequencing was performed using the Megabase 1000 (Molecular Dynamics, Sunnyvale, CA). Sequence analysis software SAGE 3.04 β was provided by K. W. Kinzler (Johns Hopkins Oncology Center, Baltimore, MD). A conservative estimate of the differential up-regulation of each gene within the given library, compared with a pool of other libraries, was calculated using a Bayesian statistics model (38).

Identification of mAbs in the serum

Serum concentrations of JES5 and 11B11 were determined by ELISA. 96-well plates were coated with 10 μg/ml JES5 or 11B11 in 0.1 M $NaHCO_3$, and blocked with 50 μl PBS, 1% BSA. Serum samples from the mice were diluted 1/20 in PBS, 1% BSA containing either rIL-10 (10 ng/ml) or rIL-4 (2 ng/ml), and preincubated for 60 min at room temperature before being transferred into the JES5- or 11B11-coated plates, where the samples were incubated for a further 60 min. After washing with PBS, 0.05% Tween, the plates were incubated 60 min with anti-IL10 (SXC-1) biotin or anti-IL-4 (BVD6-24G2) biotin (both from BD Biosciences). The plates were then incubated with extravidin-peroxidase (Sigma-Aldrich, Poole, U.K.) for 30 min, and developed with substrate buffer and absorbance at 492 nm analyzed with a microplate reader (Labsystems, Helsinki, Finland). Serum 4F10 was determined by incubating serum samples in anti-hamster IgG (HIG-29) (BD Biosciences) coated plates, and detecting any bound hamster Ab with biotin-conjugated HIG-29. Serum PC61 was determined by flow cytometry by incubating CD25$^+$ Con A blasts with the experimental serum diluted 1/5 in PBS, 1% BSA for 30 min at 4°C, followed by addition of anti-CD25 (PC61) PE-conjugated (BD Biosciences), as well as anti-CD4 CyChrome. Three-color FACSCaliber analysis was performed using CellQuest software. In all cases, the experimental serum samples were compared with serum from untreated animals, and serum spiked with mAb to known concentrations.

Results and Discussion

Spleen cells from "tolerized" mice abrogate skin graft rejection by spleen cells from naive mice

Regulatory cells that suppress graft rejection by naive spleen cells can be found in the spleens of mice made tolerant to an allograft with therapeutic mAbs (3). We confirmed that 10^7 spleen cells from naive CBA/Ca mice were, upon adoptive transfer, sufficient to reject B10.BR skin grafts in T cell-depleted hosts (39). An equal number of spleen cells from mice tolerant to B10.BR skin grafts could prevent graft rejection when coadministered with the naive cells.

Tolerance was induced in CBA/Ca mice by treatment with 3 mg of the combination of nondepleting CD4 and CD8 mAbs administered over 1 wk following the transplantation of B10.BR skin grafts. We used CP1-CBA mice as T cell-depleted hosts for cell transfusion (27). This transgenic strain is histocompatible with CBA/Ca and allows selective T cell depletion with the human-CD52 mAb CAMPATH-1H (7), as human CD52 is expressed under the control of the CD2 promoter in all T cells. CP1-CBA mice were thymectomized at 4 wk of age, and depleted of T cells with

CAMPATH-1H 1 wk before cell transfer (designated as "empty" mice). The empty mice were transfused with 10^7 spleen cells from tolerant mice, 10^7 spleen cells from naive CBA/Ca, or an equal number (10^7) of spleen cells from both tolerant and naive mice. All animals were grafted with B10.BR skin the following day. Rejection was only observed in the mice transfused with cells from naive CBA/Ca (Fig. 1). Spleen cells from tolerant mice not only failed to reject the skin grafts, but also abrogated rejection by naive T cells, so demonstrating dominant tolerance. Similar results were obtained in experiments where 2×10^7 and 4×10^7 spleen cells from both tolerant and naive mice were transfused. We decided to use 10^7 spleen cells from naive CBA/Ca as the target population to assess the number and phenotype of regulatory cells able to prevent rejection.

$CD4^+CD25^+$ cells from naive mice prevent graft rejection upon adoptive transfer

We investigated whether the capacity to suppress transplant rejection pre-existed in naive mice. We isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from the spleens of naive CBA/Ca mice. Empty CP1-CBA mice were injected with 10^7 unsorted spleen cells from naive CBA/Ca, together with 10^6 of either $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells, also from naive CBA/Ca. All mice were transplanted with B10.BR skin on the following day. Delayed graft rejection was observed in the group transferred with the $CD4^+CD25^+$ cells, with 6 of the 10 mice showing indefinite graft survival (Fig. 2). The animals injected with unsorted spleen cells alone rejected the skin grafts at a rate similar to the group receiving $CD4^+CD25^-$ T cells. To rule out an artifact of the sorting procedure, a control experiment was performed where spleen cells from naive CBA/Ca were sorted and subsequently remixed to the exact starting proportions. These cells failed to prevent skin graft rejection upon adoptive transfer (data not shown). One interpretation for these results is that aggression and

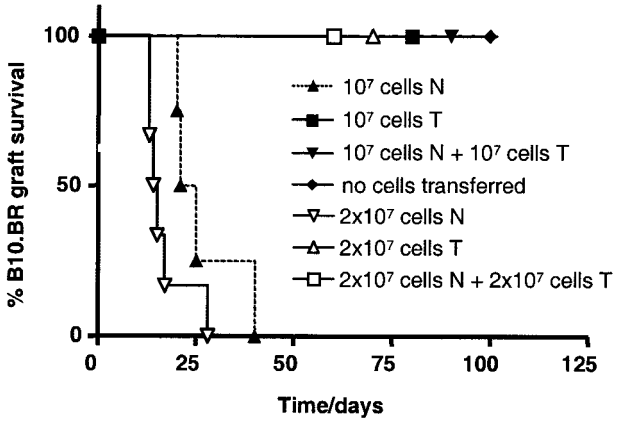

FIGURE 1. Spleen cells from tolerant mice prevent rejection mediated by nontolerant cells. CP1-CBA mice were thymectomized at 4 wk of age, and depleted of T cells with 0.1 mg CAMPATH-1H at days −7 and −8. At day −1, these mice received an i.v. injection of 10^7 (▲) or 2×10^7 (▽) spleen cells from naive CBA/Ca;10^7 (■) or 2×10^7 (△) spleen cells from CBA/Ca mice tolerant of B10.BR skin grafts; 10^7 or 2×10^7 spleen cells from the same naive CBA/Ca donors, together with an equal number of spleen cells tolerant donors (▼ and □); and a final group was not injected (◆). All animals received a B10.BR skin graft on the following day (day 0). Only mice transfused with cells from naive donors rejected the skin grafts, the rejection being slightly faster when more cells were transfused. The spleen cells from tolerant donors not only allowed indefinite graft survival, but were also able to suppress rejection mediated by the naive cells injected at the same time.

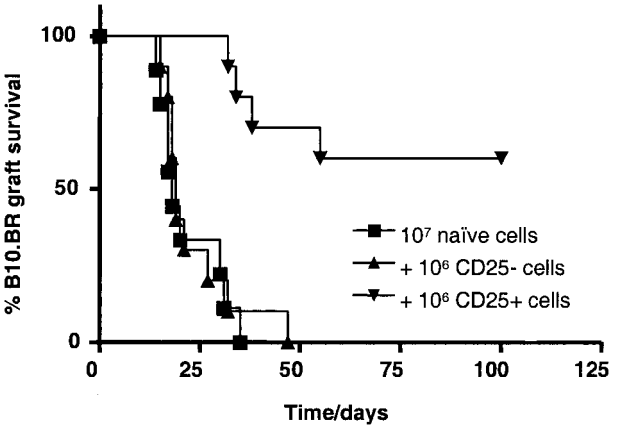

FIGURE 2. $CD4^+CD25^+$ cells from naive mice prevent graft rejection upon adoptive transfer. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were sorted from spleens of CBA/Ca mice. Empty CP1-CBA mice were injected i.v. with 10^6 of either $CD4^+CD25^+$ or $CD4^+CD25^-$ cells together with 10^7 unsorted spleen cells. All mice were transplanted with B10.BR skin the following day. The group injected with $CD4^+CD25^-$ cells (▲, $n = 10$, median survival time (MST) = 19 day) rejected the skin grafts at a similar rate as the control group where only 10^7 unsorted spleen cells were transferred (■, $n = 9$, MST = 18 days). However, when the $CD4^+CD25^+$ cells were coinjected with the unsorted cells, graft rejection was significantly delayed, and several mice accepted the transplanted skin indefinitely (▼, $n = 10$, MST > 100, $p < 0.0001$). This figure represents pooled results from two different experiments.

tolerance are the outcome of situations dictated by the numerical balance between regulatory and effector cells.

This outcome differs from previously published data (24, 25, 40). However, in our experiments, the number of $CD4^+CD25^+$ cells injected into each recipient was much higher than the numbers used in those studies. We suggest that the optimal regulator-effector ratio can only be reached when such high numbers of regulatory cells are transferred. In fact, one previous study demonstrated that purified $CD4^+CD25^+$ from naive animals were incapable of making a graft vs host allogeneic response upon transfer into a mismatched immunodeficient recipient (40). In another study, analyzing islet graft rejection, $CD4^+CD45RB^{low}$ cells from naive donors could, when coadministered at an appropriate ratio, prevent graft rejection by $CD4^+CD45RB^{high}$ cells (41). This is also consistent with the observation of Sakaguchi et al. (19) that depletion of the $CD25^+$ T cells increases the speed of first-set allograft rejection.

The Ag specificity of the $CD4^+CD25^+$ T cells from naive animals that suppress transplant rejection is currently unknown. $CD4^+CD25^+$ cells have been shown to inhibit the proliferation of $CD4^+CD25^-$ cells to different alloantigens in vitro, as long as the $CD4^+CD25^+$ cells are themselves preactivated (42). It may be that their repertoire contains receptors directed toward self-Ags (43). If so, then there is the possibility that self-reactive regulators mediate graft acceptance through "linked suppression", where they are brought into the local microenvironment of the alloreactive cell. Alternatively, the receptor repertoire of $CD4^+CD25^+$ T cells may show cross-reactivity to alloantigens present in the graft. Given the finding that suppression involves indirect presentation of Ag (44), the "alloantigens" in question are likely to be donor-type peptides presented in conjunction with host-type MHC.

$CD4^+CD25^+$ T cells from tolerized mice are more efficient than $CD4^+CD25^-$ cells as mediators of dominant transplantation tolerance

Having established that $CD4^+CD25^+$ T cells from naive animals suppress graft rejection, we compared the potency of both

$CD4^+CD25^+$ and $CD4^+CD25^-$ populations from tolerized mice in preventing rejection. The $CD4^+CD25^+$ and $CD4^+CD25^-$ populations were purified from the spleens of CBA/Ca mice made tolerant to B10.BR skin transplants 100–120 days earlier (Fig. 3*A*). Different numbers of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were transferred with the fixed number 10^7 of naive spleen cells into empty CP1-CBA recipients. All recipients received B10.BR skin grafts on the following day. When 10^5 $CD4^+CD25^+$ cells were transferred together with 10^7 spleen cells from naive CBA/Ca, a delay in graft rejection was observed when compared with the groups transferred with the same number of $CD4^+CD25^-$ cells, or with controls which had received naive spleen cells only (Fig. 3*B*). However, when the number of $CD4^+CD25^-$ T cells was increased 10-fold to 10^6, graft rejection was delayed to an extent comparable to the 10^5 $CD4^+CD25^+$ group. No skin graft rejection by the naive cells was observed in the groups transferred with 10^6 $CD4^+CD25^+$ or 10^7 $CD4^+CD25^-$ T cells. These results suggest that both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells can mediate transplantation tolerance, the $CD4^+CD25^+$ T cells being 10 times more potent than $CD4^+CD25^-$ cells. However, as the number of $CD4^+CD25^-$ T cells in tolerant mice is ~10 times higher than that of $CD4^+CD25^+$ cells, it is likely that both populations have a significant role in maintaining transplantation tolerance.

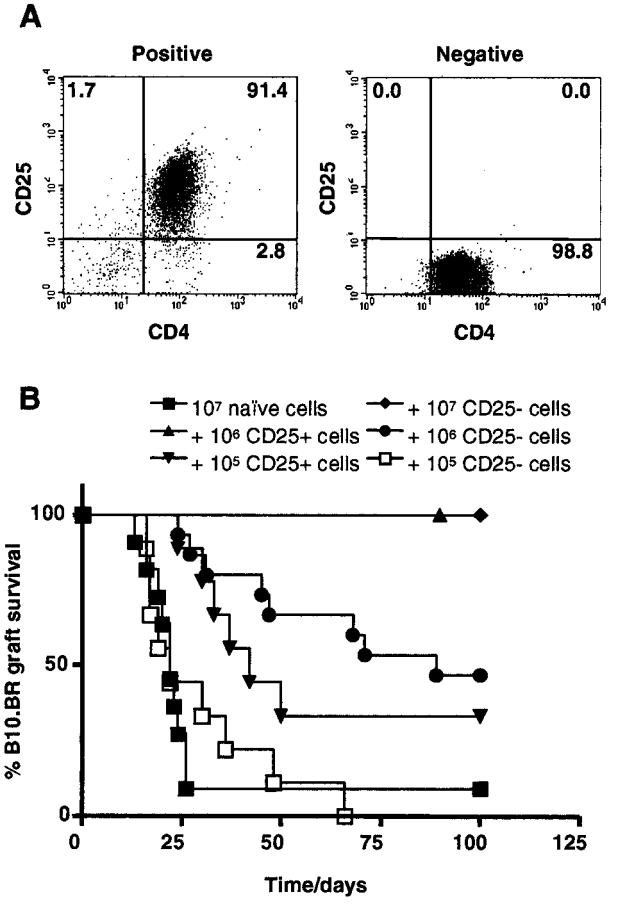

FIGURE 3. $CD4^+CD25^+$ T cells from tolerant mice are more efficient than $CD4^+CD25^-$ cells as mediators transplantation tolerance. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were sorted from the spleens of CBA/Ca mice tolerant to B10.BR skin transplants as described in *Materials and Methods*. Different numbers of these cells were cotransferred i.v. with 10^7 unsorted spleen cells from naive CBA/Ca into empty CP1-CBA mice. *A*, FACS profiles of the transferred $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. *B*, All mice were transplanted with B10.BR skin the day following cell transfer. When unsorted spleen cells were transferred in the absence of cells from tolerant mice, the grafts were readily rejected (■, $n = 11$, MST = 22 days). A similar rate of rejection was observed when 10^5 $CD4^+CD25^-$ cells were added (□, $n = 9$, MST = 22 day). When the number of $CD4^+CD25^-$ cells was increased to 10^6 (●, $n = 15$, MST = 89 day), or when 10^5 $CD4^+CD25^+$ cells were transferred (▼, $n = 9$, MST = 42 day), rejection was significantly delayed and several animals accepted the grafts indefinitely. No rejection was observed in the groups injected with 10^6 $CD4^+CD25^+$ cells (▲, $n = 13$, MST > 100) or 10^7 $CD4^+CD25^-$ cells (◆, $n = 4$, MST > 100). This figure represents pooled results from three different experiments.

These findings contrast with previous reports describing a lack of regulatory capacity within the $CD4^+CD25^-$ population in transplantation tolerance (24, 25). Although our experimental system is different to those used by Hara et al. (24) and Gregori et al. (25) in many respects, they may not have reached the appropriate cell doses of $CD4^+CD25^-$ cells required for suppression. Experiments in animal models of autoimmunity have also described a regulatory role for $CD4^+CD25^-$ peripheral T cells (14, 45–47). In one of these reports, the difference in cell numbers between $CD4^+CD25^+$ and $CD4^+CD25^-$ cells required to achieve equivalent suppression is comparable to our findings (45).

The observation that the potency of the $CD4^+CD25^+$ population seems to increase following induction of transplantation tolerance is intriguing. It is not clear at this time whether this is due to an expansion of the regulatory cells from pre-existing regulators, whether it results from de novo formation of regulatory cells, or whether this reflects selective inactivation or death of nontolerant cells; so shifting the functional bias of the population toward regulation. It is equally interesting that the $CD4^+CD25^-$ population is only seen to regulate if derived from tolerant, but not naive, populations. It may be that some "tolerant" $CD25^+$ regulatory cells loose the expression of CD25 and endow the $CD4^+CD25^-$ population with new regulatory powers, as it has been suggested following homeostatic expansion of $CD4^+CD25^+$ T cells (48). Alternatively, activation induced cell death previously reported to occur in the induction of transplantation tolerance (49, 50) may selectively remove effector cells from the $CD25^-$ population, unmasking residual regulatory cell activity.

Of interest was the finding that the "potency" of unseparated tolerized $CD4^+$ cells (containing both $CD4^+CD25^+$ and $CD4^+CD25^-$ subpopulations) seemed greater than the separated populations. $CD4^+$ cells were sorted from spleens of CBA/Ca mice tolerant to B10.BR skin grafts using magnetic microbeads, and different numbers of these cells were injected together with the fixed number of 10^7 splenocytes from naive CBA/Ca mice into empty CP1-CBA recipients (Fig. 4*A*). All animals received a B10.BR skin graft on the following day. When 10^5 or less $CD4^+$ spleen cells were transfused, the outcome was rejection (Fig. 4*B*). However, adoptive transfer of 5×10^5 or more $CD4^+$ spleen cells from tolerant mice resulted in graft acceptance. In the CBA/Ca mouse strain, ~10% of $CD4^+$ cells coexpress CD25 in both naive and tolerant animals. On the basis of these figures, we can calculate that it takes $\sim 5 \times 10^4$ $CD4^+CD25^+$ cells combined with 4.5×10^5 $CD4^+CD25^-$ cells from tolerant mice to suppress 10^7 naive spleen cells. However, we found that neither 10^5 $CD4^+CD25^+$ nor 10^6 $CD4^+CD25^-$ cells alone could provide this degree of suppression (see Fig. 3). This suggests that the "unseparated" $CD4^+$ cell population shows greater potency than the equivalent numbers of sorted $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells. Such a result could possibly reflect impairment of regulatory function from the cell separation manipulations, or perhaps the enhanced regulation from $CD4^+CD25^+$ and $CD4^+CD25^-$ regulatory cells operating together. Additional experiments are needed to clarify this.

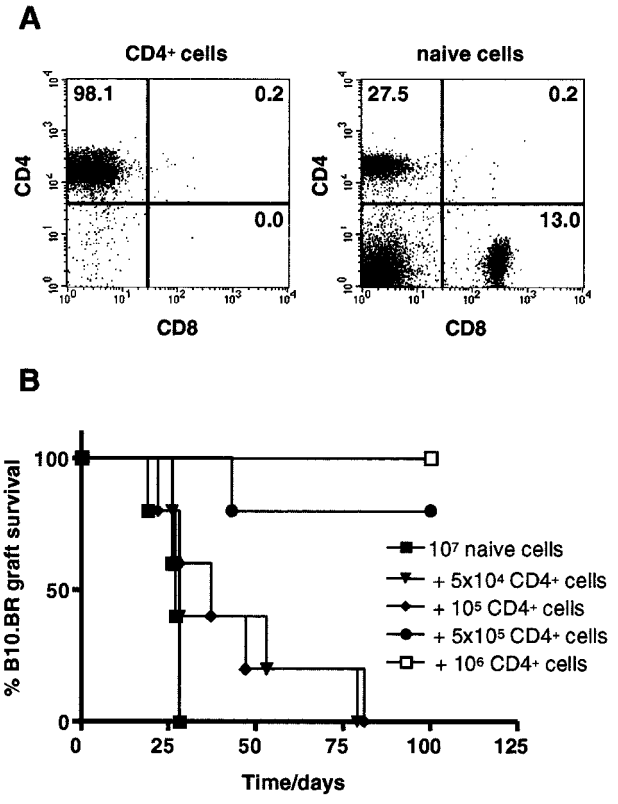

FIGURE 4. $CD4^+$ T cells from tolerant mice prevent rejection by spleen cells from naive mice in a dose-dependent way. Splenic $CD4^+$ cells were sorted from CBA/Ca mice tolerant to B10.BR skin grafts, as described in *Materials and Methods*, and injected i.v. with 10^7 spleen cells from naive CBA/Ca mice into empty CP1-CBA mice. *A*, FACS profiles of the sorted $CD4^+$ cells and the unsorted CBA/Ca spleen cells. *B*, All mice were grafted with B10.BR skin the day following adoptive cell transfer. The group injected with spleen cells in the absence of $CD4^+$ cells from tolerant mice readily rejected the skin grafts (■, $n = 5$, MST = 26 day). The rejection rate was not significantly delayed in the groups where 5×10^4 (▼, $n = 5$, MST = 28 day) or 10^5 (◆, $n = 5$, MST = 37 day) $CD4^+$ cells were added to the 10^7 spleen cells from naive CBA/Ca mice. However, when 5×10^5 (●, $n = 5$, MST>100, $p = 0.0027$) and 10^6 (□, $n = 5$, MST>100, $p = 0.0027$) $CD4^+$ cells were added, the grafts were accepted indefinitely, these results being statistically significant when compared with the control group (■).

We had observed that $CD4^+CD25^+$ T cells from tolerized mice had a greater regulatory potency than $CD4^+CD25^+$ T cells from naive donors. However, the proportion of $CD25^+$ cells among the $CD4^+$ T lymphocytes from spleen, draining lymph nodes, and peripheral blood remained constant within the two populations at any of days 7, 14, 30, and 60 into the study (our unpublished observations). Such results contrast with published data from Gregori et al. (25). This difference in outcome may be due to the different tolerance inducing protocol used, or to the greater degree of mismatch between the host and the tolerated tissue in that study.

$CD4^+CD25^+$ and $CD4^+CD25^-$ cells differ in gene expression

To validate the separation procedures adopted to isolate $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells, we examined the nature of genes expressed in each population, either resting or activated with solid-phase CD3 mAbs, using SAGE. A differential analysis of the four SAGE libraries is displayed in Fig. 5 as scatter plots comparing $CD4^+CD25^-$ spleen cells with $CD4^+CD25^+$ spleen cells before and after stimulation. A number of transcripts that do not differ between the two populations before activation are highlighted in Fig. 5*A*. These include the housekeeping genes EF-1α and GAPDH, the T cell-specific genes CD3δ and Ly116 (a Th1 marker), the activation marker OX40, together with β_2 microglobulin and MHC-I (K, D, and L, although this latter tag is slightly higher in $CD4^+CD25^-$ cells). Very few tags appear to be

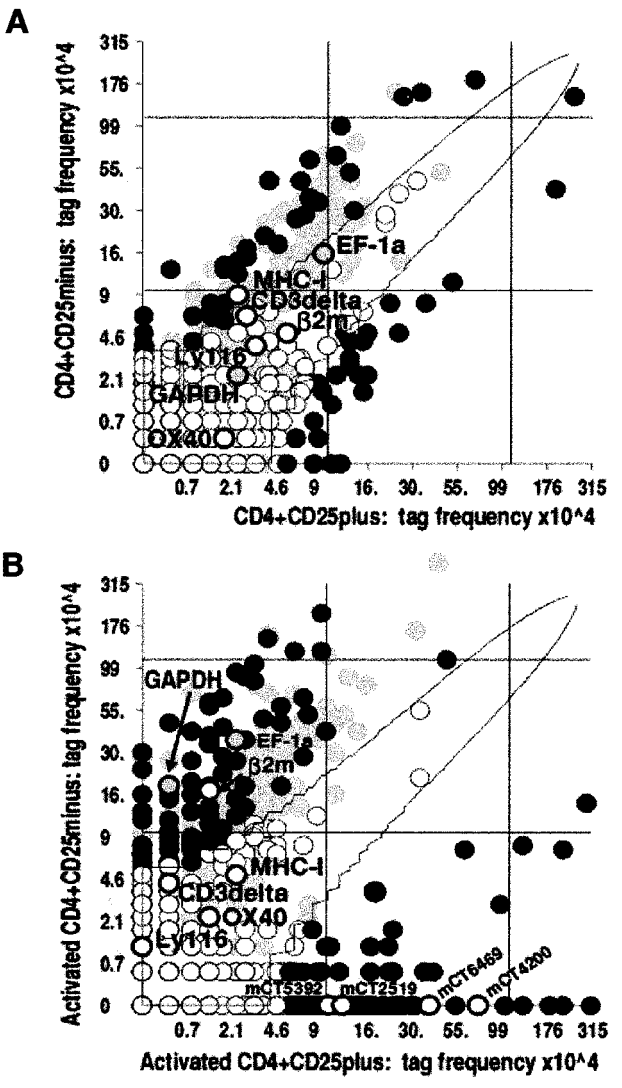

FIGURE 5. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells express different genes. *A*, Comparison of gene expression profile of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells sorted from naive CBA/Ca mice. *B*, Comparison of gene expression profile of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells following activation with solid-phase anti-CD3 mAb. SAGE libraries were compared using scatter plots where each SAGE gene tag is represented by a point plotted at the coordinates corresponding to the tag frequency per 10,000 tags (note logarithmic scale). Tags corresponding to genes whose expression is not differential are represented inside the diagonal area shown. Tags with a statistically differential expression (95% confidence of >1.2-fold up-regulation) are those plotted outside the diagonal area. Housekeeping genes are defined as tags that are nondifferentially expressed across a group of 20 SAGE libraries from different cell types (defined by SDev ≤ Mean) and are depicted in gray. For clarity, most of differentially expressed tags are represented in black, and the nondifferential in white. The following gene transcripts were identified by their SAGE tags as follows: β_2 microglobulin, TTTTCAAAAA; CD3δ, AGACCGGAAG; EF-1α, AGGCAGACAG; GAPDH, GCCTCCAAGG; Ly116, GCAGTGGTTC; MHC-I (K, D, and L), GATTGAGAAT; OX40, CTAGCAGCTG; mCT5392, CCCAGCATCC; mCT2519, AAGGCTATGT; mCT6469, CTTCTACCAA; and mCT4200, GTGGCAGGAG.

specific to either one of the populations; but 28 tags are significantly up-regulated in $CD4^+CD25^+$ cells (most of which we have as yet been unable to assign to known genes), and 97 tags significantly up-regulated in $CD4^+CD25^-$ cells. The majority of the latter tags (at least 58) map to transcripts normally considered housekeeping genes (shown in gray, and defined as being nondifferential (i.e., SD $<$ mean) across 16 other SAGE libraries, including ribosomal proteins and essential metabolic enzymes (38, 51). This relative loss of housekeeping transcripts is further exemplified after CD3 stimulation of the two populations (Fig. 5*B*), and includes GAPDH, EF-1α, and also β_2 microglobulin (while CD3δ, MHC-I, and OX40 change little). This apparent loss of housekeeping gene expression may be explained by the different capacities of the two populations to proceed through the cell cycle; it may be that $CD4^+CD25^+$ cells that do not proliferate in response to TCR ligation do not require many of the synthetic and metabolic enzymes, but express a set of new functional proteins without any cell division. It is clear that $CD4^+CD25^+$ cells have indeed expressed at least 103 new transcripts as a result of their activation (Fig. 5*B*); and therefore, are behaving in a manner that is quite distinct from $CD4^+CD25^-$ cells. Most of these tags are unique among the SAGE libraries we have constructed so far (38, 51), and have not yet been assigned to known genes. Although we used SAGE results exclusively to confirm the distinctiveness of the $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, we have thus far identified four novel candidate genes from the Celera Discovery System mouse gene database (marked as transcripts mCT5392, mCT2519, mCT6469, and mCT4200), whose roles are currently under investigation.

Dominant tolerance is not compromised by administration of mAbs targeting IL-10, IL-4, CTLA-4, and CD25

There are several conflicting reports in the literature implicating particular cytokines and cell surface molecules in dominant tolerance. In some in vivo and in vitro studies, Abs to CTLA-4 (4F10) and CD25 (PC61) have interfered with suppression (52, 53), although others did not find a role for CTLA-4 in vitro (54). We could not implicate CTLA-4 in our readout of suppression (see below). Other studies have also described a role for IL-10, IL-4, and TGFβ in suppression by regulatory T cells, where high doses of mAbs were used in an attempt to neutralize the effect of the target cytokines (24, 39, 43, 55, 56). We could not implicate these same molecules in our own studies.

We transfused 20×10^6 spleen cells from CBA/Ca mice tolerant to B10.BR skin into empty CP1-CBA mice, together with the same number of spleen cells from naive CBA/Ca animals (as described in Fig. 1). Separate groups of these animals were treated with high doses of anti-IL4 mAb (11B11), anti-IL10 mAb (JES5), or both in combination; anti-CD25 mAb (PC61), anti-CTLA-4 (4F10), or both in combination. One control group was treated with anti-canine CD8 (YCATE55), and another control group received naive spleen cells in the absence of spleen cells from tolerant mice. The mAbs were administered in doses of 2 mg at days -4, -2, 0, 5, and then weekly until rejection. The adoptive cell transfer was performed at day -1, and B10.BR skin transplants at day 0. Blood samples from all mice were collected at days 20 and 60 to determine the level of the injected mAbs in the sera. The serum concentration of the injected Abs, as determined by binding inhibition, was >100 μg/ml in all PC61-treated mice (two mice injected with PC61 + 4F10 had serum levels between 1 and 10 μg/ml at day 60); all JES5-treated mice had serum concentrations of the mAb between 10 and 100 μg/ml; all 11B11-treated mice had serum concentrations of the mAb >100 μg/ml (except four mice also injected with JES5 where the mAb concentrations were between 10 and 100 μg/ml at days 20 and 60). The serum concentration of 4F10 was determined through an anti-hamster IgG ELISA. All mice had a serum concentration of hamster Ab between 1 and 10 μg/ml at day 20, dropping to <1 μg/ml at day 60.

The group transferred with cells from naive mice readily rejected the test skin grafts (Fig. 6*A*). However, indefinite graft survival was observed in all other groups, suggesting that those particular targeted molecules do not play a critical role in dominant transplantation tolerance in this model. A similar experiment focused on the naive $CD4^+CD25^+$ population. We transferred 10^6 sorted $CD4^+CD25^+$ spleen cells from naive CBA/Ca mice, together with 10^7 splenocytes from naive CBA/Ca mice, as described. Some of the mice were treated with a combination of anti-CTLA-4 and anti-IL-10 mAbs in the doses mentioned above.

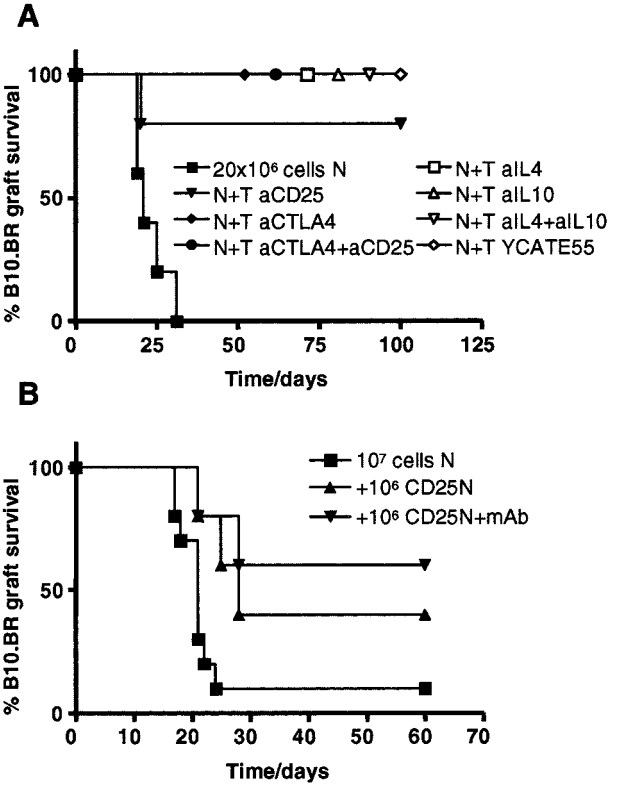

FIGURE 6. Dominant tolerance operates despite treatment with high-dose mAbs targeting IL-10, IL-4, CTLA-4, and CD25. A total of 20×10^6 spleen cells from naive CBA/Ca were injected into empty CP1-CBA mice i.v., either alone (N) or together with the same number of spleen cells from CBA/Ca tolerant to B10.BR skin (N + T). Mice were injected i.p. with 2 mg of mAbs targeting CD25, CTLA-4, IL-4, IL-10, and canine CD8 (control) at days -4, -2, 0, 2, 5, and then weekly until rejection. Cells were injected i.v. at day -1, and all animals received B10.BR skin transplants at day 0. Animals transferred with 20×10^6 spleen cells from naive CBA/Ca in the absence of mAb treatment readily rejected the grafts (■, $n = 5$, MST = 21 day). However, in all other groups, grafts were accepted indefinitely ($n = 5$, MST > 100, $p = 0.0198$ for ▼, $p = 0.0017$ for any other group). Blood samples were collected at days 20 and 60 to confirm the presence of the injected mAb in the sera. *B*, Empty CP1-CBA mice were transfused with 10^7 spleen cells from naive CBA/Ca mice, alone (■, $n = 10$, MST = 21 day) or in combination with 10^6 $CD4^+CD25^+$ spleen cells also from naive CBA/Ca donors (▼ and ▲, $n = 5$ in each group). All mice were transplanted with B10.BR skin on the following day. Some mice transfused with $CD4^+CD25^+$ T cells were treated with 2 mg of each of anti-IL10 and anti-CTLA-4 as described above (▼). There was no significant difference in graft survival between the mAb treated and untreated groups.

Treatment with these mAbs did not result in any significant difference in tolerance induced by $CD4^+CD25^+$ cells (Fig. 6*B*). A similar experiment with sorted $CD4^+CD25^+$ spleen cells from tolerized mice had a comparable result, with none of the mice treated with the mAbs rejecting their grafts (data not shown). Therefore, we can exclude a role for the targeted molecules as mediators of regulation in the experimental system we studied. Previous demonstrations of roles for these molecules in other experimental readouts suggest: 1) that regulation may involve diverse molecular mediators depending on the precise microenvironment where it operates; or 2) that dominant tolerance exploits multiple redundant suppressive pathways where blockade of any one would not impact the outcome; or 3) that some of the effects seen are on the effector population in rendering them more sensitive to Ag-mediated signals–this may be a very plausible explanation for the effects of CTLA-4 mAbs, which should in principle enhance signaling (57, 58); or 4) that regulation takes place in a compartment which the injected mAb cannot easily access, such as the transplanted skin graft itself.

Our results confirm that where transplantation tolerance was induced with therapeutic mAbs, the subpopulation most potent in maintaining tolerance are the $CD4^+CD25^+$ T cells. However, regulatory activity can also be demonstrated within the $CD4^+CD25^-$ population. It is not clear whether this is a result of redundant tolerogenic strategies mediated by different cell populations, or whether it reflects the fact that a proportion of the regulatory T cell population does not express CD25 constitutively. Either way, the characterization of genes differentially expressed by $CD4^+CD25^+$ and $CD4^+CD25^-$ cells may certainly contribute to a better understanding of their physiology, may provide new markers that better define regulatory T cells, and may lead to the development of diagnostic tests for monitoring T cell population changes in autoimmunity or therapeutic tolerance.

Acknowledgments

We thank K. Nolan and P. Fairchild for helpful comments, S. Humm for preparing many of the mAbs used in vivo, and the PSB staff, especially M. Coates and S. Laynes, for excellent animal care.

References

1. Billingham, R. E., L. Brent, and P. B. Medawar. 1953. Actively acquired tolerance to foreign cells. *Nature 172:603.*
2. Qin, S. X., M. Wise, S. P. Cobbold, L. Leong, Y. C. Kong, J. R. Parnes, and H. Waldmann. 1990. Induction of tolerance in peripheral T cells with monoclonal antibodies. *Eur. J. Immunol. 20:2737.*
3. Qin, S., S. P. Cobbold, H. Pope, J. Elliott, D. Kioussis, J. Davies, and H. Waldmann. 1993. Infectious transplantation tolerance. *Science 259:974.*
4. Davies, J. D., L. Y. Leong, A. Mellor, S. P. Cobbold, and H. Waldmann. 1996. T cell suppression in transplantation tolerance through linked recognition. *J. Immunol. 156:3602.*
5. Chen, Z. K., S. P. Cobbold, H. Waldmann, and S. Metcalfe. 1996. Amplification of natural regulatory immune mechanisms for transplantation tolerance. *Transplantation 62:1200.*
6. Waldmann, H., and S. Cobbold. 1998. How do monoclonal antibodies induce tolerance: a role for infectious tolerance? *Annu. Rev. Immunol. 16:619.*
7. Graca, L., K. Honey, E. Adams, S. P. Cobbold, and H. Waldmann. 2000. Cutting edge: anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *J. Immunol. 165:4783.*
8. Hall, B. M., M. E. Jelbart, K. E. Gurley, and S. E. Dorsch. 1985. Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine: mediation of specific suppression by T helper/inducer cells. *J. Exp. Med. 162:1683.*
9. Yin, D., and C. G. Fathman. 1995. CD4-positive suppressor cells block allotransplant rejection. *J. Immunol. 154:6339.*
10. Zhai, Y., and J. W. Kupiec-Weglinski. 1999. What is the role of regulatory T cells in transplantation tolerance? *Curr. Opin. Immunol. 11:497.*
11. Waldmann, H., and S. Cobbold. 2001. Regulating the immune response to transplants: a role for $CD4^+$ regulatory cells? *Immunity 14:399.*
12. Sakaguchi, S., K. Fukuma, K. Kuribayashi, and T. Masuda. 1985. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J. Exp. Med. 161:72.*
13. Powrie, F., and D. Mason. 1990. OX-22^{high} $CD4^+$ T cells induce wasting disease with multiple organ pathology: prevention by the OX-22^{low} subset. *J. Exp. Med. 172:1701.*
14. Fowell, D., and D. Mason. 1993. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes: characterization of the $CD4^+$ T cell subset that inhibits this autoimmune potential. *J. Exp. Med. 177:627.*
15. Mason, D., and F. Powrie. 1998. Control of immune pathology by regulatory T cells. *Curr. Opin. Immunol. 10:649.*
16. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell 101:455.*
17. Shevach, E. M. 2000. Regulatory T cells in autoimmmunity. *Annu. Rev. Immunol. 18:423.*
18. Maloy, K. J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat. Immunol. 2:816.*
19. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol. 155:1151.*
20. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med. 184:387.*
21. Thornton, A. M., and E. M. Shevach. 1998. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med. 188:287.*
22. Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi. 1998. Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol. 10:1969.*
23. Piccirillo, C. A., and E. M. Shevach. 2001. Cutting edge: control of $CD8^+$ T cell activation by $CD4^+CD25^+$ immunoregulatory cells. *J. Immunol. 167:1137.*
24. Hara, M., C. I. Kingsley, M. Niimi, S. Read, S. E. Turvey, A. R. Bushell, P. J. Morris, F. Powrie, and K. J. Wood. 2001. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J. Immunol. 166:3789.*
25. Gregori, S., M. Casorati, S. Amuchastegui, S. Smiroldo, A. M. Davalli, and L. Adorini. 2001. Regulatory T cells induced by 1α,25-dihydroxyvitamin D_3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J. Immunol. 167:1945.*
26. Velculescu, V. E., L. Zhang, B. Vogelstein, and K. W. Kinzler. 1995. Serial analysis of gene expression. *Science 270:484.*
27. Gilliland, L. K., L. A. Walsh, M. R. Frewin, M. P. Wise, M. Tone, G. Hale, D. Kioussis, and H. Waldmann. 1999. Elimination of the immunogenicity of therapeutic antibodies. *J. Immunol. 162:3663.*
28. Monaco, A. P., M. L. Wood, J. G. Gray, and P. S. Russell. 1966. Studies on heterologous anti-lymphocyte serum in mice. II. Effect on the immune response. *J. Immunol. 96:229.*
29. Peto, R., M. C. Pike, P. Armitage, N. E. Breslow, D. R. Cox, S. V. Howard, N. Mantel, K. McPherson, J. Peto, and P. G. Smith. 1977. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. *Br. J. Cancer 35:1.*
30. Riechmann, L., M. Clark, H. Waldmann, and G. Winter. 1988. Reshaping human antibodies for therapy. *Nature 332:323.*
31. Bhattacharya, A., M. E. Dorf, and T. A. Springer. 1981. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol. 127:2488.*
32. Yelton, D. E., C. Desaymard, and M. D. Scharff. 1981. Use of monoclonal anti-mouse immunoglobulin to detect mouse antibodies. *Hybridoma 1:5.*
33. Cobbold, S. P., A. Jayasuriya, A. Nash, T. D. Prospero, and H. Waldmann. 1984. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. *Nature 312:548.*
34. Lowenthal, J. W., R. H. Zubler, M. Nabholz, and H. R. MacDonald. 1985. Similarities between interleukin-2 receptor number and affinity on activated B and T lymphocytes. *Nature 315:669.*
35. Krummel, M. F., and J. P. Allison. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J. Exp. Med. 182:459.*
36. Sander, B., I. Hoiden, U. Andersson, E. Moller, and J. S. Abrams. 1993. Similar frequencies and kinetics of cytokine producing cells in murine peripheral blood and spleen: cytokine detection by immunoassay and intracellular immunostaining. *J. Immunol. Methods 166:201.*
37. Ohara, J., and W. E. Paul. 1985. Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. *Nature 315:333.*
38. Zelenika, D., E. Adams, S. Humm, C.-Y. Lin, H. Waldmann, and S. P. Cobbold. 2001. The role of $CD4^+$ T cell subsets in determining transplantation rejection and tolerance. *Immunol. Rev. 182:164.*
39. Davies, J. D., G. Martin, J. Phillips, S. E. Marshall, S. P. Cobbold, and H. Waldmann. 1996. T cell regulation in adult transplantation tolerance. *J. Immunol. 157:529.*
40. Taylor, P. A., R. J. Noelle, and B. R. Blazar. 2001. $CD4^+CD25^+$ immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J. Exp. Med. 193:1311.*
41. Davies, J. D., E. O'Connor, D. Hall, T. Krahl, J. Trotter, and N. Sarvetnick. 1999. $CD4^+$CD45RB low-density cells from untreated mice prevent acute allograft rejection. *J. Immunol. 163:5353.*
42. Thornton, A. M., and E. M. Shevach. 2000. Suppressor effector function of $CD4^+CD25^+$ immunoregulatory T cells is antigen nonspecific. *J. Immunol. 164: 183.*
43. Seddon, B., and D. Mason. 1999. Regulatory T cells in the control of autoimmunity: the essential role of transforming growth factor β and interleukin 4 in the

prevention of autoimmune thyroiditis in rats by peripheral $CD4^+CD45RC^-$ cells and $CD4^+CD8^-$ thymocytes. *J. Exp. Med. 189:279.*
44. Wise, M. P., F. Bemelman, S. P. Cobbold, and H. Waldmann. 1998. Linked suppression of skin graft rejection can operate through indirect recognition. *J. Immunol. 161:5813.*
45. Stephens, L. A., and D. Mason. 2000. CD25 is a marker for $CD4^+$ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function are found in both $CD25^+$ and $CD25^-$ subpopulations. *J. Immunol. 165:3105.*
46. Olivares-Villagomez, D., A. K. Wensky, Y. Wang, and J. J. Lafaille. 2000. Repertoire requirements of $CD4^+$ T cells that prevent spontaneous autoimmune encephalomyelitis. *J. Immunol. 164:5499.*
47. Shevach, E. M. 2001. Certified professionals: $CD4^+CD25^+$ suppressor T cells. *J. Exp. Med. 193:F41.*
48. Gavin, M. A., S. R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of $CD4^+CD25^+$ suppressor T cells in vivo. *Nat. Immunol. 3:33.*
49. Wells, A. D., X. C. Li, Y. Li, M. C. Walsh, X. X. Zheng, Z. Wu, G. Nunez, A. Tang, M. Sayegh, W. W. Hancock, et al. 1999. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat. Med. 5:1303.*
50. Li, Y., X. C. Li, X. X. Zheng, A. D. Wells, L. A. Turka, and T. B. Strom. 1999. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat. Med. 5:1298.*
51. Zelenika, D., E. Adams, S. Humm, L. Graca, S. Thompson, S. P. Cobbold, and H. Waldmann. 2002. Regulatory T cells over-express a subset of Th2 gene transcripts. *J. Immunol. 168:1069.*
52. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med. 192:303.*
53. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+CD4^+$ regulatory cells that control intestinal inflammation. *J. Exp. Med. 192:295.*
54. Shevach, E. M., R. S. McHugh, C. A. Piccirillo, and A. M. Thornton. 2001. Control of T-cell activation by $CD4^+CD25^+$ suppressor T cells. *Immunol. Rev. 182:58.*
55. Powrie, F., J. Carlino, M. W. Leach, S. Mauze, and R. L. Coffman. 1996. A critical role for transforming growth factor-β but not interleukin 4 in the suppression of T helper type 1-mediated colitis by $CD45RB^{low}$ $CD4^+$ T cells. *J. Exp. Med. 183:2669.*
56. Asseman, C., S. Mauze, M. W. Leach, R. L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med. 190:995.*
57. Hurwitz, A. A., T. J. Sullivan, R. A. Sobel, and J. P. Allison. 2002. Cytotoxic T lymphocyte antigen-4 (CTLA-4) limits the expansion of encephalitogenic T cells in experimental autoimmune encephalomyelitis (EAE)-resistant BALB/c mice. *Proc. Natl. Acad. Sci. USA 99:3013.*
58. Egen, J. G., and J. P. Allison. 2002. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity 16:23.*