Differential control of allo-antigen-specific regulatory T cells and effector T cells by anti-CD4 and other agents in establishing transplantation tolerance

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Abstract

Donor-specific graft tolerance can be established by a combination of allo-antigen exposure and manipulation of T cell function, for example by donor-specific transfusion (DST) under the cover of a non-depleting anti-CD4 mAb. Yet, the cellular basis of this graft tolerance is still obscure. This report shows that T cell-deficient BALB/c nude mice reconstituted with naive unfractionated T cells are specifically tolerized to DBA/2 skin grafts by DST and anti-CD4 mAb treatment, whereas those transferred with T cell suspensions depleted of all Foxp3+CD25+CD4+ natural regulatory T cells (Tregs) are not. The treatment inhibits Mls-1a allo-antigen-specific expansion of CD4+ non-Tregs expressing Vβ6 TCR subfamily but leaves the expansion of Vβ6-expressing Tregs unaffected, allowing the latter to selectively expand and establish donor-specific tolerance. Furthermore, anti-CD4 mAb inhibits in vitro the selective expansion of allo-antigen-specific CD4+ non-Tregs but not natural Tregs, as observed with in vitro anti-CD154 [CD40 ligand (CD40L)] mAb or rapamycin treatment. The results collectively indicate that the differential effect of biologicals and pharmacological substances on the expansion of allo-antigen-specific Tregs and effector T cells and resulting dominance of the former can be a key general mechanism underlying dominant transplantation tolerance.

Introduction

Establishment of allo-antigen-specific transplantation tolerance as stable as immunological self-tolerance is an ultimate goal of organ transplantation. There is accumulating evidence that naturally arising CD25+CD4+ regulatory T cells (Tregs), which specifically express the transcription factor Foxp3, play a crucial role in the maintenance of self-tolerance and negative control of a variety of physiological and pathological immune responses including autoimmunity and tumor immunity (1). Natural Tregs can also be exploited to establish transplantation tolerance and treat graft-versus-host disease after allogeneic bone marrow transplantation (2, 3). Indeed, permanent graft tolerance can be achieved in rodents without any use of immunosuppressant when antigen-specific natural Tregs are allowed to expand to the extent that they are sufficient in number and suppressive activity to control the expansion/activation of allo-reactive effector T cells (4). Tregs also play key roles in allograft tolerance induced and maintained by T cell-targeting biological agents or immunosuppressive drugs (2, 5). For example, exposure to allograft under the cover of cell-non-depleting anti-CD4 mAb or blocking anti-CD154 [CD40 ligand (CD40L)] mAb leads to the appearance of allo-antigen-specific CD25+CD4+ Tregs with the ability to suppress allograft rejection (6-9). Rapamycin has been shown to enrich CD25+CD4+ Tregs in vitro and in vivo (10).
Yet, it is still unclear how these treatments induce allo-antigen-specific Tregs capable of suppressing donor T cells, whether such antigen-specific Tregs have expanded from natural Tregs or induced from naive T cells or whether the treatments with different molecular targets share a common cellular basis for the establishment of allograft tolerance.

We show in this report that combined therapy of cell-non-depleting anti-CD4 mAb and donor-specific transfusion (DST) allows the selective expansion of donor-specific Tregs while inhibiting expansion of antigen effector T cells both in vivo and in vitro. In addition, anti-CD4, anti-CD154 mAb and rapamycin similarly inhibit in vitro expansion of effector T cells, but not Tregs, albeit by different molecular mechanisms. This differential effect on the expansion of antigen-reactive Tregs and effector T cells, with resulting selective expansion of the former, can be exploited to design new agents and regimen for establishing antigen-specific dominant tolerance toward non-self- and also self-antigen.

Materials and methods

Mice

Female BALB/c, DBA/2, C57BL/6 (B6), C3H and BALB/c athymic nude mice of 4–6 weeks of age were purchased from Clea Japan (Tokyo, Japan). BALB/c-Thy1.1 congenic mice were established in our laboratory (11). All these mice were maintained in our animal facility and cared for in accordance with the institutional guidelines for animal welfare.

Antibodies and reagents

FITC-, PE-, CyChrome or biotin-labeled mAbs to CD25 (7D4), CD4 (RM4-5 and H129.19), Vδ6 (RR4-7), Vβ10 (B21.5) and PE–, allophycocyanin (APC)– or CyChrome–streptavidin, as a secondary reagent, were all purchased from PharMingen (San Diego, CA, USA). Rat anti-mouse glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) mAb (DTA-1) was made from ascites in SCID mice and purified with the addition of recombinant mouse granulocyte macrophage colony-stimulating factor and rmIL-4 (10 ng ml⁻¹ final concentration for both cytokines) (Peprotech, Rocky Hill, NJ, USA). On day 3, supernatants and non-adenherent cells were aspirated and replaced by fresh cytokine-supplemented medium. Mature DCs were generated by the overnight addition of 1 μg ml⁻¹ of LPS (S. enterica; Sigma) on either day 5 or 6 of culture. DCs prepared in this way were typically 60–70% CD11c⁺. Purified cells were routinely analyzed by flow cytometry using a FACSCalibur™ (BD Biosciences, San Jose, CA, USA).

Skin transplantation and cell transfer

As previously described (4, 15), full thickness tail skin was removed from euthanized donors, placed on sterile filter paper moistened with PBS and kept at 4°C until use, usually within 30 min. The dorsal surfaces of anesthetized recipient mice were washed with 70% ethanol. A graft bed was prepared with fine scissors by removing an area of epidermis and dermis down to the level of the muscle fascia. Skin grafts (0.5 cm²) were placed into the prepared bed without suturing and then covered with Vaseline-impregnated gauze and an adhesive plastic bandage. After 7 days, the bandage was removed. Cell suspensions in 0.2 or 0.4 ml of RPMI1640 medium (Gibco BRL) were intravenously (i.v.) administered through a tail vein in the cell transfer. Skin graft survival was assessed four times a week by visual and tactile examination. Rejection was defined as the first day on which the entire epidermal surface of the graft became necrotic. Statistical analysis of graft survival was made using the log-rank method.
Allogenic expansion of carboxyl fluorescent succinimidyl ester-labeled CD25+CD4+ and GITRlowCD4+ T cells

For in vitro studies, CD25+ or GITRlowCD4+ T cells were collected from BALB/c-Thy1.1 congenic mice and were incubated with 1 μM carboxyl fluorescent succinimidyl ester (CFSE) for 10 min at 37°C for CFSE labeling. CD25+CD4+ or GITRlowCD4+ responders (2.5 × 10^4) were mixed with 2.5 × 10^4 DCs from DBA/2 mice in round-bottom 96-well tissue culture plates (Falcon, Franklin Lakes, NJ, USA) in RPMI1640 (Gibco BRL) medium containing 10% FCS (PAA Laboratories, Newport Beach, CA, USA), penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) and 50 μM 2-ME. On the seventh day of culture, cells were collected, blocked with Fc receptor antibody (2.4G2) and stained for Thy1.1 and Vβ6 or Vβ10. Cell proliferation was quantified using a FACSCalibur™ (BD Biosciences) with dead cell exclusion by 7-AAD.

Apoptosis analysis

Apoptotic cells were identified using flow cytometric analysis of cells stained with APC-labeled Annexin V to identify apoptotic cells and 7-AAD to label permeable (dead) cells (BD PharMingen), according to the manufacturer’s instructions using a FACSCalibur™ (BD Biosciences).

Results

Donor-specific tolerance to skin allografts can be induced by anti-CD4–DST combined therapy in minor histocompatibility mismatch between BALB/c and DBA/2

Infusion of donor allo-antigen under the cover of a cell-non-depleting anti-CD4 mAb (YTS177) has been reported to generate allo-antigen-specific CD25+CD4+ Tregs with the ability to regulate rejection of skin allografts (6, 7, 16, 17). These studies, which used CBA Rag-1−/− mice reconstituted with syngeneic T cells as recipients and C57BL/10 mice as donors, do not allow the detection of allo-antigen-specific Tregs. To detect Tregs specific for donor allo-antigens, we took advantage of the anti-Mls-1 (Mtv-7 endogenous retroviral antigen) response of BALB/c mice, which are Mls-1b,t to DBA/2,nontolerant mice in Groups III, IV and V that had rejected DBA/2 skins. Graft survival (%)

To induce graft rejection of DBA/2 skin graft, third party B6 skin fragment was transplanted onto the flank of DBA/2-tolerized mice in Group I in (A) or non-tolerized mice in Groups III, IV and V that had rejected DBA/2 skins.

We have previously reported that Foxp3+ natural Tregs, whether they are CD25+ or CD25−, express GITR at higher levels than other T cells, and GITRlowCD4+ T cells in normal naïve mice are free of Foxp3+ Tregs (13). We therefore analyzed the tolerizing capacity of anti-CD4/DST with GITRlowCD4+ T cells to determine whether the treatment directly tolerizes the effectors (or effector precursors) of graft rejection. Nude mice reconstituted with GITRlowCD4+ cells (Group II) could not be effectively tolerized to DBA/2 skin
grafts even with anti-CD4–DST combined therapy although graft survival was modestly but significantly extended in comparison with the untreated control group (MST: 24 days versus 14.5 days, \( n = 7 \) and 6, respectively, \( P < 0.05 \)). In addition, BALB/c nude mice reconstituted with GITR\textsubscript{low}CD4\textsuperscript{+} cells without anti-CD4–DST combined therapy rejected DBA/2 skin grafts as rapidly as Group V mice reconstituted with whole T cells (data not shown).

Next, by observing the survival of third party allografts, we examined whether tolerance induced by anti-CD4–DST combined therapy was donor specific. B6 skin was grafted onto the flank of mice retaining DBA/2 grafts and those mice that had rejected DBA grafts in control groups. B6 skin grafts were rejected normally, regardless of whether the recipient mice were tolerant or non-tolerant of DBA/2. MST of 25 days (DBA/2-tolerant mice) versus 22.5 days (DBA/2-non-tolerant mice), \( n = 7 \) and 18, respectively (Fig. 1C). The result indicates that the DBA/2-tolerant mice were immunocompetent and the tolerance was antigen specific.

Thus, combined therapy with anti-CD4 mAb plus DST can induce donor-specific graft tolerance in BALB/c nude mice reconstituted with unfractionated whole T cells, but not in those transferred with GITR\textsubscript{low}CD4\textsuperscript{+} cells that are devoid of natural Tregs. Both anti-CD4 and DST treatments are essential for the induction of tolerance.

Anti-CD4 mAb inhibits in vivo expansion of allo-antigen-specific GITR\textsubscript{low}CD4\textsuperscript{+} T cells but not the expansion of CD25\textsuperscript{+}CD4\textsuperscript{+} Tregs

The above results indicate that natural Foxp3\textsuperscript{+}CD25\textsuperscript{+}CD4\textsuperscript{+} Tregs present in the GITR\textsubscript{low}CD4\textsuperscript{+} population play an essential role for the establishment of graft tolerance by DST-anti-CD4 treatment. It is also possible that effector T cells (or their precursors) might differentiate into Tregs during in vivo allo-antigen exposure and contribute to tolerance induction (4, 6, 18). We therefore set out to investigate the antigen-specific expansion/contraction of Tregs or GITR\textsubscript{low}CD4\textsuperscript{+} effector T cells in response to anti-CD4–DST treatment. In order to follow the in vivo kinetics of transferred cells, we isolated T cells from naive BALB/c-Thy1.1\textsuperscript{+} congenic mice and used them to reconstitute BALB/c nude recipients, which are Thy1.2\textsuperscript{+}. Donor-specific (anti-Mls-1) responses were followed by tracing the expansion of V\beta6\textsuperscript{+} T cells in transferred Thy1.1\textsuperscript{+} Tregs or GITR\textsubscript{low}CD4\textsuperscript{+} effector T cells under anti-CD4–DST combined therapy. By comparing the frequencies of V\beta6\textsuperscript{+} cells to those of V\beta10\textsuperscript{+} cells (which do not respond to Mls-1\textsuperscript{a}), we could exclude the possibility of any non-specific homeostatic proliferation arising from transfer into a lymphopenic environment. Prior to transfer, CD25\textsuperscript{+}CD4\textsuperscript{+} T cells and naive GITR\textsubscript{low}CD4\textsuperscript{+} T cells contained very similar frequencies of V\beta6 (~7%) and V\beta10 (~3%) (Fig. 2A).

Following adoptive transfer, analysis of V\beta6\textsuperscript{+} cell frequency in lymph nodes demonstrated that Tregs had expanded to similar extents regardless of whether mice were treated with anti-CD4 or isotype control mAb (Fig. 2A and B). In contrast, anti-CD4–DST significantly inhibited GITR\textsubscript{low}CD4\textsuperscript{+} cell expansion when compared with the isotype control. Similarly, the expansion of V\beta6 Tregs but not V\beta6GITR\textsubscript{low}CD4\textsuperscript{+} cells was observed in the spleens after anti-CD4–DST treatment (Supplementary Figure S1, available at International Immunology Online). In contrast with V\beta6\textsuperscript{+} cells, V\beta10\textsuperscript{+} Tregs and GITR\textsubscript{low}CD4\textsuperscript{+} T cells expanded neither in any of the treatment groups nor at any of the time points (Fig. 2B).

In addition, to determine whether the lymphopenic environment of nude mice influenced the effects of anti-CD4–DST on each T cell population, we transferred CFSE-labeled CD25\textsuperscript{+}CD4\textsuperscript{+} Tregs or GITR\textsubscript{low}CD4\textsuperscript{+} cells to normal BALB/c mice and assessed their expansion as the degree of CFSE dilution. As in lymphopenic hosts, anti-CD4–DST treatment selectively inhibited the antigen-specific expansion of GITR\textsubscript{low}CD4\textsuperscript{+} effector T cells, but not Tregs (Fig. 2C).

Thus, anti-CD4/DST allows in vivo expansion of antigen-specific Tregs but inhibits the expansion of antigen-specific effector T cells.

Donor-specific CD25\textsuperscript{+}CD4\textsuperscript{+} Tregs expand and regulate antidonor activity of GITR\textsubscript{low}CD4\textsuperscript{+} effector cells in allograft-tolerized mice

To confirm that the allo-antigen-specific Treg expansion is a key to donor-specific tolerance induced by anti-CD4–DST combined treatment, we attempted to determine whether the expansion of donor-specific (V\beta6\textsuperscript{+}) Tregs actually occurred in mice which had been completely tolerized to DBA/2 skin grafts (Fig. 3). To distinguish between in vivo-expanded regulatory populations arising from the original CD25\textsuperscript{+}CD4\textsuperscript{+} Tregs and those from GITR\textsubscript{low}CD4\textsuperscript{+} cells, we again employed Thy1.1 congenic mice. BALB/c nude mice were reconstituted with a mixture of an equal number of Thy1.1\textsuperscript{+}CD25\textsuperscript{+}CD4\textsuperscript{+} Tregs and Thy1.2\textsuperscript{+}GITR\textsubscript{low}CD4\textsuperscript{+} effector T cells, treated with anti-CD4 (or control mAb) and DST and transplanted with DBA/2 skin grafts according to the basic protocol in Fig. 1(A).

All mice that had received anti-CD4/DST were tolerized to DBA/2 skins (Fig. 3A, Group I), but those treated with control mAb/DST were not (Group II) (MST: >100 days and 20 days, respectively, \( P < 0.005 \)).

In tolerant mice (Group I), the frequencies of V\beta6\textsuperscript{+} Tregs were significantly higher than before transfer (20.8 versus 8.2%, respectively, \( n = 5 \), \( P < 0.02 \)), whereas the frequencies of GITR\textsubscript{low}-derived V\beta6\textsuperscript{+}CD4\textsuperscript{+} T cells were significantly lower than before transfer (2.4 versus 8.3%, respectively, \( n = 5 \), \( P < 0.05 \)) (Fig. 3B). On the other hand, in rejecting mice (Group II), the frequencies of both V\beta6\textsuperscript{+} Tregs and GITR\textsubscript{low}-derived V\beta6\textsuperscript{+}CD4\textsuperscript{+} cells were higher than before transfer (Tregs: 39.4 versus 8.2%, respectively, \( P < 0.001 \); GITR\textsubscript{low}CD4\textsuperscript{+} T cells: 49.9 versus 8.3%, respectively, \( P < 0.0001 \)).

Staining of intracellular Foxp3 revealed that donor Thy1.1\textsuperscript{+}CD25\textsuperscript{+}CD4\textsuperscript{+} T cells retained Foxp3 expression and that both tolerant mice and graft-rejecting mice harbored similar frequencies of Foxp3\textsuperscript{+} cells with similar proportions of V\beta6\textsuperscript{+} and V\beta6\textsuperscript{−} cells (Fig. 3C). V\beta6\textsuperscript{−} T cells constituted ~40% of Foxp3\textsuperscript{+} cells in both tolerant and rejecting mice, showing allo-antigen-specific expansion of Foxp3\textsuperscript{+} cells in both mice (see also Fig. 4). Among Foxp3\textsuperscript{−} cells in the Thy1.2\textsuperscript{+} population, V\beta6\textsuperscript{−} cells increased in graft-rejecting mice but not in tolerant mice, correlating with the results in Fig. 3B. In addition, transferred GITR\textsubscript{low}CD4\textsuperscript{+} T cells, which are almost completely depleted of Foxp3\textsuperscript{+} cells (11), appeared to give rise to a small number of Foxp3\textsuperscript{+} cells. Some of such Thy1.2\textsuperscript{+}Foxp3\textsuperscript{+} cells could, however, be
derived from nude mice because aged nude mice harbored a small but detectable number Foxp3+CD4+ T cells (1% of CD4+ cells) (data not shown).

Thus, the selective expansion of allo-antigen-reactive Foxp3+ Tregs correlates with the establishment of stable graft tolerance. Antigen-specific Foxp3+ Tregs also expand in graft-rejecting mice; however, the expansion of effector T cells appears to surpass that of Tregs in the absence of anti-CD4–DST treatment.

Donor-specific Tregs expanded by anti-CD4–DST treatment actively maintain graft tolerance

We next attempted to determine whether graft tolerance induced by anti-CD4–DST treatment was actively maintained.
Fig. 3. The expansion of donor-specific CD25+CD4+ or GITRloCD4+ T cells in graft-tolerant or rejecting mice. (A) BALB/c nude mice were reconstituted with a mixture of an equal number (1 × 10⁵) of Thy1.1+CD25+CD4+ T cells and Thy1.2+CD4+GITRlo cells, treated with anti-CD4/DST or control Ab/DST as in Fig. 1(A) and grafted with DBA/2 skins. (B) One hundred days later, lymph node and spleen cells from mice retaining skin grafts or having rejected grafts were assessed for the percentages of Vβ6+ or Vβ10+ cells among Thy1.1+ or Thy1.2+CD4+ cells. A representative staining (upper figures) and percentages of Vβ6+ or Vβ10+ cells in individual mice in (A) (lower figures). Dotted lines indicate average percentages of Vβ6+ and Vβ10+ cells among Thy1.1+ or Thy1.2+ cells in donor cell inocula before cell transfer. (C) Lymph node and splenocytes were gated for Thy1.1+ or Thy1.2+ cells and assessed for the expression of Vβ6 and intracellular Foxp3. The numbers in quadrants are percentages of cells among Thy1.1+ or Thy1.2+ cells. A representative of three independent experiments.
by the regulatory activity of CD25+CD4+ Tregs directed against GITRlowCD4+ cell-derived effector cells rather than simply by a reduction in cell number of the latter. To test whether the removal of Tregs unmasked the activity of GITRlowCD4+ cells and triggered rejection of grafts, we collected lymph node cells and spleenocytes from individual tolerant mice in Group I in Fig. 3 and the same number of cell suspensions were transferred to the second recipient after depletion of donor-derived Tregs by anti-Thy1.1 mAb and C, and 1 × 10^7 treated cells were transferred to one each of nude mouse bearing DBA/2 graft. Group A: recipients of control mAb + C-treated cells; Group B: recipients of anti-Thy1.1 + C-treated cells. (B) Graft survival in Groups A and B. (C) Lymph node cells and spleenocytes were collected from Group A when the graft was rejected or Group B when graft survival was >100 days and analyzed by FACS for the percentages of Vβ6+ or Vβ10+ cells among Thy1.1−CD4+ T cells in Group A or either Thy1.1+ or Thy1.1−CD4+ T cells in Group B. The percentages were compared with those of donor T cells before transfer. Asterisk means *P < 0.00005 by paired t-test.

Fig. 4. Graft survival and the change of donor-specific population of CD25+CD4+ or GITRlowCD4+ T cells in the secondary recipients. (A) Lymph node and spleen cells collected from each tolerant mouse in Fig. 3(A) (total five mice) were treated with anti-Thy1.1 or control mAb and C, and 1 × 10^7 treated cells were transferred to one each of nude mouse bearing DBA/2 graft. Group A: recipients of control mAb + C-treated cells; Group B: recipients of anti-Thy1.1 + C-treated cells. (B) Graft survival in Groups A and B. (C) Lymph node cells and spleenocytes were collected from Group A when the graft was rejected or Group B when graft survival was >100 days and analyzed by FACS for the percentages of Vβ6+ or Vβ10+ cells among Thy1.1−CD4+ T cells in Group A or either Thy1.1+ or Thy1.1−CD4+ T cells in Group B. The percentages were compared with those of donor T cells before transfer. Asterisk means *P < 0.00005 by paired t-test.

by the regulatory activity of CD25+CD4+ Tregs directed against GITRlowCD4+ cell-derived effector cells rather than simply by a reduction in cell number of the latter. To test whether the removal of Tregs unmasked the activity of GITRlowCD4+ cells and triggered rejection of grafts, we collected lymph node cells and spleenocytes from individual tolerant mice in Group I in Fig. 3 and the same number of cell suspensions were transferred to the second recipient after depletion of donor-derived Tregs by in vitro treatment either with anti-Thy1.1 mAb and C or with C alone (Groups A and B, respectively, in Fig. 4A). All the grafts in Group A were swiftly rejected (MST: 40 days), whereas all the grafts in Group B were retained for >100 days (*P < 0.005) (Fig. 4B).

The frequencies of Vβ6+ and Vβ10+ populations were measured to assess the sizes of allo-antigen-reactive Tregs and effector T cells (Fig. 4C). In rejecting mice (Group A), the Vβ6 frequency among GITRlow-derived CD4+ cells, which were Thy1.1−, was much higher than before transfer (35.6 versus 2.5%, n = 5, *P < 0.00005). On the other hand, in tolerant mice (Group B), the frequencies of Vβ6+ GITRlow-derived CD4+ cells remained low and unchanged following transfer (mean: 2.4
Fig. 5. Expansion of allo-antigen-specific CD25^{+}CD4^{+} Tregs or GITR^{low}CD4^{+} non-Tregs stimulated with allogeneic DCs in the presence of anti-CD4 mAb. (A and B) In order to detect expansion of responders separately from stimulators, CD25^{+}CD4^{+} or GITR^{low}CD4^{+} T cells were collected from naive BALB/c-Thy1.1^{+} congenic mice. Then, either of the populations was CFSE labeled and co-cultured with freshly prepared mature DCs.
versus 4.2%, \( n = 5 \), \( P = 0.1357 \)), with similar or slightly elevated frequencies of V\( \text{J}6^+ \) Tregs, which were Thy1.1\(^+ \), following transfer (30.2 versus 20.8%, \( n = 5 \), \( P < 0.02 \)).

Thus, once tolerance is established by anti-CD4–DST treatment, allo-antigen-reactive Tregs actively and continuously suppress the expansion of effector T cells, which are not deleted upon exposure to allo-antigen via direct or indirect pathway but able to mediate graft rejection once Tregs are depleted or reduced.

**CD25\(^+\)CD4\(^+\) Tregs can expand to allogeneic DCs in the presence of anti-CD4 mAb but the expansion of GITR\(^{low}\)CD4\(^+\) T effector cells is blocked**

We next investigated whether the relative resistance of Tregs to \( \textit{in vivo} \) anti-CD4 treatment could also be observed in an \( \textit{in vitro} \) mixed lymphocyte reaction (MLR). It has been shown that naturally arising CD25\(^+\)CD4\(^+\) Tregs readily expand to bone marrow-derived dendritic cells (DCs) \( \textit{in vitro} \) (19–21). We therefore examined the expansion of Tregs or GITR\(^{low}\)CD4\(^+\) cells from BALB/c mice to \( \textit{in vitro} \)-matured bone marrow-derived DCs from DBA/2 mice in the presence of titrated doses of anti-CD4. Consistent with the \( \textit{in vivo} \) experiments, anti-CD4 but not the isotype control clearly inhibited the expansion of GITR\(^{low}\)CD4\(^+\) cells in a dose-dependent manner as measured by CFSE dilution and cell counts (Fig. 5B and C) but had no effect on Treg expansion whatsoever (Fig. 5A and B). These results collectively indicate that the susceptibility to anti-CD4 is fundamentally different between natural Tregs and non-Treg CD4\(^+\) cells.

Furthermore, not only in a mismatch of minor histocompatibility antigen but also in a fully MHC-mismatched response observed between BALB/c (H-2\(^b\)) T cells and B6 (H-2\(^b\)) DCs, anti-CD4 allowed the expansion of CD25\(^+\)CD4\(^+\) Tregs but inhibited that of GITR\(^{low}\)CD4\(^+\) T cells (Supplementary Figure S2, available at International Immunology Online). This indicates that the differential effect of anti-CD4–DST treatment on T cell sub-populations in BALB/c anti-DBA/2 responses is not unique to the direct pathway of antigen presentation in anti-Mls responses.

**CD25\(^+\)CD4\(^+\) Tregs can expand to allogeneic DCs in the presence of anti-CD40L mAb but the expansion of GITR\(^{low}\)CD4\(^+\) T cells is blocked**

In addition to anti-CD4–DST, combined therapy with anti-CD40L mAb (clone MR1) and DST has also been reported to tolerize recipients to allografts in a variety of murine models (2, 5, 8, 9). Yet, it remains to be determined whether the mAb differentially affects antigen-specific expansion of Tregs and effector T cells. In \( \textit{in vitro} \) MLR, anti-CD40L indeed inhibited antigen-dependent proliferation of GITR\(^{low}\)CD4\(^+\) cells but not Tregs, assessed by the percentages of CFSE-labeled cells and cell numbers in culture wells (Fig. 6A and B). Thus, non-Tregs are sensitive to anti-proliferative effect of the mAb, whereas Tregs are resistant, allowing the expansion of the latter but not the former.

**Rapamycin induces apoptosis/necrosis of GITR\(^{low}\)CD4\(^+\) effector cells but not CD25\(^+\)CD4\(^+\) Tregs during stimulation by allogeneic DCs**

It was reported that T cells activated in the presence of rapamycin, an IL-2 signaling inhibitor, were highly enriched for CD4\(^+\)CD25\(^{bright}\) T cells (11). However, the susceptibility of antigen-induced expansion of natural Tregs and Treg-free effector T cells to rapamycin has not been separately assessed and compared. In MLR using allogeneic DCs in the presence of rapamycin, the frequencies of viable Tregs and GITR\(^{low}\)CD4\(^+\) cells were both substantially low and cell proliferation measured by CFSE dilution was similarly reduced, suggesting that the agent inhibited the antigen-dependent expansion of both cell types (Fig. 7A). However, cell morphology (as denoted by forward and side scatters) after culture with rapamycin was noticeably different between Tregs and GITR\(^{low}\)CD4\(^+\) cells. Upon allo-stimulation, Tregs enlarged in size regardless of the presence of rapamycin, in contrast to the distinct reduction in cell volume of GITR\(^{low}\)CD4\(^+\) cells. This difference was also reflected in cell viability as measured by trypan blue exclusion (data not shown). This showed unaltered numbers of viable Tregs following rapamycin treatment but dramatically fewer GITR\(^{low}\)CD4\(^+\) cells (Fig. 7B). Notably, Annexin V–7-AAD staining in the same MLR system revealed that rapamycin induced the dramatic apoptosis/necrosis in GITR\(^{low}\)CD4\(^+\) cells but has no effect on Treg viability compared with the control cultures (Fig. 7C) or with each population before culture (Supplementary Figure S3, available at International Immunology Online). The resistance of Tregs to rapamycin-induced apoptosis/necrosis did not appear to be simply a delay of the effect since it could be observed at two separate time points (days 3 and 6). Thus, Tregs are resistant, while allo-reactive effector T cells are sensitive, to rapamycin-induced apoptosis/necrosis, rather than anti-proliferative effect, resulting in a selective enrichment of allo-reactive Tregs by rapamycin treatment.

**Discussion**

A number of reports have shown that naturally occurring Foxp3\(^+\)CD25\(^+\)CD4\(^+\) Tregs are essential for induction of allo-graft tolerance in rodents and suggested to play a key role in clinical transplantation in humans (2, 7, 17, 22–25). However, these studies have been neither directly related the donor-specific expansion of Tregs to the donor-specific tolerance nor clearly demonstrated the manner in which antigen-specific Tregs are induced. In this study, we were able to detect and trace donor-specific expansion of both Tregs and effector T cells by observing their responses to Mls allo-antigen in an experimental skin transplant model. By
comparing the frequency of Mls-1-specific Vb6+ cells with that of Mls-1-non-reactive Vb10+ cells, we were also able to exclude the possibility of antigen-non-specific expansion of Tregs and non-Tregs due to homeostatic proliferation. This approach has revealed that Tregs expand specifically to the donor graft and their continued presence is required for the establishment of long-term allograft tolerance. Furthermore, there is a fundamental difference between allo-responsive Treg and effector T cells in the susceptibility to a diverse set of immunosuppressive agents such as anti-CD4 mAb, anti-CD40L mAb and the IL-2 signal inhibitor rapamycin. These agents commonly lead to selective expansion of antigen-specific natural Tregs because of higher susceptibility of effector T cells than Tregs or higher resistance of Tregs than effector T cells to an anti-proliferative or apoptosis-inducing effect of the agents.

The differential effects of a variety of agents on the activation, proliferation and survival of natural Tregs and effector T cells can be attributed to possible differences in their sensitivity to antigenic stimulation and co-stimulation and also in their mode of intracellular signal transduction. For example, Foxp3+ Tregs are naturally in an ‘antigen-primed’ state already in the thymus before actual antigen encounter in the periphery; they are more easily activated by antigenic stimulation than naive T cells presumably because of their high expression of T cell accessory molecules including adhesion molecules such as leukocyte function-associated antigen (LFA)-1 (26). Indeed, Foxp3+ Tregs physically outcompete non-Tregs in aggregating around antigen-presenting cells in vitro by an LFA-1-dependent mechanism and suppress their up-regulation of CD80/CD86 expression by a cytotoxic T lymphocyte-associated antigen-4-dependent mechanism (27, 28). These findings suggest that antigen-stimulated Tregs may be more resistant than naive T cells to co-stimulation blockade, for example by anti-CD4 or anti-CD40L, leading to a predominant expansion of antigen-specific Tregs (29). Anti-CD4 and anti-CD40L may also directly enhance suppressive activity of Tregs (8, 30). On the other hand, the enrichment of Tregs by rapamycin treatment can be attributed to their relative resistance to apoptosis/necrosis because of their different mode of activation via the PI3 (phosphatidylinositol-3)-Akt-mTOR (mammalian target of rapamycin) signaling pathway (31–33). Further study of the molecular basis of the different sensitivity of Tregs and effector T cells to activating, proliferative or apoptosis-inducing stimuli is required to determine which molecules are suitable targets for differential control of the number or the function of the two populations to establish dominant graft tolerance.

In our experiments utilizing a Mls-mismach combination, it could be argued that in contrast with Mls-mismatched stimulation through the direct recognition pathway, in vivo T cell activation by major MHC-mismatch stimulation may be mainly through the indirect pathway (34). It is highly likely, however, that allogeneic MHC stimulation can expand Tregs through the indirect pathway as well, since in vivo stimulation of TCR-transgenic mouse-derived CD25*CD4+ Tregs with a specific antigen and CFA or with antigen-loaded mature DCs can evoke their proliferation (35–37). It is also generally accepted that the direct pathway predominates in the immediate aftermath of transplantation when graft-resident donor-type antigen-presenting cells migrate to the surrounding lymphoid tissue, where they stimulate...
Taken together, assuming that both direct and indirect pathways can contribute to Treg expansion, our findings indicate that in vivo expansion of antigen-specific Tregs at an early phase and the maintenance of their increased numbers in a chronic phase are essential for the establishment and maintenance of stable graft tolerance.

Tregs can be classified into two groups, so called natural Tregs and adaptive Tregs such as Tr1, Th3 and also Foxp3+ T cells derived from naive T cells in the periphery (39). It is not yet clear which Treg population is more important for tolerance induction in organ transplant models, partly because there is no specific cell surface marker to absolutely distinguish between naturally occurring Tregs from other T cell subsets. While Foxp3 determines the development of natural Tregs, it is likely not required for the development of either T1, T3 or Tr1 cells (40). A previous report suggested that Tregs could develop from CD25− naive precursors using anti-CD4/DST in thymectomized mice and that these cells could suppress skin allograft rejection (41). However, this study used PC61 (anti-CD25) mAb to deplete endogenous CD25+CD4+ Tregs thus one cannot exclude the possibility that Foxp3+CD25+ Tregs remained despite depletion of CD25+ Tregs (13). We were able to improve on this previous study by using GITRlowCD4+ T cells as effectors that express the lowest levels of Foxp3 in any T cell and thus should harbor few, if any, naturally preexisting Foxp3+ Tregs (13). We observed that anti-CD4/DST was unable to elicit long-term graft tolerance in the absence of natural Tregs, suggesting that anti-CD4/DST cannot induce the appearance of therapeutically relevant Tregs from GITRlow T cells. The slight prolongation of graft survival under these conditions (Fig. 2B, Group II) probably only reflects a transient receptor blockade of effector T cells by anti-CD4. Importantly, we could not find any evidence of sufficient induction of Foxp3+ cells in transferred GITRlowCD4+ effector populations, even in the presence of co-transferred Tregs (Fig. 3C). The finding apparently rules out the ‘infectious induction’ of naturally occurring Tregs in this model. Yet, it remains to be determined under what condition adaptive Tregs are induced if ever from naive T cells that otherwise may differentiate to graft-destructive effector T cells.

We previously showed by similar cell transfer experiments utilizing nude mice that in vivo exposure of naturally arising Foxp3+CD25−CD4+ Tregs to MHC-mismatched skin grafts in the absence of other T cells elicited spontaneous expansion of allo-antigen-specific Tregs, which suppressed allograft rejection mediated by subsequently transferred naive T cells and thereby established long-term graft tolerance (4). In the present study, permanent graft tolerance can indeed be established by preparing in vivo dominance of allo-antigen-specific natural Foxp3+ Tregs over antigen-reactive effector T cells by treatment with a variety of agents for a limited period at an initial phase of organ transplantation. Antigen-specific Tregs can also be expanded and enriched ex vivo by in vitro allo-antigen stimulation of T cells in the presence of those agents that exhibit differential effect on Treg and effector T cells to enrich the former. After further removing effector T cells, such in vitro-expanded antigen-specific Tregs can be used as adoptive immunotherapy in organ
transplantation (2–5, 38). Further elucidation of the molecular basis of the differential effect will help to design new biological or chemical agents for establishing graft tolerance in organ transplantation and reestablishing self-tolerance in treating autoimmune disease.

Supplementary data
Supplementary figures are available at International Immunology Online.

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Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>C</td>
<td>complement</td>
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<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
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<tr>
<td>CFSE</td>
<td>carboxyl fluorescent succinimidyl ester</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DST</td>
<td>donor-specific transfusion</td>
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<tr>
<td>GITR</td>
<td>glucocorticoid-induced tumor necrosis factor receptor</td>
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<tr>
<td>i.v.</td>
<td>intravenously</td>
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<tr>
<td>LFA</td>
<td>leukocyte function-associated antigen</td>
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<td>MLR</td>
<td>mixed lymphocyte reaction</td>
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<td>MST</td>
<td>median survival time</td>
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<tr>
<td>Treg</td>
<td>regulatory T cell</td>
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References


