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Targeted CTLA-4 Engagement Induces CD4\(^+\)CD25\(^+\)CTLA-4\(\text{high}\) T Regulatory Cells with Target (Allo)antigen Specificity\(^1\)

Chenthamarakshan Vasu,\(^*\) Bellur S. Prabhakar,\(^†\) and Mark J. Holterman\(^2*\)

CTLA-4 (CD152) is actively involved in down-regulating T cell activation and maintaining lymphocyte homeostasis. Our earlier studies showed that targeted engagement of CTLA-4 can down-modulate T cell response and suppress allo- and autoimmunee responses. In this study, we report that targeted CTLA-4 engagement can induce immune tolerance to a specific target through selective induction of an Ag-specific CD4\(^+\)CD25\(^+\)CTLA-4\(\text{high}\) regulatory T cell (Treg cell) population. Allogeneic cells coated with anti-CTLA-4 Ab induced immune hyporesponsiveness through suppression of proinflammatory cytokines IFN-\(\gamma\) and IL-2, and up-regulation of the regulatory cytokines IL-10, TGF-\(\beta\)1, and IL-4, presumably through the engagement of CTLA-4 on activated T cells. Although rechallenge with alloantigen failed to break the unresponsiveness, a transient recovery from tolerance was observed in the presence of high concentrations of exogenous IL-2, saturating concentrations of neutralizing anti-TGF-\(\beta\)1 and anti-IL-10 Abs, and blocking anti-CTLA-4 Ab, and upon depletion of CD4\(^+\)CD25\(^+\) Treg cells. The CD4\(^+\)CD25\(^+\)CTLA-4\(\text{high}\) Treg cells from tolerant mice suppressed the effector function of CD25\(^-\) T cells from Ag-primed mice. Adoptive transfer of these Treg cells into Ag-primed mice resulted in a significantly reduced alloantigen-specific response. Further characterization demonstrated that the Treg cells with memory phenotype (CD62L\(^-\)) were more potent in suppressing the alloantigen-specific T cell response. These results strongly support that the targeted engagement of CTLA-4 has therapeutic potential for the prevention of transplant rejection. *The Journal of Immunology, 2004, 173: 2866–2876.

Allotageng-specific T cell tolerance is one of the main goals in preventing graft rejection. Although the search for more directed therapies that target alloreactive T cells in an Ag-specific fashion continues, nonspecific immunosuppressive drugs dominate the current therapeutic strategies. Induction of T cell hyporesponsiveness or tolerance holds potential in promoting long-lasting graft survival without the need for chronic immunosuppressive drugs.

Activation of mature T lymphocytes is a multistep process requiring both Ag-specific triggering of the TCR complex and costimulation mediated through the CD28-B7.1/B7.2 pathway (1, 2). The CD28 ligands, CDB8 (B7.1) and CDB86 (B7.2), are found on APCs, and their ligation to CD28 on T cells leads to IL-2 up-regulation and progression through the cell cycle. CTLA-4 is a critical inhibitor of T cell activation as evidenced by the lethal lymphoproliferation seen in CTLA-4 knockout mice (3, 4). The B7/CD28 pathway plays a primary role in T cell homeostasis, and manipulating this pathway has emerged as a powerful strategy to modulate the immune response with clinical applications. The inhibition of the costimulatory pathway in the presence of antigenic stimulation may result in T cell anergy (5–8). This has been successfully used in animal models to prevent allograft rejection by blocking CD86 and/or CD80, thereby leading to prolonged graft survival (9–14). We and others have demonstrated that engagement of CTLA-4, concomitant with Ag stimulation, can suppress T cell function or result in T cell hyporesponsiveness (15–18). This observation supports the earlier view that engagement of CTLA-4 concomitant with TCR-MHC Ag complex interaction can result in inhibition of T cell activation despite simultaneous costimulation through CD28 (19). Most interestingly, CTLA-4-Ig that can prevent or suppress transplant rejection and autoimmune conditions by blocking costimulation (9–12) has not been shown to promote generation of regulatory T cells (Treg cells).\(^3\) In fact, recent studies have shown that CD28/B7 costimulation is necessary for generation and maintenance of Ag-specific Treg cells (20–23).

Previously, we reported that engagement of CTLA-4 on alloreactive T cells that are in contact with their target could down-modulate the T cell response (15). We subsequently showed that targeted CTLA-4 engagement can down-modulate autoreactive T cells in a model of autoimmune thyroiditis (16). This approach induced thyroid Ag-specific tolerance that resulted in disease suppression. In this current study, we explored the mechanism of Ag-specific tolerance induction upon CTLA-4 engagement during alloantigen recognition. In this tolerance induction system, thyroid-stimulating hormone (TSH) receptor-expressing M12 (H2\(\text{d}\)) cells (mM12 cells) were used as an alloantigen source for stimulating H2 k mice (mM12) and vice versa. Previous studies have shown that the allotageng-specific T cell response is dependent on TCR-MHC Ag interaction (24). Engagement of the CD28 pathway with APCs results in IL-2 production and progression of T lymphocytes through the cell cycle (25). Our earlier studies demonstrated that the targeted engagement of CTLA-4 on alloantigenic T cells may result in immune hyporesponsiveness through suppression of proinflammatory cytokines IFN-\(\gamma\) and IL-2 (26). The addition of anti-CTLA-4 Ab induced immune hyporesponsiveness through suppression of proinflammatory cytokines IFN-\(\gamma\) and IL-2, and up-regulation of the regulatory cytokines IL-10, TGF-\(\beta\)1, and IL-4, presumably through the engagement of CTLA-4 on activated T cells. Although rechallenge with alloantigen failed to break the unresponsiveness, a transient recovery from tolerance was observed in the presence of high concentrations of exogenous IL-2, saturating concentrations of neutralizing anti-TGF-\(\beta\)1 and anti-IL-10 Abs, and blocking anti-CTLA-4 Ab, and upon depletion of CD4\(^+\)CD25\(^+\) Treg cells. The CD4\(^+\)CD25\(^+\)CTLA-4\(\text{high}\) Treg cells from tolerant mice suppressed the effector function of CD25\(^-\) T cells from Ag-primed mice. Adoptive transfer of these Treg cells into Ag-primed mice resulted in a significantly reduced alloantigen-specific response. Further characterization demonstrated that the Treg cells with memory phenotype (CD62L\(^-\)) were more potent in suppressing the alloantigen-specific T cell response. These results strongly support that the targeted engagement of CTLA-4 has therapeutic potential for the prevention of transplant rejection.

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had no effect on this tolerant state. Furthermore, this tolerance induction was associated with selective expansion of a subpopulation of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells that could down-modulate the allosresponse of T cells from nontolerant mice.

Materials and Methods

**Mouse strains**

Six-week-old female CBA/J, BALB/c, and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in the biological resources laboratory at the University of Illinois at Chicago and allowed food and water ad libitum. All mice were used at 8 wk of age and cared for in accordance with the guidelines set forth by the Animal Care and Use Committee at University of Illinois at Chicago.

**Abs and cell lines**

M12, a B cell lymphoma of BALB/c origin (H-2<sup>k</sup>), M12 cells stably transfected with the murine TSHR cDNA (mM12 cells) (24), and Ag8 mouse B cell lymphoma (H-2<sup>d</sup>) were used in this study. Hamster anti-mouse CTLA-4 hybridoma (UCI0-4-F-I0-11; American Type Culture Collection, Manassas, VA) and mouse anti-mouse TSHR hybridoma (D6-4), were grown in DMEM/F12 medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), fungizone (1 µg/ml), and 10% FBS. The D6-4 hybridoma was prepared by fusing spleen cells from BALB/c mice with autoimmune Grave’s disease and the mouse B cell myeloma Sp2/0 (25). Abs were purified from the spent medium using Protein L (Sigma-Aldrich, St. Louis, MO) and normal rat IgG1 isotype control were purchased from R&D Systems (Minneapolis, MN); magnetic bead-conjugated, anti-PE, anti-mouse CD4, CD8a; and PE-labeled anti-mouse CD3, CD4, CD8, CD25, CTLA-4, CD62L, CD69, IL-2, IL-4, IL-10, IFN-γ, and TGF-β (Caltag Laboratories, San Francisco, CA, and BD Pharmingen, San Diego, CA) were used in flow cytometry and required cytokine-speciﬁc standards for detecting mouse IL-2, IL-4, and IFN-γ (Caltag Laboratories) and for detecting IL-10 and TGF-β (BD Pharmingen) in ELISA. Neutralizing Abs to mouse IL-4 (rat IgG1; clone 11B11) and IL-10 (rat IgG1; clone JES5-2A5) were purchased from eBioscience (San Diego, CA). Recombinant mouse IL-2 (rIL-2; neutralizing Ab to mouse TGF-β1 (rat IgG1; clone 1D11), and normal rat IgG1 isotype control were purchased from R&D Systems (Minneapolis, MN); magnetic bead-conjugated, anti-PE, anti-mouse CD4, CD62L Abs, Pan T cell isolation kit, CD4<sup>+</sup>CD25<sup>+</sup> T cell isolation kits (Miltenyi Biotec, Auburn, CA) were used to isolate CD<sup>+</sup>, CD<sup>4</sup><sup>+</sup>, CD<sup>4</sup><sup>-</sup>CD<sup>25</sup><sup>-</sup>, CD<sup>4</sup><sup>-</sup>CD<sup>25</sup><sup>+</sup>CD<sup>62</sup>L<sup>+</sup>, and CD<sup>4</sup><sup>-</sup>CD<sup>25</sup><sup>+</sup>CD<sup>62</sup>L<sup>-</sup> cells. The Fab of anti-CTLA-4 was prepared by papain digestion using papain-linked agarose beads (Pierce, Rockford, IL) according to the manufacturer’s instructions.

**Tolerance induction by CTLA-4 engagement**

mM12 cells were treated with mitomycin C (50 µg/10<sup>6</sup> cells/ml) and 1 × 10<sup>7</sup> cells were incubated with either cBiAb or tBiAb (100 µg) for 30 min on ice. Eight-week-old female CBA/J mice (H-2<sup>b</sup>) were then injected with these cells i.p. twice at 10-day interval (on day 0 and 10). Although control mice (mM12 immune group) received mM12 cells coated with cBiAb prepared from control Abs (anti-TSHR anti-hamster IgG BIAb), test mice (BiAb-treated group) received mM12 cells coated with tBiAb prepared by linking anti-CTLA-4 and anti-TSHR Abs throughout this study unless specified. Hereafter, mice that received cBiAb-coated mM12 cells and tBiAb-coated are referred as control group and BiAb-treated group, respectively. Mice were sacrificed 10 days after the booster injection (day 20), and lymphocytes were collected from spleens and lymph nodes. For some studies, tolerized mice were rechallenged with mM12 cells on day 60 and sacrificed on day 70.

**Immunization/challenge with alloantigens**

Mice were tolerized by CTLA-4 engagement as described above. On day 20, these mice were immunized (i.p.) with mitomycin C-treated spleen cells (1 × 10<sup>7</sup> cells/mouse) from C57BL/6 (H<sup>2</sup><sup>b</sup>) or BALB/c (H<sup>2</sup><sup>d</sup>) mice, or Ag8 (H<sup>2</sup><sup>d</sup>) cells (2 × 10<sup>6</sup> cells/mouse). These mice were sacrificed on day 30 and tested for their ability to respond to M12 and other alloantigens by lymphocyte proliferation and IL-2 production assays.

**Proliferation assay**

Effector cells (spleen cells or purified T cell populations; 0.5 × 10<sup>6</sup> cells) and mitomycin C-treated target cells (M12 cells; 0.5 × 10<sup>6</sup> cells/well; spleen cells; 5 × 10<sup>5</sup>) were plated in 96-well flat-bottom tissue culture plates in triplicate in RPMI 1640 containing 2% normal mouse serum at a final volume of 0.25 ml/well. In some experiments, the assay was conducted in the presence of varying concentrations (5–200 U/ml) of rIL-2. After 48 h, cells were pulsed with 1 µCi/well [<sup>3</sup>H]thymidine for 18 h in 100 µl of the above medium supplemented with 2% normal mouse serum. Cells were harvested, and [<sup>3</sup>H]thymidine incorporation was measured using Microbeta scintillation counter (PerkinElmer Wallac, Gaithersburg, MD). Background counts of effector cell cultures containing no M12 cells were subtracted from test values to calculate the actual counts.

**CFSE staining**

Single-cell suspensions of spleen or purified lymphocytes from either immune or tolerant mice were suspended in HBSS at a concentration of 1 × 10<sup>6</sup> cells/ml and labeled with the tracking fluorochrome CFSE (Molecular Probes, Eugene, OR). Cells were incubated with CFSE at a final concentration of 1 µM in HBSS for 5 min, and labeling was terminated by the addition of FCS (10% of the total volume). Cells were washed twice in complete RPMI 1640 medium and used in proliferation assays as described above. The dilution of CFSE was measured by flow-cytometric analysis after 7 days of incubation.

**Flow cytometry**

Single-cell suspensions of spleen and lymph nodes were washed with PBS supplemented with 2% FBS (pH 7.4) and blocked with anti-CD16/CD32 Fc block on ice for 15 min. Cells were stained with FITC-, PE-, and cytokine-labeled appropriate Abs in various combinations on ice for 30 min, washed, and analyzed in a FACS analyzer (BD Biosciences, San Jose, CA), and the data were analyzed using the CellQuest or WinMDI software. Control cells were stained with isotype-matched control Abs and analyzed. At least 10,000 cells were analyzed in all experiments.

**Cytokine production**

A total of 5 × 10<sup>6</sup> spleen cells/well (12-well plate) was incubated in the presence of 0.5 × 10<sup>6</sup> mitomycin C-treated target M12 cells in 1.5 ml of RPMI 1640 medium supplemented with 2% normal mouse serum for 48 h. Cell-free supernatants (in culture supernatants; 100 µl) were collected after 48 h and the cytokine levels were assayed by ELISA using paired Abs and respective cytokine standards for the detection of IL-2, IL-4, IL-10, IFN-γ, and TGF-β1 according to the manufacturer’s directions. Cytokines were detected by adding HRP-labeled streptavidin followed by washing and addition of tetramethyl benzidine-H<sub>2</sub>O<sub>2</sub> substrate (BD Pharmingen) for 5–10 min. The OD<sub>450</sub> was read using a Microplate reader (Bio-Rad Laboratories, Hercules, CA). The amount of cytokine was determined using an appropriate cytokine-specific standard curve. Background cytokine levels of effector cell cultures containing no M12 cells were subtracted from test values to calculate the actual cytokine response.

For intracellular cytokine expression analysis, spleen cells collected at the 0 or 21 day experiment were either activated with mitomycin C-treated M12 cells or allowed to rest for 36 h. Cells were stained with FITC-conjugated Ab to CD3 and were then fixed and permeabilized using Cytofix/perm reagent (BD Pharmingen). Subsequently, these cells were stained with PE-labeled specific anti-cytokine Abs, washed, and analyzed in a FACS analyzer as described above. In some experiments, cytokines were detected by multiplex cytokine assay using Luminex (Austin, TX) technology at the Luminex core facility of University of Pittsburgh Cancer Center (Pittsburgh, PA).

**Cytokine neutralization and CTLA-4 blockade assays**

T cells were cultured in the presence of mitomycin C-treated M12 cells as described above for CFSE dilution assay. To these cultures, varying concentrations of neutralizing anti-mouse IL-4 (1–2000 ng/ml), anti-mouse IL-10 (10–5000 ng/ml), anti-mouse TGF-β1 (10–2000 ng/ml), and/or isotype-matched control Abs were added and incubated for 7 days. Varying concentrations of neutralizing anti-mouse IL-4 (1–2000 ng/ml), anti-mouse IL-10 (10–5000 ng/ml), anti-mouse TGF-β1 (10–2000 ng/ml), and/or isotype-matched control Abs were added and incubated for 7 days. Varying...
concentrations (100 ng to 10 μg/ml) of Fab of anti-mouse CTLA-4 or isotype control Ab were added to some cultures to block CTLA-4 interactions. The dilution of CFSE was measured by flow-cytometric analysis after 7 days of incubation.

Isolation of CD4⁺CD25⁺ T cells and other T cell subpopulations

CD4⁺CD25⁺ cells were isolated using Abs conjugated to magnetic beads and magnetic separation column according to manufacturer’s directions. Pooled mouse spleen and lymph node cells were incubated with anti-CD16/32 Ab for 15 min on ice to block FcRs, and subsequently, CD3⁺ T cells were isolated using a pan T cell isolation (negative selection) kit by following manufacturer’s instructions. CD4⁺CD25⁺ T cells were isolated from spleen cells or the CD3⁺ population using either PE-labeled anti-mouse CD25 and magnetic bead-labeled anti-PE Abs, or Cy5⁺CD25⁺ T cell isolation kit. Cells were incubated with PE-labeled anti-mouse CD25 Ab for 30 min on ice. Cells were then incubated with magnetic bead-conjugated anti-PE Ab for 15 min on ice, washed, and sorted using LS magnetic columns or an automated machine (Automacs; Miltenyi Biotec). For some experiments, CD4⁺CD25⁺ cells were enriched for CD62L⁺ and CD62L⁻ populations using magnetic separation procedures. For this, CD3⁺ cells were isolated using the pan T cell isolation kit, and CD62L⁺ and CD62L⁻ cells were enriched using magnetic bead-labeled anti-mouse CD62L Ab. CD25⁺ cells from CD62L⁻ fraction were enriched as described above. CD62L⁺ T cells were washed using acidified PBS to remove bound beads, and the CD25⁺ population was enriched.

Coculture of CD25⁺ and CD25⁻ T cells

CD4⁺CD25⁻ cells from control mice (mM12 immune) were mixed with CD4⁺CD25⁺ cells from tolerant mice and vice versa at a ratio of 10:1. In some assays, CD4⁺CD25⁺CD62L⁻ or CD4⁺CD25⁺CD62L⁺ enriched populations were used instead of CD4⁺CD25⁺ cells. These mixtures at various ratios or individual cell populations were used in T cell proliferation assays (total of 0.6 × 10⁶ cells/well) that were conducted either in the presence or absence of M12 cells, as described above.

Transwell T cell inhibition assays

CD4⁺CD25⁻ cells from tolerant mice were isolated as described above. CD4⁺CD25⁺ cells (2.5 × 10⁶) plus mitomycin C-treated M12 cells (0.5 × 10⁶) in the well insert (upper compartment) and CD25⁻ T cells plus M12 cells (2.5 × 10⁶) in the well (lower compartment) were cultured in a Transwell assay system using Falcon Transwell tissue culture well inserts (BD Biosciences) in a 24-well plate format. After 48 h, cells were pulsed with 2.5 μCi of [³H]thymidine for 18 h. Cells from the upper and lower compartments were pooled, and proliferation was measured as [³H]thymidine incorporation in a scintillation counter as described above.

Adoptive transfer of Treg cells

CD4⁺CD25⁻ T cells were isolated from both control and test mice (CBA/J; H₂ª) as described above. Recipient mice were immunized with M12 cells (H₂ª) on day 0 and C57BL/6 spleen cells (H₂ª) on day 10. Approximately 5 × 10⁶ CD4⁺CD25⁻ T cells were adoptively transferred (i.v.) to recipient mice on day 20, 2 h before challenge injection with M12 cells. These mice were sacrificed on day 30 posttransfer, and tested for T cell-proliferative and IL-2 responses to M12 cells and C57BL/6 spleen cells as described above.

Statistical analysis

Mean, SD, and statistical significance (p value) were calculated using SSSS statistical application (SSPS, Chicago, IL). The p value was determined using the nonparametric Wilcoxon signed test. In most cases, values of BiAb-treated group (mice that received anti-CTLA-4 Ab-coated mM12 cells) were compared with that of mM12 immune group (mice that received isotype control Ab-coated mM12 cells) unless specified. Differences in the percentage of fluorescence-positive cells between test and control groups were tested using the nonparametric sign test. A p value of ≥0.05 was considered significant.

Results

Engagement of CTLA-4 upon allore cognition suppresses alloimmune response

To address whether targeted CTLA-4 engagement will inhibit an in vivo allogeneic T cell response, control Ab (cBiAb-) or CTLA-4 Ab (tBiAb)-coated allogeneic mM12 cells were injected into CBA/J mice. In one group (BiAb-treated group 2) received tBiAb-coated mM12 cells only during the challenge immunization to test whether the T cell response can be turned down during the secondary immune response. As shown in Fig. 1, spleen cells from control mice showed strong T cell-proliferative, IL-2, and IFN-γ responses upon ex vivo restimulation with mM12 cells. Treated mice, immunized either both times or only during the second immunization with BiAb-coated mM12 cells, however, showed significantly reduced T cell proliferation, IL-2, and IFN-γ responses. Interestingly, alloimmune suppression induced upon CTLA-4 engagement was associated with increased production of IL-4, IL-10, and TGF-β (Fig. 1b). Spleen cells from mice that received BiAb-coated mM12 cells showed ~3- to 4-fold higher levels of these cytokines compared with spleen cells from mice that received control Ab-coated mM12 cells.
Alloimmune suppression induced upon CTLA-4 engagement is persistent

To test whether restimulation of lymphocytes from BiAb-treated mice in vitro can break tolerance, spleen cells were stimulated with M12 cells in vitro for 5 days. Cells were collected from these cultures and tested for their ability to respond to a further restimulation by M12 cells. These cells showed no sign of recovery from tolerance upon ex vivo rechallenge and failed to proliferate or produce significant amounts of IL-2 in response to tertiary stimulation with M12 cells (Fig. 2, a and b). However, cells from both test and control groups of mice responded to mitogenic stimulation (Con A), clearly indicating that the cells were viable and able to proliferate. These results suggest that tolerance to alloantigen induced upon targeted CTLA-4 engagement is not readily reversible with ex vivo alloantigen challenge.

Next, the duration and reversibility of the CTLA-4 engagement-induced tolerance in vivo was tested. Control and BiAb-treated mice were rested for 50 days and challenged with M12 cells, and spleen cells from these mice were tested for their ability to respond to M12 restimulation ex vivo. As shown in Fig. 2, c and d, spleen cells from tolerant (BiAb-treated) mice showed no increase in their response to M12 cells, suggesting that the tolerance induced upon CTLA-4 engagement is maintained even after in vivo challenge with the alloantigen.

FIGURE 2. Lasting effect of tolerance induced upon CTLA-4 engagement. CBA/J (H2k) mice were immunized with BiAb-coated mM12 cells on days 0 and 10 as described in Materials and Methods. These mice were divided into two sets and sacrificed on either day 20 or 70. On day 20, spleen cells from naive mice (naive) or mice immunized with cBiAb-coated (control) or tBiAb-coated (BiAb-treated) mM12 cells were stimulated in triplicate wells (0.5 × 10^6 spleen cells/well) with mitomycin C-treated M12 cells (0.5 × 10^3/well) for up to 5 days. a, Lymphocytes were washed, counted, and restimulated with M12 cells or Con A (1 μg/ml) for 48 h for T cell proliferation assay by the [3H]thymidine incorporation method. b, Spent media collected after 48 h were tested for IL-2 by ELISA. c and d, On day 60, the second set of mM12 immune as well as BiAb-treated mice were challenged with M12 cells, sacrificed on day 70 along with naive mice, and tested for T cell proliferative and IL-2 responses to M12 cells. Background counts per minute (<200) and cytokine (<10 pg/ml) values in spleen cell cultures containing no M12 cells or Con A were subtracted from respective test values. Results are expressed as mean ± SD of triplicate samples obtained from five individual mice. This experiment was repeated with similar results. The p value was calculated by comparing treated group values with the corresponding values for the control group. *, p ≤ 0.05.

Tolerance induced upon targeted CTLA-4 engagement is target specific

The specificity of suppression of the anti-H2d immune response upon CTLA-4 engagement was tested by challenging BiAb-treated CBA/J (H2d) mice with different cells. On day 20 after treatment, these mice received either spleen cells from a different mouse strain (C57BL/6 mice, H2b), or spleen cells from BALB/c mice or Ag8 cell line, both syngeneic to M12 cells (H2d). On day 30, these mice were tested for T cell proliferation and IL-2 production in response to restimulation by spleen cells from C57BL/6 and BALB/c mice, Ag8 and M12 cells. Control as well as BiAb-treated mice showed the same levels of T cell-proliferative and IL-2 responses to C57BL/6 spleen cells (Fig. 3a). However, the pattern of responses to BALB/c spleen and Ag8 cells was more or less similar to that noted when stimulated with M12 cells (Fig. 3, b and c). These results suggest that T cell tolerance induced upon CTLA-4
engagement during the immune response to H2d alloantigen is target specific and effective against cells bearing the same Ag (i.e., H2d) but not other Ags (i.e., H2b).

**CD4+CD25+ T cells are selectively expanded in tolerant mice**

Next, we tested to see whether the tolerance induction was associated with changes in CD4+CD25+ T cells. To address this question, naïve, control, and BiAb-treated mice were sacrificed on day 20, and lymph node and spleen cells were analyzed by flow cytometry for the CD4+CD25+ population. We found a significant increase in the percentage of CD4+CD25+ T cells in BiAb-treated (tolerant) mice in both spleen and lymph nodes, but not in the control mice (Fig. 4). To test whether the increase in the percentage of CD4+CD25+ T cells in lymphoid cells from tolerant mice was simply due to the decrease in CD4+CD25− T cells, we measured the percentage of both CD4+CD25+ and CD4+CD25− T cells within the CD4+ T cell population as well as absolute numbers of CD4+CD25+ and CD4+CD25− T cells in the spleens. As shown in Fig. 4a, there was a significant increase (p < 0.034) in CD4+CD25+ T cells within the CD4+ T cells in tolerant mice relative to controls. However, the number of CD4+CD25− T cells within the T cell population was not significantly different in treated and untreated mice (not shown). We also found a significant increase (p < 0.021) in the absolute numbers of CD4+CD25− T cells in spleens from BiAb-treated mice compared with those from control mice. For example, while control mice showed an average of 1.74 × 10^7 CD4+CD25− cells and 1.62 × 10^7 CD4+CD25+ T cells per spleen, tolerant mice showed an average of 1.76 × 10^7 CD4+CD25− cells and 2.5 × 10^6 CD4+CD25+ T cells. A similar increase in CD25+ T cells was also observed in the lymph nodes of tolerant mice (not shown).

It has been reported that CTLA-4 is constitutively expressed on CD4+CD25+ T cells and involved in mediating its regulatory activity in vitro and in vivo (27, 28). As shown in Fig. 4b, CD4+CD25+ T cells from tolerant mice showed an increased expression of CTLA-4 compared with CD4+CD25− cells from control or naïve mice. However, CD4+CD25+ T cells from all three groups showed little or no surface CTLA-4 expression (not shown).

**T cell phenotypes in BiAb-treated mice upon ex vivo challenge**

As a first step toward understanding the mechanism of T cell hyporesponsiveness to allogeneic target and Treg cell generation in BiAb-treated mice, we tested the phenotypic changes of T cells from BiAb-treated mice upon ex vivo challenge with target (M12) cells. Spleen cells were incubated with M12 cells and tested for various markers at different time points (Fig. 5). The number of T cells expressing the early activation marker CD69 was significantly less in spleen cells from BiAb-treated mice after 24 h of stimulation compared with control immune mice (Fig. 5a). T cells secreting various cytokines were detected by intracellular staining after 36-h stimulation. While the number of IFN-γ-secreting T cells was significantly higher in m12 immune mice compared with tolerant mice, an increase in IL-4-, IL-10-, and TGF-β1-producing cells was observed in tolerant mice (Fig. 5b). Because low levels or no expression of CD62L is correlated with the memory phenotype, we tested for its expression after 5 days of stimulation. The number of T cells expressing low levels of CD62L was significantly lower in CD3+ cells from BiAb-treated mice compared with controls (Fig. 5c). To test the Treg cell phenotype, cells from similar cultures were washed thoroughly, rested for 3 days, and analyzed for CD25 expression. Very importantly, the number of CD4+ cells expressing CD25 in spleen cells from tolerant mice was almost twice that of control immune mice (Fig. 5d). CFSE-stained spleen cells were incubated for 7 days with M12 cells and analyzed for CFSE dilution. As shown in Fig. 5e, the proliferative responses of both CD4+ and CD8+ T cells were significantly lower in spleen cells from tolerant mice compared with immune controls. However, the number of divisions undergone by these cells from both tolerant and control mice appeared to be more or less the same.

**Exogenous IL-2 partially reversed the unresponsiveness to alloantigen but not tolerance**

To test whether exogenous IL-2 can reverse the tolerance induced upon CTLA-4 engagement, M12 cells and varying amounts of rmIL-2 were added to spleen cells from different mice. Fig. 6a shows the proliferative response of spleen cells after antigenic re-stimulation either in the absence or presence of exogenously added rmIL-2. Although addition of small amounts of rmIL-2 did not induce a significant proliferation of T cells from tolerized mice, a partial recovery (up to 60% compared with that of control mice) was observed in the presence of rmIL-2 at higher concentrations. Furthermore, the hyporesponsive state was reversed only in the presence of both IL-2 and alloantigen, but not with either alloantigen or IL-2 alone, suggesting that hyporesponsiveness can be reversed only if the Ag and excessive levels of IL-2 are present at the same time.

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**FIGURE 4.** CD4+CD25+ Treg cells are induced upon targeted CTLA-4 engagement. CBA/J mice (H2b) were immunized with allogeneic (H2d) mM12 cells along with cBiAb or tBiAb as described in Materials and Methods. a. Spleen and lymph node cells were collected from these mice along with naive mice, and stained with FITC-conjugated anti-mouse CD4 Ab and PE-labeled anti-mouse CD25 Ab for FACS analysis. The CD4+ cell population was gated for the above graphs. The percentage of CD25+ cells is indicated in the inner rectangle, and the range of percentages of CD4+CD25− cells in five individual mice from each group is indicated in the main rectangle. The p value was calculated by comparing each value for the treated group with the corresponding value of the control group. *, p < 0.05. b. Expression of CTLA-4 on CD4+CD25+ cells isolated from naive mice (naive), mice immunized with cBiAb-coated mM12 (H2d) (control), or tBiAb-coated mM12 cells (BiAb-treated) was tested by FACS analysis after staining with anti-CTLA-4-PE Ab.
To assess the tertiary response to alloantigen by T cells from tolerant mice, effector cells from the above culture (Fig. 6a) were collected on day 5, washed, rested, and incubated with fresh target M12 cells. Interestingly, tolerant T cells, which had proliferated in the presence of IL-2 and alloantigen, were nonresponsive during a subsequent encounter with alloantigen in a tertiary stimulation (Fig. 6b), indicating that these cells continue to remain tolerant and that the reversal of the proliferative response does occur only in the presence of high levels of IL-2 and alloantigen.

IL-10 and TGF-β1 play an important role in the hyporesponsiveness of T cells from tolerant mice

Upon tolerance induction by targeted CTLA-4 engagement, we found an increase in IL-4, IL-10, and TGF-β1, along with a decrease in proinflammatory cytokines such as IFN-γ. To determine whether the suppressive effect induced by CTLA-4 engagement is dependent on these inhibitory cytokines, neutralizing Abs against them were added to ex vivo cell cultures. As shown in Fig. 6c, whereas blockade of IL-4 did not show any significant effect on the M12 cells. Interestingly, tolerant T cells, which had proliferated in the presence of IL-2 and alloantigen, were nonresponsive during a subsequent encounter with alloantigen in a tertiary stimulation (Fig. 6a), indicating that these cells continue to remain tolerant and that the reversal of the proliferative response does occur only in the presence of high levels of IL-2 and alloantigen.

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FIGURE 6. Role of cytokines and CTLA-4 in CTLA-4 engagement-induced tolerance. a, Spleen cells collected from tolerant (left panel) or immune (right panel) mice were incubated alone or with M12 cells (1:10 target:effector ratio). Cultures of tolerant cells received varying concentrations of rmIL-2 and were incubated for up to 5 days. a, On day 2, one set of cultures was pulsed with [3H]thymidine for 18 h to test for T cell proliferation. b, On day 5, the remaining cells were washed and rested for 48 h, and equal numbers of viable cells were further incubated with M12 cells in the absence of rmIL-2 for 48 h and tested for T cell proliferation by the [3H]thymidine incorporation method. c, Aliquots of cells were stained with CFSE before incubation with M12 cells, supplemented with saturating concentrations of neutralizing anti-mouse IL-4, IL-10, TGF-β1, or the Fab of anti-mouse CTLA-4 Ab as described in Materials and Methods. Cells were tested for CFSE dilution on day 7 after staining with PE-labeled anti-mouse CD3 Ab. This experiment was repeated with similar results. Percentage of cells of the selected region is indicated for each graph.

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proliferative response of tolerant cells to M12 stimulation, neutralization of IL-10 resulted in partial recovery (compared with control spleen cells in the absence of neutralizing Abs) from hyporesponsiveness. However, neutralizing Ab to TGF-β1 alone or in combination with anti-IL-10 Ab resulted in almost complete recovery of T cells from hyporesponsiveness (Fig. 6c). As seen with exogenous IL-2, subsequent incubation of cells collected from these cultures with target cells in the absence of neutralizing Abs showed that reversal of proliferative response in the presence of cytokine-neutralizing Abs was also transient (results not shown).

**CTLA-4 on CD4^+CD25^+ T cells is important in maintaining tolerance**

As observed in Fig. 4b, CD4^+CD25^+ T cells from tolerant mice expressed increased levels of CTLA-4 on their surface. To test the role of increased CTLA-4 expression on tolerance, Fab of a blocking anti-CTLA-4 Ab was added at various concentrations. Addition of anti-CTLA-4 Fab at saturating concentration resulted in almost complete recovery of T cells from hyporesponsiveness (Fig. 6c), suggesting that engagement of CTLA-4 on target cells may be necessary to initiate hyporesponsiveness to alloantigen.

**Role of CD4^+CD25^+ T cells in targeted CTLA-4 engagement-induced tolerance**

To see whether CD4^+CD25^+ Treg cells have a role in down-regulating alloantigen-specific T cell responses, CD4^+CD25^+ and CD25^- T cells from both tolerant and control mice were isolated using magnetic cell separation systems (Fig. 7a). CD4^+CD25^-depleted CD3^+ cells from tolerant mice, but not control mice, showed a significantly enhanced proliferative response to M12 cells (Fig. 7b). Cultures devoid of CD25^- cells from tolerized

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**FIGURE 7.** Role of CD4^+CD25^+ T cells in CTLA-4 engagement-induced tolerance. a, Spleen and lymph node cells collected from cBiAb- or tBiAb-coated mM12 cell-immunized mice were combined and then fractionated into CD25^- and CD25^+ T cell populations as described in Materials and Methods. CD25^- and CD25^+ (5 × 10^5 cells/well) cells were incubated individually (b−d) or in combinations (f) with M12 cells (0.5 × 10^5 cells/well) in 96-well plates or (g) in 24 Transwell plates with 0.1-μm pore size inserts. After 48 h, cells were pulsed with [3H]thymidine for 18 h to measure T cell proliferation. Spent media from the assay described for b were tested for various cytokines using multiplex cytokine assay using Luminex technology (c−e). Results are expressed as mean ± SD of triplicate values, and the assay was repeated three times.
mice responded normally and showed little or no regulatory cytokine responses upon restimulation with allogeneic (Fig. 7, c-e). Although both IL-10 and TGF-β1 responses were mainly restricted to CD25+ T cells, the major source of IL-4 was found to be CD25− T cells. When CD4+CD25+ cells from tolerant mice were cocultured with CD25− T cells from immune mice, we noted that the response to M12 cells was significantly suppressed (Fig. 7f). However, addition of CD4+CD25+ T cells from immune mice to CD25− T cells from tolerant mice failed to suppress the T cell response to M12 cells.

Contact dependence is the predominant mechanism in maintaining tolerance

Next, we performed a Transwell chamber assay to investigate whether the tolerance induction by CD4+CD25− cells is mediated primarily by soluble factors or requires T-T cell contact. As shown in Fig. 7f, CD4+CD25− T cells from tolerant mice suppressed the proliferation of lymphocytes from control mice. Separation of the two populations (CD4+CD25− cells and CD25+ T cells) in Transwell chamber showed virtually the same effect noted when CD25− cells were depleted (Fig. 7g). Furthermore, although CD4+CD25− cells from tolerant mice induced unresponsiveness of CD25− T cells, maintaining them in separate chambers with M12 cells did not show a similar suppression. However, the presence of M12 cells in both chambers resulted in ~30% reduction in the proliferation, suggesting that cytokine production by CD4+CD25− T cells requires alloantigen stimulation, and that these cytokines have an effect, albeit low, on CD25− cells that are in contact with alloantigen. As observed in Fig. 7f, coculturing CD4+CD25− and CD25+ T cells in the same chamber resulted in maximum suppression. These results suggest that direct cell-cell contacts (target-regulatory, target-effector, and regulatory-effector contacts) may be required for optimal inhibition by CD4+CD25+ T cells from tolerant mice.

Tolerance induced upon CTLA-4 engagement can be adoptively transferred

M12 cells (H2k) and C57BL/6 mice (H2b) spleen cell–primed mice were injected i.v. with CD4+CD25+ T cells from tolerant and control mice. These mice were challenged with M12 cells after 2 h, and 15 days later were sacrificed and tested for their ability to respond to target cells. In this experiment, spleen cells from recipient mice that received CD4+CD25+ T cells from tolerant mice showed significantly reduced proliferative and IL-2 responses to M12 cells relative to spleen cells from nonrecipient and control CD4+CD25+ T cell recipients (Fig. 8a). Spleen cells from recipients were also challenged ex vivo with C57BL/6 spleen cells. As shown in Fig. 8a, CD4+CD25+ T cells from neither tolerant nor control mice had any effect on proliferative or IL-2 responses to C57BL/6 spleen cells and showed the target specificity of transferred tolerance.

CD4+CD25+ T cells with the memory phenotype are more potent in inducing hyporesponsiveness

To localize this regulatory effect to the memory or naive populations, CD4+CD25+ T cells from tolerant mice were further separated into CD62L+ and CD62L− fractions (Fig. 9a) and tested for their surface CTLA-4 expression, and their ability to produce IL-10 and TGF-β1, and to suppress T cell-proliferative responses. Relative to CD25+CD62L+ T cells, CD25+CD62L− cells expressed higher levels of CTLA-4, and produced significantly higher amounts of IL-10 and TGF-β1 upon exposure to M12 cells (Fig. 9c and d). Both populations failed to produce significant amounts of IL-4, IL-2, and IFN-γ (not shown). Although both CD25+CD62L+ and CD25+CD62L− Treg cells showed the potential to suppress the effector T cell responses to allogeneic, the CD25+CD62L− population with the memory phenotype (CD25+CD62L−) was more potent (Fig. 9e).
injected with tBiAb-coated allogeneic cells failed to break the hyporesponsive state, suggesting that the suppression observed in these mice was not a temporary effect solely mediated by inhibitory cytokines, but was long-lasting and the Treg cells may play a critical role.

To test the specificity of tolerance generated upon targeted CTLA-4 engagement, we used stimulator cells carrying the same (H2\(^d\)) or a different (H2\(^b\)) alloantigen. In our experimental system, the H2\(^b\) mice showed tolerance to different cells bearing the H2\(^d\) alloantigens but responded normally to cells bearing the H2\(^b\) alloantigens. These results clearly showed that only a subset of T cells with specificity toward a particular target Ag was tolerant, and the remaining cells were unaffected and were able to respond to unrelated alloantigens. Importantly, T cells could be induced to recover from hyporesponsiveness by antigenic restimulation in the presence of a high dose of exogenous IL-2. However, this did not lead to a permanent reversal of the anergic state, because a subsequent antigenic restimulation of these IL-2-treated cells, in the absence of IL-2, still failed to show a significant response.

Our studies support the notion that Treg cells mediate the persistent hyporesponsiveness to specific Ags after targeted CTLA-4 engagement. Earlier studies have shown that CTLA-4 engagement can induce lower IL-2 (29, 30) and higher IL-10 and TGF-\(\beta\) production (31, 32), conditions conducive to the development of Treg cells (33, 34). Consistent with this, earlier we demonstrated that CTLA-4 engagement in a tissue/target-specific manner could induce target autoantigen-specific immune tolerance (16). Previously, we demonstrated that CTL response to alloantigen-bearing target cells is significantly diminished in mice that received BiAb-coated allogeneic cells (15). In this study, we observed an increase in the number of CD4\(^+\)CD25\(^+\) Treg cells in both spleen and lymph nodes of tolerant mice relative to the control and naive mice, without a proportionate reduction in CD4\(^+\)CD25\(^-\) T cell numbers.

Treg cells are often characterized by their constitutive expression of cell surface proteins such as CD25, CTLA-4, and glucocorticoid-induced TNFR family-related gene (34–37). However, these proteins are also expressed on activated T cells, and using these molecules as definite markers is not reliable, unless their ability to suppress effector T cell function is determined. Other studies have shown that the Forkhead transcription factor Foxp3 is specifically expressed in CD4\(^+\)CD25\(^+\) Treg cells and is required for their development (38). An essential role for surface-expressed TGF-\(\beta\) in Treg cell functioning has also been reported (39). The importance of CD4\(^+\)CD25\(^+\) Treg cells in preventing the development of autoimmune disease and their property as anergic and suppressive cells have been reported extensively (34–38). Depletion of these cells can render animals more susceptible to autoimmune diseases (40, 41). However, the precise mechanism of their induction and action in vivo has not been well defined. Unlike other T cell populations, CD4\(^+\)CD25\(^+\) Treg cells constitutively express CTLA-4 on their surface. In this context, it is very interesting to note that CD4\(^+\)CD25\(^+\) Treg cells from allotolerant mice expressed increased levels of CTLA-4 on their surface relative to CD4\(^+\)CD25\(^+\) T cells from immune controls or naive mice. Although several studies have demonstrated that CTLA-4 is important in CD4\(^+\)CD25\(^+\) Treg cell function (27, 28, 42), the role CTLA-4 plays in CD4\(^+\)CD25\(^+\) Treg cell-mediated tolerance remains controversial. However, in the present study, treatment with Fab of anti-CTLA-4 could temporarily restore the immune response of effector T cells, similar to that noted after exogenous IL-2 treatment. This showed that CTLA-4 is essential for maintaining the tolerogenic function of Treg cells induced upon targeted CTLA-4 engagement.
The reversal of hyporesponsiveness upon CTLA-4 blockade suggests the engagement of constitutively expressed CTLA-4 on Treg cells. In fact, recent studies have demonstrated using B7.1 and B7.2 knockout mice that CTLA-4 engagement by its ligands is necessary for the maintenance and homeostasis of naturally existing CD4\(^+\)CD25\(^+\) Treg cells (20–23, 43). In this context, it is important to note that M12 cells, the target cell line used in this study, expresses both B7.1 and B7.2. In addition, our observations suggest that contact with target cells is necessary for Treg cells to produce regulatory cytokines and maintain their Ag specificity through a mechanism involving TCR engagement. Our results also suggest that alloreactive T cells exist in a hyporesponsive state in the tolerant mice that can be reversed upon removal of CD4\(^+\)CD25\(^+\) Treg cells, or in the presence of excessive IL-2 or IL-10 and TGF-\(\beta\)1 neutralizing Abs. These observations strongly suggest that Ag-specific T cells are maintained in an anergic state, perhaps by IL-10 and TGF-\(\beta\), but not deleted.

One of the major characteristics of Treg cells is their ability to produce IL-10 and TGF-\(\beta\), and the role of these cytokines play in Treg cell-induced suppression (26, 44). In this study, we noted that tolerized mice produced higher levels of IL-10 and TGF-\(\beta\), and that CD4\(^+\)CD25\(^+\) T cells were the major source of these cytokines. Although T cells from tolerized mice in our study produced higher amounts of IL-4, CD25\(^-\) cells, and not the CD4\(^+\)CD25\(^+\) Treg cells, were the source of this cytokine. Neutralization of IL-4 had no significant effect on T cell hyporesponsiveness, whereas neutralization of IL-10 and TGF-\(\beta\) restored, albeit transiently, the ability of effector T cells to respond to alloantigen. However, results from Transwell experiments suggested a lesser role for these cytokines when the Treg cells were not in direct contact with the effector cells. Although we do not know the reasons for this apparent discrepancy, we believe that it could be that the effector cells (CD25\(^-\)) need to simultaneously interact with the Treg cells (CD25\(^+\)) and the target cells (alloantigen). Alternatively, the presence of these cells in the same chamber might prevent dilution of cytokines released by the Treg cells and allow them to more readily bind and act on effector cells that are in close proximity.

Our observations are consistent with earlier reports (45, 46) suggesting that CD4\(^+\)CD25\(^+\) Treg cells may originate from naive or Ag-specific T cells. In fact, studies have shown that Ag-specific Treg cells can be generated from pre-existing Ag-nonspecific CD4\(^+\)CD25\(^+\) populations as well as from Ag-specific effector T cells (47, 48). Two major subsets of Treg cells have been reported (48). The first subset of naturally existing CD4\(^-\)CD25\(^+\) T cells do not show Ag specificity and act through TGF-\(\beta\) (49). The second subset of adaptive Treg cells, which can be induced ex vivo, develop as a consequence of activation of mature T cells by suboptimal Ag exposure and/or costimulation (50). Studies have shown that adaptive Treg cells mainly act through secreted factors such as IL-10 (50, 51). Our studies and other previous reports (16, 44–47, 52) show that distinct CD4\(^+\)CD25\(^+\) T cell populations with different expression levels of surface molecules, cytokine secretion patterns, and mode of action may be activated depending on the method used for tolerance induction.

It has been reported previously that signaling through CD28 and CTLA-4 controls two distinct forms of anergy in vitro (53). One form apparently results from TCR occupancy in the absence of CD28 costimulation and CTLA-4 signaling, and can be reversed by IL-2. The other form of anergy is associated with the failure to proliferate after activation, which occurs despite the presence of CD28 costimulatory signals and cannot be reversed by IL-2. The potential of the first form of anergy (lack of CD28 signaling) to induce a long-term tolerance has not been well established. However, this study and other earlier reports (16, 45) suggest that CTLA-4 signaling concurrent with CD28 activation and TCR engagement arrests cells from further proliferation and allows them to acquire Ag specificity and, perhaps, differentiate into Treg cells.

Most interestingly, we observed that the CD62L\(^-\) Treg cells were more potent in suppressing the effector T cell response against M12 cells relative to CD62L\(^+\) Treg cells. Moreover, the better inhibitory nature of CD62L\(^-\) Treg cells correlated with their relatively higher surface expression of CTLA-4 and production of IL-10 and TGF-\(\beta\)1 upon exposure to alloantigen. This is contrary to earlier findings (54, 55), which showed that both subsets are anergic but the CD62L\(^-\) population is more potent on a per-cell basis, and can proliferate and maintain suppressive function far better than the CD62L\(^+\) population or unseparated CD4\(^+\)CD25\(^+\) Treg cells. These studies also demonstrated that the CD62L\(^-\) subset can be expanded far more easily in culture and is more responsive to chemokine-driven migration to secondary lymphoid organs. This discrepancy could be due to the difference in strategies used for inducing tolerance. In the present study, CD4\(^+\)CD25\(^+\) Treg cells are most likely Ag specific, and thus, the CD62L\(^-\) subset might represent a memory phenotype.

Collectively, our study demonstrates that Treg cells generated by targeted CTLA-4 engagement will have therapeutic potential. The ultimate aim of our approach is to induce alloantigen-specific T cell tolerance to transplants. Allografts such as pancreatic islets can be coated with anti-CTLA-4 Ab coupled to islet or MHC-specific Ab before transplantation. Alternatively, cells that are syngeneic to the MHC Ags of the transplant can be coated with an anti-CTLA-4 Ab coupled to the alloantigen-specific Ab and injected into the recipient to induce tolerance before transplantation. Further studies are required to test the feasibility of targeted CTLA-4 engagement strategy in preventing transplant rejection.

References


