Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3+CD25+CD4+ regulatory T cells

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Abstract

Naturally arising CD25+CD4+ regulatory T (T_R) cells can be exploited to establish immunologic tolerance to non-self antigens. In vivo exposure of CD25+CD4+ T cells from normal naive mice to alloantigen in a T cell-deficient environment elicited spontaneous expansion of alloantigen-specific CD25+CD4+ T_R cells, which suppressed allograft rejection mediated by subsequently transferred naive T cells, leading to long-term graft tolerance. The expanded T_R cells, which became CD25low in the absence of other T cells, stably sustained suppressive activity, maintained expression levels of other T_R cell-associated molecules, including Foxp3, CTLA-4 and GITR, and could adoptively transfer tolerance to normal mice. Furthermore, specific removal of the T_R cells derived from originally transferred CD25+CD4+ T_R cells evoked graft rejection in the long-term tolerant mice, indicating that any T_R cells deriving from CD25+CD4+ naive T cells minimally contribute to graft tolerance and that natural T_R cells are unable to infectiously confer significant suppressive activity to other T cells. Similar antigen-specific expansion of T_R cells can also be achieved in vitro by stimulating naturally present CD25+CD4+ T cells with alloantigen in the presence of IL-2. The expanded CD25+CD4+ T cells potently suppressed even secondary MLR in vitro and, by in vivo transfer, established antigen-specific long-term graft tolerance. Thus, in vivo or in vitro, direct or indirect ways of antigen-specific expansion of naturally arising Foxp3+CD25+CD4+ T_R cells can establish antigen-specific dominant tolerance to non-self antigens, and would also be instrumental in re-establishing self-tolerance in autoimmune disease and antigen-specific negative control of pathological immune responses.

Introduction

It is to be hoped that immunologic tolerance to certain non-self antigens, such as alloantigens in organ transplants, can be established as stably as natural tolerance to self-constituents. Accumulating evidence indicates that naturally arising CD25+CD4+ regulatory T cells (T_R cells) play a crucial role in the maintenance of immunologic self-tolerance and negative control of immune responses (1–3). Elimination of CD25+CD4+ T_R cells, for example, leads to spontaneous development of various autoimmune diseases in normal animals and provokes effective tumor immunity in otherwise non-responding animals.
Immunologic tolerance induced by regulatory T cells (4–8). The elimination also enhances immune responses to non-self antigens including allogenic transplantation antigens (4). In this report we have addressed the issue of how natural CD25+Cd4+ T_R cells can be expanded in vivo and in vitro in an antigen-specific manner and what condictions are required to establish long-term tolerance to non-self-antigens with such antigen-specific T_R cells.

Natural CD25+Cd4+ T_R cells are at least in part produced by the normal thymus as a functionally mature T-cell subpopulation (9). They are functionally unique in that they proliferate poorly in response to antigenic stimulation in vitro unless IL-2 is provided and in that, upon in vitro stimulation with specific antigens, they potentely suppress the activation/proliferation of other T cells in an antigen-nonspecific manner, seemingly through cell to cell interactions on antigen-presenting cells (APC) (9–12). Phenotypically, they constitutively express CTLA-4, certain members of Toll-like receptors, CD103 (αE integrin) and GITR (glucocorticoid-induced TNF receptor family-related gene) at high levels (13–19). They specifically express the transcription factor Foxp3, which appears to act as a master control gene for their development and function (20–22). These findings collectively indicate that naturally arising CD25+Cd4+ T_R cells are a developmentally, phenotypically and functionally distinct subpopulation of T cells (1). Furthermore, recent studies have shown by utilizing CD25+Cd4+ T cells from TCR transgenic mice that antigen-specific T_R cells can expand in vivo upon antigen stimulation along with potent adjuvant or mature DCs (23–25). Natural CD25+Cd4+ T_R cells also show homocytic proliferation in a T cell-deficient environment (26,27), and a fraction of them are proliferating in normal animals presumably by recognizing self-antigens (28,29). It remains to be determined, however, how this in vivo proliferative capacity of natural T_R cells can be exploited for inducing antigen-specific tolerance to non-self-antigens and negative control of immune responses. It is also unclear whether natural T_R cells should play key roles in long-term immunologic tolerance induced to non-self antigens, such as allografts, by short-term administration of monoclonal antibodies or drugs that control the activation/proliferation of T cells, or whether antigen-specific T_R cells generated by these treatments should originate from natural T_R cells or naive T cells (30–35). Furthermore, it should be determined whether natural T_R cells can confer a suppressive activity to naive T cells in an ‘infectious’ manner in immunologic tolerance (36–38).

In this report, we demonstrate that alloantigen-specific T_R cells present in the naturally arising CD25+Cd4+ T_R cell population can be expanded in vivo by sensitization to alloantigens without any adjuvant, in contrast to their in vitro hypoproliferation upon allogeneic stimulation. Notably, permanent graft tolerance can be achieved if this antigen-specific expansion of natural T_R cells is allowed to the extent that they are sufficient in number and suppressive activity to control the expansion/activation of allo-reactive effector T cells. Alloantigen-specific CD25+Cd4+ T_R cells can also be expanded in vitro in the presence of IL-2 and used for inducing similar antigen-specific graft tolerance in vivo. We further assess the stability of the phenotype and function of these antigen-expanded T_R cells in vivo, in particular whether they can confer regulatory activity to non-T_R cells or whether other T cells can give rise to T_R cells. This simple way of antigen-specific T_R cell expansion, either by directly stimulating them in vitro with specific antigens or indirectly preparing an in vivo immunological condition that allows their spontaneous antigen-specific expansion, can also be used for re-establishing self-tolerance in autoimmune disease.

**Methods**

**Mice**

Seven-week-old female BALB/c, DBA/2, C57BL/6 (B6), C3H and BALB/c athymic nude mice were purchased from Clea Japan (Tokyo, Japan). BALB/c-Thy 1.1 congenic mice were established in our laboratory (5). 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-labeled, or biotinylated mAbs to CD25 (7D4), CD4 (RM4-5 and H129.19), CD8 (H129.19, Vj6 (RR4-7) and Vj10 (B21.5) were purchased from PharMingen (San Diego, CA). FITC-labeled or biotinylated anti-Thy 1.1 mAbs were from Serotec (Oxford, UK). PE- or CyChrome-streptavidin, as secondary reagents, were also from PharMingen. Anti-GITR mAb, DTA-1 (16) was made from ascites in SCID mice and purified by 40% ammonium sulfate precipitation twice. Affinity-purified goat anti- rat IgG (specific for whole IgG molecule) and purified normal hamster IgG were purchased from ICN Pharmaceuticals (Aurora, OH). Normal rat IgG was purchased from Sigma (St Louis, MO). Murine recombinant IL-2 (rIL-2) (3.89 × 10^6 U/mg) was a gift from Shionogi Co. (Osaka, Japan).

**Preparation of T cell subpopulations**

To enrich CD4+ T cells, spleen and lymph node cells were treated with anti-CD8 (3.155) and anti-CD24 (J11d) mAbs and incubated on plastic dishes pre-coated with goat anti-rat IgG at 37°C for 30 min. Non-adherent cells (>85% of which were CD4+) were stained with biotin-anti-CD25 mAb, then with PE-streptavidin and FITC-anti-CD4 mAb; and CD25+ or CD25−CD4+ T cells were purified by Epics–ALTRA cell sorter (Beckman Coulter, Miami, FL). Purity of the CD25+ or CD25−CD4+ population was >98% and ~99%, respectively. Cells were also stained with biotin-anti-CD25 mAb, then with PE-streptavidin, CyChrome-anti-CD4 and FITC-anti-Thy 1.1 mAb; and CD25hi, CD25int or CD25−CD4+ Thy 1.1+ T cells were purified by cell sorter. In some experiments, CD25+ cells were enriched by the MACS system (Magnetic Cell Sorting; Miltenyi Biotech). Briefly, enriched CD4+ T cells were stained with biotin-anti-CD25 mAb, PE-streptavidin and then incubated with anti-PE microbeads according to the manufacturer's instruction. Cells were positively selected on an LS separation column. CD25+CD4+ population was further prepared from the positive fraction by using an LS column again. The negative fraction was incubated with anti-CD4 microbeads and the CD25−CD4+ population positively selected on an LS column. Purity of CD25+ or CD25−CD4+ population was >93% and ~99%, respectively. To prepare unfractonated whole T cells, spleen and lymph node cells were depleted of B cells and adherent cells by treatment with J11d mAb and panning on anti-rat IgG-coated plastic dishes. Non-adherent
cells were further treated with rabbit complement (C) (Cederlane Laboratories, Ontario, Canada). CD8+ T cells were enriched by incubation with J11d and anti-CD4 (GK1.5) mAbs, panning and C treatment as described above. In some experiments, lymphocytes were depleted of CD25+ cells by treatment with anti-CD25 (7D4) mAb and C, as previously described (4,5).

Skin grafting and cell transfer

Full thickness B6, C3H, DBA/2 or BALB/c tail skin (0.5 cm²) was grafted onto the backs of BALB/c nude mice. The grafted sites were wrapped for 7 days with gauze and bandages. After a further 7 days, the mice were reconstituted with 2 × 10⁵ unfractionated T cells from naive BALB/c mice. Some mice received 2–6 × 10⁵ BALB/c CD25+CD4+ T cells at the same time or 1 week before reconstitution with normal, untreated T cells from naive BALB/c mice. Grafts were inspected four times a week and considered rejected when no viable donor skin was present. Statistical analysis of graft survival was made by the log-rank method.

Mixed lymphocyte reaction (MLR)

BALB/c whole, CD4+, CD8+ or CD25+CD4+ T cells (5 × 10⁵/well) together with various numbers of CD25+CD4+ T cells (0–5 × 10⁵/well) were cultured with RBC-lysed and X-irradiated (20 Gy) B6 or C3H splenocytes (1 × 10⁵/well) as stimulators for 6 days in 96-well round-bottom plates (Corning Costar, Cambridge, MA) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (PAA Laboratories, Newport Beach, CA), penicillin (100 U/ml), streptomycin (100 μg/ml) and 50 μM 2-mercaptoethanol. Cultures were pulsed with 3[H]thymidine ([3H]TdR) (37 kBq/well) (Du Pont/NEN) for the last 16 h. To assess the secondary MLR, BALB/c CD25+CD4+ T cells (5 × 10⁵/well) were pre-cultured with irradiated B6 splenocytes for 7 days, washed, and then cultured with X-irradiated fresh B6splenocytes for 5 days in the presence or absence of CD25+CD4+ T cells. To assess anti-Mls-1 responses, BALB/c T cells were cultured with mitomycin C (MMC)-treated DBA/2 splenocytes for 5 days.

In vitro stimulation of CD25+CD4+ T cells

BALB/c CD25+CD4+ T cells (2.5 × 10⁶/well) were co-cultured with X-irradiated B6 or C3H splenocytes, or with MMC-treated DBA/2 splenocytes (1 × 10⁵/well) for 7 days in the presence of 100 U/ml rIL-2. Cells were washed and added to the MLR or inoculated to the skin-grafted nude mice. For analysis of Vβ6 or Vβ10-expressing cells, freshly prepared BALB/c CD4+ T cells or CD25+CD4+ T cells were co-cultured with DBA2 splenocytes plus IL-2 (100 U/ml) for 7 days, stained with biotin-anti-CD25 mAb and FITC-anti-Vβ6 or Vβ10 mAb, then with PE-streptavidin. The proportion of Vβ6- or Vβ10-expressing cells was analyzed by flow cytometry (Epics-XL, Beckman Coulter).

RT-PCR

Total cellular RNA was extracted from 1–5 × 10⁵ sorted cells supplemented with 10 μg glycogen using Isogen reagent (Nippon Gene, Tokyo, Japan). The total amount of RNA was reverse transcribed using Superscript II reverse-transcriptase and oligo(dT)12–18 primer (Invitrogen Japan, Tokyo, Japan) in a final volume of 20 μl. Foxp3 mRNA levels were quantified by real-time PCR as described previously (20). Normalized value for Foxp3 mRNA expression in each sample was calculated as the relative quantity of Foxp3 divided by the relative quantity of HPRT (×100). All samples were run in triplicate.

Results

In vivo expansion of antigen-specific CD25+CD4+ T<sub>R</sub> cells upon sensitization to allografts

To determine whether natural CD25+CD4+ T<sub>R</sub> cells could expand upon allogeneic antigen stimulation without any adjuvant, we took advantage of the anti-Mls-1<sup>a</sup> (Mtv-7) response of BALB/c (Mls-1<sup>a</sup>) mice; i.e. expansion of BALB/c Vβ6+ T cells is stimulated specifically by exposure to the Mls-1 antigen of DBA/2 mice (39). BALB/c-Thy1.1+CD25+CD4+ T cells were transferred to nude mice with DBA/2 or BALB/c skin grafts, and the percentage and the number of Thy1.1+CD4+ splenic T cells expressing Mls-1-reactive Vβ6 or non-reactive Vβ10 was assessed every week after transfer (Fig. 1A and B). Analysis 1 week after transfer showed that both the percentage and number of Vβ6+ cells increased (~5-fold and ~20-fold, respectively) in the nude mice with DBA/2 grafts but not in those with BALB/c grafts, contrasting with no increase of the percentage or number of Vβ10+ cells in both groups (Vβ6+ or Vβ10+ cells constituted ~10% and ~6%, respectively, of CD25+CD4+ T cells in normal naive BALB/c mice; see legend to Fig. 1 for detailed percentage and number). The time course study revealed that maximal Vβ6+ cell expansion occurred 1 week after transfer and showed significant differences from the expansion in nude mice with syngeneic BALB/c grafts (P < 0.05) (Fig. 1B). Although it was difficult to obtain a sufficient number of Vβ6+ T cells from these nude mice to assess their in vitro Mls-1-specific suppressive activity (see below), they exerted potent suppression upon anti-CD3 stimulation (data not shown).

To determine then whether CD25+CD4+ T<sub>R</sub> cells could generate an antigen-specific suppressive activity upon exposure to alloantigen, we transferred CD25+CD4+ T cells, prepared from BALB/c-Thy-1<sup>a</sup> congenic mice, to BALB/c nude mice with B6 or C3H skin grafts, and assessed 1 week later whether Thy-1.1+ cells recovered from the mice suppressed the in vitro MLR responses of BALB/c naive T cells to B6 or C3H stimulator cells (Fig. 1C). Although the total numbers of Thy-1.1+ T cells recovered in each group of mice were comparable (data not shown; see also Fig. 6B), the Thy-1.1+ cells from either group of mice exhibited ~4-fold more potent MLR suppression when the stimulator cells were from the same strain as the graft donor, compared with the stimulator cells from the other strain.

These results taken together indicate that antigen-specific T<sub>R</sub> cells in the CD25+CD4+ T<sub>R</sub> cell population can substantially expand in vivo within a week upon sensitization to allo-antigens and that such stimulated T<sub>R</sub> cells show antigen-specific suppression.

Induction of allograft tolerance by naturally occurring CD25+CD4+ T<sub>R</sub> cells

We subsequently examined whether CD25+CD4+ T<sub>R</sub> cells can suppress in vivo allogeneic immune responses more effectively after expansion. We transferred 2 × 10⁵ naive
T cells to B6 skin-grafted BALB/c nude mice with an equal number of CD25+CD4+ T cells either simultaneously or 7 days after the transfer of the CD25+CD4+ population (Fig. 2A). Although the co-transfer of CD25+CD4+ T cells and naive T cells significantly prolonged graft survival compared with transfer of naive T cells alone (median survival time (MST): 42 days vs 31 days, n = 12 and 29, respectively; P = 0.002), inoculation of CD25+CD4+ T cells 7 days prior to naive T-cell transfer prolonged graft survival further (MST: 64 days, n = 13, P = 0.0002) with 23% of the mice showing long-term (>100 days) graft acceptance. Furthermore, when a 3-fold excess (6 × 10^5) of CD25+CD4+ T cells to naive T cells was transferred 7 days apart, 73% of the recipients showed long-term acceptance of the grafts (MST: >100 days, n = 15, P < 0.00001).

Grafting of B6 or C3H skin grafts on each flank of these tolerant mice to determine the antigen-specificity of tolerance resulted in rapid rejection of C3H grafts in all the mice, and, interestingly, gradual rejection of the fresh B6 grafts in 60% of recipients while all the mice retained the original B6 grafts (Fig. 2B).

We then attempted to determine whether the graft tolerance can be adoptively transferred to other mice (Fig. 2C). When spleen and lymph node cells were transferred from the mice with long-term surviving (>100 days) B6 grafts, shown in Fig. 2(A), to naive nude mice with B6 or C3H skin grafts; 1 week later graded numbers of Thy-1.1+ cells recovered from the nude mice were co-cultured at indicated ratios with CD25+CD4+ T cells freshly prepared from normal BALB/c mice, and stimulated with B6 or C3H stimulator cells. A representative result of two independent experiments is shown.
Thus, CD25+CD4+ T cells in normal naive mice can induce transplantation tolerance by suppressing graft-rejecting T cells, especially when the former is allowed to expand for a limited period in the absence of the latter; and this dominant tolerance has antigen-specificity and can be adoptively transferred to other mice by T cells.

Phenotypic and functional stability of CD25+CD4+ T<sub>R</sub> cells

The result in Fig. 2 that CD25<sup>-</sup> T cells were able to adoptively transfer allograft tolerance indicate that some, at least, of naturally arising CD25<sup>-</sup>CD4+ T<sub>R</sub> cells may lose their CD25 expression while retaining suppressive function; alternatively, CD25<sup>-</sup>CD4+ T cells may differentiate to functional T<sub>R</sub> cells. To assess these possibilities, we first examined the stability of the expression of T<sub>R</sub> cell-associated molecules, the suppressive activity in vivo, and the level of Foxp3 gene expression, when CD25<sup>-</sup>CD4+ T cells from naive mice were transferred to nude mice (Fig. 3).

When CD25<sup>-</sup> or CD25<sup>-</sup>CD4+ T cells prepared from BALB/c-Thy1.1 mice were transferred to BALB/c nude mice with B6 skin grafts, the levels of CD25 expression on the inoculated CD25<sup>-</sup>CD4+ T cells were much lower 7 days after transfer than pre-transfer while the inoculated CD25<sup>-</sup>CD4+ T cells up-regulated CD25 expression to a similar level to that of the CD25<sup>-</sup>CD4+<sup>-</sup>-derived T cells (Fig. 3A and B). The inoculated CD25<sup>-</sup>CD4+<sup>-</sup> T cells also gave rise to a small population, constituting ~5% of the CD25<sup>-</sup>CD4+<sup>-</sup>-derived cells, that expressed high levels of CD25 and moderately down-regulated CD4 expression (Fig. 3B, upper panel). Although the expression of GITR and CTLA-4 was up-regulated in both populations after transfer, the expression patterns, which are normally higher on CD25<sup>-</sup>CD4+ T cells than CD25<sup>-</sup>CD4+ T cells (13–17), were well preserved. Expression of CD103, which is expressed by a fraction of CD25<sup>-</sup>CD4+ T cells in normal naive mice (18), was also retained after transfer.

To assess the in vitro suppressive activity of CD25<sup>-</sup> and CD25<sup>-</sup>CD4+<sup>-</sup>-derived T cells in nude mice, the CD25-positive or CD25-negative fraction was co-cultured with freshly prepared BALB/c CD25<sup>-</sup>CD4+ T cells and stimulated with anti-CD3 (Fig. 3B, lower panel). The CD25<sup>-</sup>CD4+<sup>-</sup>-derived cells, either CD25-positive or -negative, showed suppressive activity equivalent to that of freshly prepared BALB/c CD25<sup>-</sup>CD4+ T cells; and they themselves scarcely proliferated upon TCR stimulation (Fig. 3B). Furthermore, they sustained the levels of Foxp3 mRNA expression equivalent to 50~110% of the natural CD25<sup>-</sup>CD4+ T-cell population present in normal mice.
On the other hand, the CD25\(^{+}\)/CD4\(^{+}\)-derived T cells, either CD25-positive or -negative, were non-suppressive, proliferated upon anti-CD3 stimulation, and scarcely expressed Foxp3 (Fig. 3B and C). Interestingly, however, CD25\(^{\text{high}}\)/CD4\(^{\text{low}}\) cells, a fraction that had developed from CD25/CD4\(^{+}\) T cells and constituted ~5% of the CD4\(^{+}\) T cells (fraction c' in Fig. 3B and C), was hypoproliferative upon TCR stimulation, exhibited a suppressive activity equivalent to natural CD25\(^{+}\)/CD4\(^{+}\) TR cells, and expressed Foxp3 at a high level. This TR population appeared to have differentiated/expanded from CD45RBlow GITR\(^{\text{high}}\) cells in the CD25/CD4\(^{+}\) population since transfer of CD25/CD4\(^{+}\) T cells after depletion (Fig. 3C). On the other hand, the CD25\(^{-}\)/CD4\(^{+}\)-derived T cells, either CD25-positive or -negative, were non-suppressive, proliferated upon anti-CD3 stimulation, and scarcely expressed Foxp3 (Fig. 3B and C). Interestingly, however, CD25\(^{\text{high}}\)/CD4\(^{\text{low}}\) cells, a fraction that had developed from CD25/CD4\(^{+}\) T cells and constituted ~5% of the CD4\(^{+}\) T cells (fraction c' in Fig. 3B and C), was hypoproliferative upon TCR stimulation, exhibited a suppressive activity equivalent to natural CD25/CD4\(^{+}\) TR cells, and expressed Foxp3 at a high level. This TR population appeared to have differentiated/expanded from CD45RBlow GITR\(^{\text{high}}\) cells in the CD25/CD4\(^{+}\) population since transfer of CD25/CD4\(^{+}\) T cells after depletion (Fig. 3C).
of CD45RB<sup>low</sup> cells or GITR<sup>high</sup> cells to nude mice resulted in the development of lower numbers (i.e., 1/4~1/5) of such CD25<sup>high</sup_CD4<sup>low</sup> T<sub>R</sub> cells than the transfer of whole CD25<sup>+</sup_CD4<sup>+</sup> T cells (Supplementary fig. 1, available at International Immunology Online).

These results collectively indicate that CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells may lose their CD25 expression presumably in the absence of other T cells while still retaining their suppressive activity, and that a small subpopulation of CD25<sup>+</sup_CD4<sup>+</sup> T cells can differentiate to CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells which are functionally similar in vitro to natural CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells although the majority of CD25<sup>+</sup_CD4<sup>+</sup> T cells are kept non-regulatory.

**Potent and persistent suppressive activity of antigen-stimulated CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells**

To determine then which T<sub>R</sub> cells, either derived from CD25<sup>+</sup> or CD25<sup>+</sup_CD4<sup>+</sup> T cells, are responsible for maintaining allograft tolerance (Figs 2 and 3), we prepared tolerant mice by transferring BALB/c-Thy-1.1<sup>+</sup_CD25<sup>+</sup_CD4<sup>+</sup> T cells to B6-skin-grafted BALB/c nude mice 7 days prior to inoculation of normal BALB/c (Thy-1.2<sup>+</sup>) CD25<sup>+</sup> cell-depleted T cells in the ratio of 10:1. Eighty percent of the mice stably retained the skin grafts 100 days after the transfer of CD25<sup>+</sup> T cells. These mice also retained both Thy-1.2<sup>+</sup> cells and Thy-1.1<sup>+</sup> cells; two-thirds of the Thy-1.1<sup>+</sup> T cells expressed CD25 at levels as high as naturally present CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells. Since nude mice develop some endogenous CD4<sup>+</sup> cells (4,40), the Thy-1.2<sup>+</sup> fraction contained a larger number of cells than Thy-1.1<sup>+</sup> cells (3.4% vs 1.1%), irrespective of the transfer of a larger number of the latter cells (Fig. 4A). Transfer of lymphocytes from these tolerant mice to newly prepared B6-skin-grafted BALB/c nude recipients resulted in no graft rejection in the majority, whereas transfer of lymphocytes after elimination of Thy-1.1<sup>+</sup> cells by anti-Thy1.1 mAb and C-treatment led to rapid graft rejection in all the recipients (Fig. 4B). The result indicates that T<sub>R</sub> cells derived from natural CD25<sup>+</sup_CD4<sup>+</sup> T cells are mainly responsible for the maintenance of dominant transplantation tolerance in the donor mice and that other T<sub>R</sub> cells, including those derived from CD25<sup>+</sup_CD4<sup>+</sup> T cells (Fig. 3), may not be sufficiently potent per se to be able to suppress rejection. Furthermore, judging from the result that one-third of CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells had lost CD25 expression in tolerant mice (Fig. 4A), successful transfer of tolerance by CD25<sup>+</sup> cells in Fig. 2(C) can be attributed to the original CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells that had lost CD25 expression, rather than T<sub>R</sub> cells derived from CD25<sup>+</sup_CD4<sup>+</sup> T cells.

**In vitro expansion of alloantigen-specific CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells**

Naturally present CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells exhibit poor proliferative responses in primary allogeneic MLR in vitro, and suppress the allo-reactive proliferation of both CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells; and once T<sub>R</sub> cells are activated by alloantigen stimulation, they suppress allo-responses of third party T cells as well (Supplementary fig. 1). Thus, the mode of in vitro CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cell-mediated suppression is similar between T cell responses to histocompatibility antigens and soluble protein antigens (10,12).

Such alloreactive CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells expanded in vitro upon allo-antigen stimulation in the presence of IL-2 (Fig. 5A). BALB/c CD25<sup>+</sup_CD4<sup>+</sup> T cells stimulated with X-irradiated B6 splenocytes along with a high dose IL-2 (e.g. 100 U/ml) expanded ~2-fold in 1 week, and ~4-fold in 4 weeks. These expanded cells sustained Foxp3 expression (Fig. 5B). When these cells were washed and restimulated with B6 cells, they scarcely proliferated but potently suppressed the primary MLR of freshly prepared BALB/c CD25<sup>+</sup_CD4<sup>+</sup> T cells (Fig. 5C). Compared with freshly prepared CD25<sup>+</sup_CD4<sup>+</sup> T cells, much

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**Fig. 4. Persistence of in vivo suppressive activity of CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells.** BALB/c nude mice with B6 skin grafts were made tolerant by transfer of 1 x 10<sup>6</sup> BALB/c-Thy-1.1 CD25<sup>+</sup_CD4<sup>+</sup> T<sub>R</sub> cells 7 days prior to inoculation of 1 x 10<sup>9</sup> BALB/c (Thy-1.2<sup>+</sup>) CD25<sup>-</sup>depleted T cells. (A) After 100 days, the expression level of CD25 on Thy-1.1<sup>+</sup> or Thy-1.1<sup>+</sup_CD4<sup>+</sup> T cells from graft-tolerant nude mice was analyzed. Control staining is shown by shadow. (B) Spleen and lymph node cells from graft-tolerant nude mice were treated with anti-Thy-1.1 and C or C only; 2 x 10<sup>6</sup> treated cells were transferred to naive nude mice with B6 skin grafts and graft survival was monitored. The data presented are the total of two independent experiments.
Fig. 5. In vitro expansion of allo-antigen-specific CD25\(^+\)CD4\(^+\) T\(_R\) cells and resulting enhancement of their antigen-specific suppression. (A) BALB/c CD25\(^-\)/CD4\(^-\) T cells, CD25\(^+\)/CD4\(^+\) T cells, or the two populations mixed at the ratio of 1:1 were stimulated with B6 splenocytes in the presence of various concentrations of IL-2. (B) Foxp3 expression of BALB/c CD25\(^-\)/CD4\(^-\) T cells freshly prepared, prestimulated with B6 splenocytes plus IL-2 (100 U/ml) for 1 or 4 weeks were examined by quantitative RT–PCR. (C) BALB/c CD25\(^+\)/CD4\(^+\) T cells freshly prepared, or prestimulated with B6 splenocytes plus IL-2 for 1 or 4 weeks were mixed with BALB/c CD25\(^-\)/CD4\(^-\) T cells either freshly prepared (left) or prestimulated with B6 splenocytes for 7 days (right). The cell mixtures were stimulated with X-irradiated B6 splenocytes. (D) BALB/c CD25\(^+\)/CD4\(^+\) T cells either freshly prepared or prestimulated with B6 or C3H spleen cells plus IL-2 for 7 days were mixed with freshly prepared BALB/c CD25\(^+\)/CD4\(^+\) T cells at various ratios and stimulated with either X-irradiated B6 (left) or C3H (right) spleenocytes. (E) Percentage of V\(_{\beta}6\) or V\(_{\beta}10\) cells in the BALB/c CD25\(^+\)/CD4\(^+\) population unstimulated or stimulated with MMC-treated DBA/2 splenocytes plus IL-2 for 7 days. (F) BALB/c CD25\(^+\)/CD4\(^+\) T cells freshly prepared or prestimulated with DBA/2 splenocytes plus IL-2 for 7 days were mixed with freshly prepared BALB/c CD25\(^+\)/CD4\(^+\) T cells at various ratios and stimulated with MMC-treated DBA/2 splenocytes for 5 days. A representative result of more than three independent experiments is shown in (A–F).
smaller numbers of these prestimulated T cells sufficed to suppress the MLR completely. Furthermore, 1- or 4-week-prestimulated TR cells could potently suppress a secondary MLR, whereas the same number of freshly prepared CD25+CD4+ T cells could not (Fig. 5C). The suppressive activity of the 4-week-stimulated CD25+CD4+ T cells was much higher than the activity of the 1-week-stimulated ones. Furthermore, titration of the number of TR cells required for a particular degree of suppression revealed that B6-prestimulated BALB/c CD25+CD4+ T cells were more (nearly 8-fold) potent than C3H-prestimulated BALB/c CD25+CD4+ T cells in suppressing the response of BALB/c CD25+CD4+ T cells to B6-stimulation; the C3H-prestimulated CD25+CD4+ T cells showed a comparable suppressive activity to that of freshly prepared BALB/c CD25+CD4+ T cells. Moreover, compared with non-stimulated BALB/c CD25+CD4+ T cells, 16- to 32-fold fewer of the DBA/2-prestimulated BALB/c CD25+CD4+ T cells sufficed to completely suppress anti-Mls-1 response of BALB/c CD25+CD4+ T cells (Fig. 5F). This marked increase in the magnitude of suppression, compared with increase in the percentage of Vβ6+ cells, indicates that the suppressive activity of individual antigen-specific CD25+CD4+ TR cells also increased.

Thus, in vitro stimulation of CD25+CD4+ T cells with alloantigenic cells and IL-2 can enhance their suppressive activity in an alloantigen-specific fashion. Judging from the increase in suppressive activity and the cell number, this enhancement can be mainly attributed to the expansion of antigen-specific TR cells and to a lesser degree to an increase in the suppressive activity of individual TR cells.

Immunologic tolerance induced by regulatory T cells

Antigen-specific suppression of graft rejection by ex vivo stimulated CD25+CD4+ TR cells

To examine the ability of the ex vivo prestimulated CD25+CD4+ TR cells to suppress graft rejection in vivo, BALB/c nude mice were grafted with B6 skin, and 2 × 10^5 B6 or C3H-prestimulated BALB/c CD25+CD4+ T cells, 1 × 10^6 B6-prestimulated BALB/c CD25+CD4+ T cells, or PBS alone were transferred. Seven days later, all mice received 2 × 10^5 naive BALB/c whole T cells. The result is the total of five independent experiments. (B) BALB/c nude mice engrafted with C3H skin grafts received 2 × 10^5 B6 or C3H-prestimulated BALB/c CD25+CD4+ T cells, or PBS alone as control. Seven days later, all mice received 2 × 10^5 naive BALB/c whole T cells. The data shown are the total of six independent experiments. (C) BALB/c nude mice engrafted with B6 skin grafts received 2 × 10^5 BALB/c-Thy1.1 CD25+CD4+ T cells freshly prepared or prestimulated with B6 splenocytes and 100 U/ml rIL-2 for 1 week. Seven days later, spleen and lymph node cells were harvested and the number of Thy1.1+CD4+ cells was calculated from the total number of harvested cells and the percentage of Thy1.1+CD4+ cells among them. The result shown is the total of three independent experiments.
CD25+CD4+ T cells in vitro stimulated with B6 APCs with 100 U/ml IL-2 for 1 week were transferred to BALB/c nude mice with B6 skin grafts (Fig. 6A). The transfer significantly prolonged graft survival in a cell dose-dependent fashion (MST = 67 days at 1:1 ratio of pre-stimulated CD25+CD4+ T cells vs freshly prepared whole T cells, n = 10, and MST >100 days at 5:1 ratio, n = 9; P = 0.00004 and 0.00003 vs control, respectively). This prolongation appeared to be antigen-specific since B6-prestimulated CD25+CD4+ T cells significantly prolonged the survival of B6 grafts at 1:1 ratio of T_R cells and naive T cells; the survival was significantly longer compared with the transfer of C3H-prestimulated CD25+CD4+ T cells significantly prolonged the survival of C3H grafts than B6-prestimulated CD25+CD4+ T cells (MST = 62 vs 40 days, P = 0.03) (Fig. 6A). Similarly, C3H-prestimulated CD25+CD4+ T cells significantly prolonged the survival of C3H grafts from B6-prestimulated or freshly prepared BALB/c-Thy1.1 CD25+CD4+ T cells to BALB/c nude mice with B6 skin grafts revealed that, in contrast to the 2-fold expansion of freshly prepared CD25+CD4+ T cells within 1 week after transfer, the in vitro pre-stimulated CD25+CD4+ T cells were reduced to half the number of the inoculated cells.

Taken together, the in vitro pre-stimulated CD25+CD4+ T cells can effectively prolong the graft survival in an antigen-specific manner, although a significant fraction of them may die within 1 week after transfer or, as another possibility, they might migrate into non-lymphoid tissues.

**Discussion**

A key finding in this report is that in vivo exposure of naturally arising Foxp3+CD25+CD4+ T_R cells to alloantigen in the absence of other T cells elicited spontaneous expansion of alloantigen-specific T_R cells, which suppressed allograft rejection mediated by subsequently transferred naive T cells and established long-term graft tolerance. In addition, in vitro allogeneic stimulation of natural T_R cells in the presence of IL-2 also elicited their antigen-specific proliferation and apparently enhanced suppressive activity in individual antigen-specific T_R cells. This in vivo or in vitro, direct or indirect induction of antigen-specific expansion of natural Foxp3+CD25+CD4+ T_R cells would be instrumental for establishing transplantation tolerance and re-establishing self-tolerance in autoimmune disease.

We took advantage of Mls-specific expansion of T cells that express a particular Vβ TCR subfamily to monitor in vivo and in vitro T_R cell expansions and differentiate their antigen-specific expansion from possible antigen-nonspecific homeostatic proliferation in the T-cell-deficient environment of nude mice. This approach clearly showed alloantigen-specific expansion of natural T_R cells. It can be argued that in contrast with this Mls-disparate sensitization, in vivo T-cell activation by MHC-disparate allo-stimulation may be mainly through the indirect pathway (41). It is highly likely, however, that allogeneic MHC stimulation can expand T_R cells through the indirect pathway as well, since in vivo stimulation of TCR-transgenic mice-derived CD25+CD4+ T_R cells with a specific antigen and CFA or with antigen-loaded mature DCs can evoke their proliferation (23–25). Although the peak of T_R cell expansion with Mls stimulation was ~1 week in nude mice, the precise kinetics of T_R cell expansion upon allo-MHC stimulation must be determined to confer more distinct antigen specificity to T_R cells and establish more stable antigen-specific tolerance.

Given that natural CD25+CD4+ T_R cells are pre-committed to be suppressive in function before antigen exposure, this in vivo and in vitro antigen-specific T_R cell expansion has the following implications. First, the expansion of antigen-specific T_R cells forms the cellular basis of antigen-specificity in T_R cell-mediated dominant tolerance, even when the expanded T_R cells may exert antigen-nonspecific or bystander suppression of other T cells to a certain extent (Supplementary fig. 2). Second, if alloantigen-specific CD25+CD4+ T_R cells are allowed to expand in the absence of allo-reactive effector T cells (as the transfer of CD25+CD4+ T cells alone to T cell-deficient nude mice), they can expand in a short time to the extent that they are sufficient in number and suppressive activity to stably control allo-reactive effector T cells and thereby to establish dominant transplantation tolerance. Third, a dynamic balance is attained and maintained between such allo-specific CD25+CD4+ T_R cells and allo-reactive effector T cells in graft-tolerant animals, with the former continuously and actively suppressing the latter. Supporting this notion, removal of T_R cells from long-term tolerant mice evoked graft rejection (Fig. 4B), and inoculation of normal naive T cells failed to elicit rejection in tolerant animals (Fig. 2C). Furthermore, the expanded natural T_R cells are apparently unable to confer significant suppressive activity, if at all, to naive T cells in an 'infectious' manner during their co-existence in the tolerant host, since specific removal of the expanded T_R cells derived from CD25+CD4+ T_R cells sufficed to evoke graft rejection in tolerant animals (Fig. 4B). The result, however, does not exclude the possibility that, while the expanded CD25+CD4+ T_R cells restrain the activation of allo-reactive T cells, CD25+CD4+ T cells may give rise to certain T_R cells (Fig. 3; and see discussion below).

It was noted in our experiments that the long-term tolerant animals gradually rejected secondary grafts while stably retaining the original grafts (Fig. 2B). This illustrates several key features of dominant graft tolerance. First, allo-reactive effector T cells potentially capable of rejecting graft have persisted in tolerant animals under the dominant control by T_R cells. Second, activation of graft-rejecting effector T cells by secondary grafts is insufficient to trigger rejection of the original graft. Third, the maintenance of graft tolerance may partly depend on the local activity of T_R cells (42) or stable settlement of T_R cells in their 'niche' (43). Thus, at the original graft site, a stable local balance appears to have been established between T_R cells and effector T cells that have been recruited to the site, whereas the two populations may be in a more delicate and vulnerable balance at the site of secondary graft. Monitoring the recruitment and persistence...
of T_R cells, e.g. as Foxp3+ cells (see discussion below), and effector T cells at the graft site may help to assess the local balance between the two populations and thereby the local state of graft tolerance.

The antigen-expanded CD25+CD4+ T_R cells are functionally and phenotypically stable, more potent in suppression than T_R cells derived from CD25−CD4+ T cells, and able to adoptively transfer graft tolerance to naive mice. For example, expression of Foxp3, which appears to be a master control gene for the development and function of natural CD25+CD4+ T_R cells, is stably maintained and shows a good correlation with the level of in vitro suppressive activity irrespective of the phenotypes of cell surface molecules or the origin of T_R cells (see below). The expression levels of CTLA-4, GITR and CD103 were also kept higher in CD25+CD4+ T_R cells or a fraction of them, compared with T cells derived from CD25−CD4+ T cells. These findings indicate that CTLA-4, GITR, CD103 and Foxp3 in particular, are good markers for monitoring natural T_R cells. In contrast to these molecules, natural T_R cells may decrease the level of CD25 expression under certain conditions, such as their transfer to nude mice (Fig. 3A) or SCID mice (26,27). Noteworthy, however, is that CD25 expression by CD25+CD4+ T_R cells was moderately maintained when they were co-transferred with CD25−CD4+ T cells to nude mice (Fig. 4A). It is likely that IL-2 is required for the maintenance of CD25 expression on natural T_R cells and that, in the nude mice, IL-2 from the co-transferred normal T cells was responsible for the maintenance (44–46). IL-2 is also necessary for in vivo survival of CD25+CD4+ T_R cells (47). Once natural T_R cells establish transplantation tolerance, in vivo administration of IL-2 may contribute to the maintenance of their CD25 expression, survival and function, thereby to stable graft tolerance.

CD25−CD4+ T cells, especially CD45RBlowCD25−CD4+ T cells, which express Foxp3 at a low level (20), gave rise to a small number of CD25highFoxp3moderately high T_R cells with in vitro suppressive activity (Fig. 3B and C). This T_R subpopulation was, however, much less potent in suppressing graft rejection than CD25+CD4+ T_R cells (Fig. 4B). Similar findings were also made in other experimental systems; e.g. the CD45RBlowCD25−CD4+ T cell population contains T_R cells capable of preventing autoimmune disease or colitis, or transferring transplantation tolerance induced by anti-CD4 mAb treatment. In these experiments, however, the regulatory activity of this fraction was much lower than CD25+CD4+ T_R cells, and insufficient to transfer graft tolerance (26,32,34,48–50). Moreover, such CD25+CD4+ T_R cells became CD25+ upon antigenic stimulation in vitro and in vivo (Fig. 3B; M. Ono and S. Sakaguchi, unpublished data). The origin of these CD25+ T_R cells remains to be determined; e.g. they may be derived from natural CD25+CD4+ T_R cells that have lost CD25 expression or from pre-committed T_R cells naturally present in the CD25−CD4+ T cell population, especially in the CD45RBlowGITRlowCTLA-4+ fraction ([51]; Supplementary fig. 1; M. Ono, J. Shimizu, T. Yamaguchi, Z. Fehervari, Y. Miyachi and S. Sakaguchi, manuscript in preparation); alternatively, naive CD25−CD4+ non-T_R cells may differentiate to CD25+ T_R cells under certain conditions of antigen-presentation and/or cytokine milieu (37,38,52–54).

Alloantigen-specific CD25+CD4+ T cells can be expanded in vitro by allogeneic stimulation and high dose IL-2. They showed potent alloantigen-specific suppressive activity in vitro, suppressed even a secondary MLR, and could prevent graft rejection in vivo. However, they appear to have a short life span in vivo upon transfer or change their homing patterns, making them less effective in vivo compared with their potent in vitro suppressive activity, as also suggested by others in treatment of GVHD (55,56). The possible poor survival of in vitro activated CD25+CD4+ T_R cells could be attributed to their apoptosis upon sudden withdrawal of high dose IL-2 at the time of cell transfer (57). Use of other cytokines for T_R cell expansion, delivery of apoptosis-inhibitory signals to expanding T_R cells, or use of mature allogeneic DCs for stimulation without exogenous IL-2 may overcome the problem. The present results nevertheless indicate that, compared with a large number of T_R cells required for inducing tolerance to full MHC-mismatched skin grafts as shown here, a much lower number of in vitro expanded T_R cells may suffice to establish tolerance to other organs with minor histoincompatibility even if many of the transferred T_R cells may die after transfer.

Taken together, the results in this report suggest two possible ways of exploiting naturally occurring CD25+CD4+ T_R cells for induction of graft-specific tolerance, without hampering immune responses to other antigens. One is to control non-T_R cells for preparing an immunological condition facilitating antigen-specific spontaneous expansion of natural T_R cells; e.g. to reduce the number or block the activation of non-T_R cells as specifically as possible, and meanwhile to sensitize the remaining CD25+CD4+ T_R cells to alloantigens, allowing alloantigen-specific T_R cells to expand to the extent that they can dominantly suppress allo-reactive T cells recovering from the reduction or blockade. Certain monoclonal antibodies or drugs whose administration for a limited period can induce long-term T_R cell-dependent graft tolerance may have this effect as a common mechanism of tolerance induction (1,30–35,58). Another way of tolerance induction with natural T_R cells is to isolate CD25+CD4+ T_R cells from the recipient of the organ transplant, stimulate them ex vivo with donor stimulator cells, and transfer back the expanded antigen-specific T_R cells to the recipient before organ transplantation, as is possible in living donor transplantation (59). In this setting, suppression of the recipient's allo-reactive T cells by the inoculated T_R cells may allow any of the recipient's residual natural T_R cells, which may be CD25+ or CD25−, to expand spontaneously in a graft-specific fashion, potentiating graft tolerance synergistically with the inoculated T_R cells. This possible mechanism of 'infectious tolerance' is currently under investigation. These ways of inducing immunologic tolerance to organ transplants could, in principle, be applied for re-establishing self-tolerance in autoimmune disease and controlling aberrant or excessive immune responses to non-self-antigens.

Supplementary data
Supplementary data are available at International Immunology Online.

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