Targeted engagement of CTLA-4 prevents autoimmune thyroiditis

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Abstract

The CTLA-4-mediated signal is a critical step in the down-modulation of immune responses. The therapeutic potential of this signal to induce tissue-specific tolerance was investigated by using an anti-CTLA-4 antibody that was coupled to an antibody specific for the thyrotropin receptor. After in vivo administration, this bispecific antibody (BiAb) accumulated in the thyroid and prevented development of experimental autoimmune thyroiditis (EAT) in mice immunized with mouse thyroglobulin (mTg). Lymphocytes from BiAb-treated mice showed a significant reduction in their ability to proliferate, and to produce IL-2, IFN-γ and tumor necrosis factor (TNF)-α, in response to mTg re-stimulation compared to lymphocytes from untreated mice. Moreover, BiAb-treated mice showed suppressed anti-mTg antibody response, lymphocytic infiltration of the thyroid and follicular destruction. The BiAb targeted to the thyroid most likely facilitated engagement of CTLA-4, resulting in an increase in the number of CD4+CD25+ T cells. These regulatory T cells suppressed in vitro mTg-specific T cell responses, which were associated with an enhanced transforming growth factor (TGF)-β1 production. Neutralization of TGF-β1 increased mTg-specific in vitro proliferation of, and IL-2 production by, T cells from BiAb-treated mice. Our data suggest that engagement of CTLA-4 expressed on activated autoreactive T cells in close proximity to the thyroid can increase the number of regulatory T cells and their ability to produce TGF-β1, with a concomitant reduction in IFN-γ and TNF-α, resulting in suppression of EAT.

Introduction

The two-signal theory for T cell activation requires TCR engagement of its cognate antigen–MHC complex and CD28 binding to the B7 ligands (B7-1 and B7-2) on antigen-presenting cells (APC). Activation of T cells results in increased expression of the T cell surface molecule CTLA-4 that shares homology with CD28. Although B7-1 (CD80) and B7-2 (CD86) expressed on APC can bind to both CD28 and CTLA-4 (1–3), because of higher affinity, they preferentially bind to CTLA-4 (CD152) on activated T cells and attenuate the T cell response (3).

The importance of CTLA-4 in the regulation of T cell responses, and the induction of anergy and tolerance to alloantigens, tumors and pathogens has been clearly demonstrated (4–13). Of particular interest are studies in which mice devoid of CTLA-4 showed a severe inflammatory disorder due to un-regulated proliferation of T cells (8,9,14). The mechanism by which CTLA-4 down-modulates T cell responses is not yet clearly defined, but could involve at least two different non-exclusive mechanisms. One is that the CTLA-4 can bind and sequester B7 molecules from CD28, and prevent CD28 mediated co-stimulation (15,16). The second possibility is that CTLA-4 through its intracellular domain could actively transmit a negative signal causing down-regulation of activated T cells (17,18). Since CTLA-4 can maintain its regulatory function even in the absence of CD28 (19), it is likely that it primarily regulates T cell function via active signaling.

To date, a natural ligand that can selectively bind to CTLA-4 and induce signaling through its intracellular domain has not been identified. Mutant B7 molecules modified to specifically bind to CTLA-4 have been generated and can down-modulate T cell function (20,21), but their therapeutic potential has not been demonstrated. One option currently available is the use of an anti-CTLA-4 antibody that cross-links CTLA-4 and mimics the function of B7 molecules. Treatment with blocking antibodies to CTLA-4 allowed B7 molecules unfettered access to CD28 and caused an augmentation of immune responses.
(22–24). However, when these antibodies cross-link CTLa-4, concurrently and in close proximity to TCR antigen engagement, they can attenuate the T cell responses (25–28). Surface coating or expression of anti-CTLA-4 antibody on target cells can suppress alloreactive T cells by cross-linking CTLa-4 on their surface (27,28). Thus, exploiting this signaling pathway has emerged as a promising means of inducing antigen-specific tolerance.

To be able to deliver CTLa-4 signaling at close membrane proximity to the TCR–peptide–MHC complex, earlier we generated a bispecific antibody (BiAb) that can simultaneously bind to mouse thyrotropin receptor (TSHR) and CTLa-4, and showed that it can specifically inhibit an autoimmune Grave’s disease with mouse B cell myeloma, Sp2/0. Antibodies were purified from the spent medium using Protein L (Sigma, St Louis, MO) affinity columns, concentrated and dialyzed against PBS. These antibodies were digested with papain and the F(ab)2 fragments were purified by gel-filtration chromatography (41). F(ab)2 fragments were also prepared from purified mouse IgG1 and hamster IgG (BD PharMingen San Diego, CA) as described above.

The ectodomain of mouse TSHR was purified from TSHR-expressing 293 cells (mTicD8) as previously described (42). CTLa-4–lg was purchased from R & D Systems (Minneapolis, MN). FITC-conjugated anti-CD4 (Caltag, San Francisco, CA) and phycoerythrin (PE)-conjugated anti-CD25 (BD PharMingen) were used in flow cytometric analyses. Paired antibodies for mouse IL-2, IL-4, IFN-γ, IL-12, TNF-α (Caltag), IL-10 and TGF-β1 (BD PharMingen), and corresponding cytokine standards were used in ELISA for cytokine detection. Horseradish peroxidase (HRP)-labeled anti-mouse IgG, IgG1 and IgG2a (Caltag) were used to detect mtg-specific antibodies. Normal mouse thyroids were obtained from BiochemMed (Winchester, VA) and mouse thyroglobulin (mtg) was prepared as described earlier (43). Ovalbumin was purchased from Sigma. Neutralizing antibodies to mouse IL-4 (rat IgG1, clone 11B11) and IL-10 (rat IgG1, clone JES5-2A5) were purchased from eBioscience (San Diego, CA). Antibodies to mouse TGF-β1 (rat IgG1, clone 1D11) and normal rat IgG1 isotype control were purchased from R & D Systems.

**Preparation of BiAb**

Anti-CTLa-4–anti-TSHR BiAb was prepared by SPDP [N-succinimidyl-3-(2-pyridyl)dithio)propionate]–SMPB [succinimidyl-4-(p-maleimidophenyl)butyrate] chemical coupling as described earlier (23). The coupled antibodies were then purified from unlinked antibodies by passing through an anti-mouse IgG–Sepharose column that retained the BiAb and the mouse lg. The antibodies were eluted and repurified on an anti-hamster IgG–Sepharose affinity column. This step resulted in the retention of BiAb, which was subsequently eluted and stored. Isotype control BiAb was prepared using F(ab)2 fragments of purified mouse IgG1 and hamster IgG. For some experiments the BiAb and unlinked individual antibodies were conjugated to Texas Red by following a method described earlier (44).

**Specificity of BiAb binding**

The binding property of the BiAb was tested by FACS analysis. FRTL-5 (rat thyrocytes) cells were incubated with 1 μg of anti-TSHR or 2 μg of BiAb for 15 min on ice. Cells were washed in PBS containing 2% FBS, and further incubated with an optimal

**Methods**

**Mice**

Female CBA/J (H-2k) mice (6–8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). These mice were housed and treated in accordance with the animal care guidelines established at the Biological Resource Laboratories at the University of Illinois at Chicago.
dilution of FITC-labeled anti-mouse IgG and anti-hamster IgG respectively for 15 min. Cells were washed and analyzed using a FACS analyzer (Becton Dickinson, San Jose, CA) and CellQuest software. Isotype-matched antibodies (mouse IgG and hamster IgG) were used as control primary antibodies.

To test the stability, BiAb (2 mg/ml) and unlinked antibodies (1 mg/ml) were incubated in normal mouse serum for 7 days at 37°C. Aliquots were collected every 24 h and frozen until further use. Equal amounts of antibody-spiked serum samples were stored at −20°C as controls. A sandwich ELISA was carried out to test binding properties of these antibodies. Polystyrene 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with either recombinant mouse TSHR (200 ng/well) or CTLA-4-Ig (200 ng/well) in 0.1 M carbonate buffer (pH 9.5). After blocking with 1% BSA in PBS, mouse serum samples containing 'spiked' antibodies (diluted 1:1000) were incubated for 1 h at room temperature. Antibody binding to plates coated with CTLA-4-Ig was detected specifically with the addition of HRP-labeled anti-mouse IgG. Bound secondary antibodies were detected using TMB-H2O2 substrate (BD Pharmingen). The enzyme reaction was stopped using 1 N HCl and the plates were read at 450 nm in an ELISA plate reader.

Accumulation of BiAb in the thyroid

Eight-week-old CBA/J mice were injected with 50 µg of Texas Red-labeled anti-TSHR or anti-CTLA-4 antibody or 100 µg of BiAb i.v. in 100 µl of PBS. Control mice received only PBS. After 2 h, thyroids were harvested, and cryo-sections (5 µm thick) were prepared and observed under a fluorescence microscope (Nikon, Tokyo, Japan).

Induction of EAT and treatment with BiAb

Mice were divided into five groups with five mice in each group and injected i.v. with 100 µg BiAb/mouse, 50 µg of anti-CTLA-4 or anti-TSHR antibody/mouse or a mixture of 50 µg each of anti-CTLA-4 and anti-TSHR antibodies on days 0, 14 and 28. Tg control group received the same amount of BiAb prepared using isotype-matched control antibodies. All groups of mice were immunized s.c. with mTg (100 µg/mouse) emulsified in complete Freund’s adjuvant (CFA) on days 1 and 15. They were then sacrificed on day 45; lymph nodes, spleens and sera were collected to test for ex vivo T cell and cytokine responses. Thyroids were collected for histopathological examination. Since our preliminary experiments demonstrated that isotype control BiAb or individual antibodies had no effects on EAT, we used only the test BiAb (anti-TSHR-anti-CTLA-4 BiAb) for the rest of the experiments.

Treatment after disease induction

In another set of experiments, five groups of mice (five to seven mice in each group) were immunized with mTg and CFA as described above, but on days 0 and 14. Un-immunized and Tg control groups were injected i.v. with PBS on days 3, 15, 28 and 35. Mice receiving BiAb were divided into three different groups. Mice belonging to BiAb group 1 were injected with 100 µg BiAb on days 3 and 15 and PBS on days 28 and 35. Mice in BiAb group 2 were injected with PBS on days 3 and 35, but BiAb on days 15 and 28. Mice in BiAb group 3 received PBS on days 3 and 15, but BiAb on days 28 and 35. Mice were bled on day 45 to obtain sera and were then sacrificed. Lymph nodes and spleens were collected to test for ex vivo T cell proliferation and cytokine responses. In some experiments mice were immunized s.c. with ovalbumin (25 µg/mouse) emulsified in CFA, along with mTg, and treated with BiAb as described above.

T cell proliferation assay

Mouse splenocytes (5 × 10^5 cells/well) or lymph node (2 × 10^5 cells/well) cells were plated in 96-well flat-bottom tissue culture plates in triplicate with RPMI 1640 containing 1% normal mouse serum at a final volume of 0.25 ml/well. The mTg was added at a concentration of 20 µg/ml. Stimulation with concanavalin A (2 µg/ml) served as a positive control. Plates were incubated for 48 h at 37°C in a CO2 incubator, and the cells were then pulsed with 1 µCi of [3H]thymidine/well and incubated for an additional 18 h. Cells were harvested onto glass fiber filter papers using a 96-well cell harvester (Tomtec, Hamden, CT) and radioactivity was quantitated using a micro-β counter (Perkin-Elmer Wallac, Gaithersburg, MD).

Cytokine production

Cytokine production by spleen and lymph node cells, collected from mice that were sacrificed upon termination of the experiment on day 45, was tested with or without stimulation with mTg. Cells (3 × 10^5/well; 24-well plate) were maintained in the presence of mTg (20 µg/ml) in 1.5 ml RPMI 1640 medium supplemented with 2% normal mouse serum for 36 h in a CO2 incubator. Cell-free culture supernatants were collected from both un-stimulated and stimulated wells after 36 h by centrifugation. Cytokine levels in culture supernatants were assayed by ELISA using IL-2, IL-4, IFN-γ and TGF-β1 paired antibodies following the manufacturer’s instructions. The enzyme reaction was detected using TMB-H2O2 substrate (BD Pharmingen) for 5–10 min and the OD was read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA). Cell-free culture supernatants collected from T cell proliferation assay plates after 36 h of incubation were also tested for cytokines.

Cytokine neutralization assay

Cells were cultured in the presence of mTg as described above for T cell proliferation. To these cultures, varying concentrations of neutralizing anti-mouse IL-10 (10–5000 ng/ml) and/or anti-mouse TGF-β1 (10–2000 ng/ml) or isotype-matched control antibodies were added and incubated for 48 h. Spent medium was collected for cytokine analysis, and cells were pulsed with [3H]thymidine, harvested and counted as described above.

Anti-mTg antibodies

Serum levels of mTg-specific IgG, IgG1 and IgG2a antibodies were determined by ELISA. Plates (96-well; Nunc) were coated with 0.5 µg/well (100 µl) of mTg in 0.1 M carbonate/bicarbonate buffer, pH 9.6, overnight at 4°C. Wells were blocked with 300 µl PBS containing 1% BSA for 1 h at room temperature. Two-fold serial dilutions of sera were prepared, added to the wells in triplicate and incubated for 1 h at room temperature. Subsequent to washing, plates were further incubated with 100 µl/well of PBS/T containing an optimal concentration of HRP-labeled anti-mouse IgG, IgG1 or IgG2a.
for 1 h at room temperature. The enzyme reaction was developed as described above.

**Histological examination for thyroiditis**

Thyroids were fixed in formalin, embedded in paraffin, sectioned, stained with hematoxylin & eosin and subjected to microscopic examination. The disease severity was graded from 0 to 4+ according to standard pathological criteria (41).

**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 (PharMingen) on ice for 30 min. Cells were stained with FITC-labeled anti-mouse CD4 and PE-labeled anti-mouse CD25 antibodies or isotype-matched control antibodies on ice for 15 min, washed and analyzed in a FACS analyzer (Becton Dickinson, San Jose, CA). At least 10,000 cells were analyzed for each sample and all experiments were repeated 3 times to demonstrate reproducibility.

**Isolation of CD4+CD25+ T cells**

CD4+CD25+ cells were isolated using antibodies conjugated to magnetic beads and magnetic separation column by following the manufacturer’s directions. Pooled mouse spleen and lymph node cells were incubated with anti-CD16/CD32 for 15 min on ice to block Fc receptors and subsequently they were incubated with PE-labeled anti-mouse CD25 antibody for 30 min on ice. Cells were washed and incubated with magnetic bead conjugated anti-PE antibody for 15 min, washed and separated using an AutoMACS (Miltenyi Biotec, Auburn, CA). The unbound CD25+ fraction was incubated with anti-mouse CD4 antibodies coupled to magnetic beads for 30 min and fractionated as described above. Isolated cells were subjected to re-purification, washed and stained with FITC-labeled anti-CD4 and PE-labeled anti-CD25 antibodies, and tested for purity using flow cytometry. Spleen cells were depleted of CD3+ cells by negative selection using magnetic beads coated with anti-mouse CD3 antibodies.

**Co-cultivation of lymphocytes with CD4+CD25+ cells**

CD4+CD25+ cells from untreated mice were mixed with CD4+CD25+ cells from BiAb-treated mice and vice versa at a ratio of 10:1. These mixtures were used in a T cell proliferation assay that was carried out, either in the presence or absence of mTg, as described above. Mitomycin C-treated CD3+ spleen cells from naive mice (2 × 10^5 cells/well) were used as APC in all assays.

**Statistical analysis**

Mean, geometric mean titer, SD and statistical significance were calculated using SPSS application (SPSS, Chicago, IL). Statistical significance was determined using the non-parametric Wilcoxon signed test. In most cases, values for each treated and immunized group were compared with those of the untreated but immunized group. Differences in the percentage of fluorescence-positive cells between untreated and each of the treated groups were tested using the non-parametric sign test. p < 0.05 was considered as significant.

**Results**

**The BiAb binds thyrocytes and is stable in serum**

The ability of the BiAb to bind to native TSHR on thyrocytes was confirmed by FACS analysis. The BiAb bound to the FRTL-5 cells through its anti-TSHR moiety and the binding was comparable to that noted with anti-TSHR antibody (Fig. 1a). To test the stability, we incubated the BiAb with normal mouse serum at 37°C for 1 week and then tested for its binding to purified recombinant mouse CTLA-4-Ig in an ELISA. As seen in Fig. 1(b), the BiAb retained its ability to bind to CTLA-4 as well. The degree of BiAb binding to CTLA-4 was comparable to that of the anti-CTLA-4, suggesting that there was no significant loss of binding even after 1 week of incubation with normal mouse serum at 37°C. This showed that the chemical coupling did not significantly affect the binding efficiency of either the anti-TSHR (Fig 1a) or the anti-CTLA-4 (Fig. 1b) antibody components of the BiAb.

**Accumulation of BiAb in the thyroid**

As a first step in testing the effectiveness of the BiAb in immunomodulation, we tested for its ability to accumulate in

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Fig. 1. (a) Reactivity of BiAb with FRTL-5 rat thyrocytes. Cells were stained with anti-TSHR antibody and BiAb followed by FITC-labeled anti-mouse IgG or anti-hamster IgG respectively. Cells stained with isotype-matched antibodies were used as controls. (b) Stability of BiAb. Normal mouse serum was spiked with BiAb, anti-TSHR or anti-CTLA-4 antibodies and incubated at 37°C for 7 days. Aliquots were collected every 24 h, ELISA was carried out using TSHR- or CTLA-4-Ig-coated plates and the results were compared to that of the antibody-spiked serum stored at −20°C (day 0). Each value represents the mean of triplicate values. This assay was performed 3 times with similar results.
the thyroid, relative to anti-TSHR antibody. Mice were injected i.v. with Texas Red-labeled BiAb as well as individual antibodies. Mice were sacrificed after 2 h and thyroids collected. Cryosections were prepared and observed under a fluorescence microscope. As seen in Fig. 2, the fluorescence had accumulated on the surface of thyroid follicles within 2 h, indicating that both BiAb and anti-TSHR antibody can accumulate in the thyroid. In contrast, mice injected with Texas Red-labeled anti-CTLA-4 antibody showed no significant accumulation in the thyroid. We also found that 111In-labeled BiAb and anti-TSHR antibody, but not anti-CTLA-4 antibody, primarily tracks to the thyroid. The levels of these two antibodies in the thyroid were ~3–4 times their corresponding levels in blood and other tissues (e.g. spleen, heart, lung, etc.; results not shown).

Engagement of CTLA-4 by thyroid-targeted antibody can induce down-modulation of the immune response against mTg

In order to test the therapeutic potential of the BiAb we treated mTg-immunized animals with the BiAb. Initially, a series of experiments were carried out using the BiAb to optimize the effective dose required to down-regulate the immune response to mTg. We then immunized mice with mTg and treated them with an optimal dose of BiAb, isotype control BiAb or other control antibodies at different time points. Mice were sacrificed on day 45, and tested for T cell proliferation and mTg-specific antibody response. As shown in Fig. 3, there was no significant effect on anti-mTg response when mice were treated with the isotype control BiAb, the individual antibodies or a mixture of these antibodies. However, BiAb-treated mice showed significantly lower T cell proliferation and IL-2 production in response to mTg stimulation in vitro. Interestingly, treatment with BiAb had a profound suppressive effect on IFN-γ and IgG2a antibody responses, and minimal effect on IL-4 production and IgG1 antibody responses, indicating a preferential down-modulation of the Th1-type immune response. Since there was no significant effect on the anti-mTg response when mice were treated with isotype control BiAb or other control antibodies, we used only the test BiAb in the rest of the experiments.

Targeted immune down-modulation is antigen specific

The antigen specificity of BiAb-mediated antigen suppression was tested by evaluating immune responses to ovalbumin injected along with mTg. As expected, both spleen (not shown) and

![Fig. 2. In vivo sequestration of BiAb in thyroid tissue. Cryosections of thyroids obtained 2 h after injecting the indicated antibody were fixed onto glass slides and examined under a fluorescence microscope at a magnification of x40. This experiment was repeated with similar results.](image-url)
draining lymph node cells from BiAb-treated mice showed significantly lower T cell responses to mTg compared to untreated mice. However, the proliferative response to ovalbumin by cells from the same mice remained similar to that of control mice receiving only ovalbumin (Fig. 4). Antibody responses to mTg and ovalbumin mirrored the proliferation responses.

CTLA-4 engagement in the thyroid can down-regulate EAT at early and late stages of disease development

To test the potential of this BiAb in treating EAT, we immunized mice with mTg and CFA as described above, and treated them with BiAb at different stages of disease development. These mice were sacrificed on day 45, and tested for lymphocytic infiltration into the thyroid by histochemical staining and the immune response to mTg ex vivo. As seen in Fig. 5 and in Table 1, thyroids from mice belonging to BiAb-treated groups 1 and 2 showed little or no lymphocytic infiltration and follicular damage (grade 0 to 1+) and mice belonging to BiAb-treated group 3 showed a disease severity from 0 to 2+. Untreated mice, receiving mTg, showed infiltration and thyroid disease severity of 2+ to 3+. These results indicated that BiAb treatment, even when given at later stages of the disease, can either suppress the severity of thyroiditis or in some cases prevent disease development.

BiAb-treated mice showed reduced T cell proliferation (Fig. 6a) and anti-mTg antibody responses (Fig. 6b), irrespective of the time of treatment, but the effect was more profound in mice that were treated at early stages of disease development. Relative to mice from the mTg control group (mean titer $1.2 \times 10^5$), BiAb-treated mice produced a significantly reduced amount of IgG2a antibody (mean titer $1.9-2.6 \times 10^4$). In contrast, the reduction in IgG1 response, although significant, was less profound in treated mice. In response to mTg stimulation, lymph node and spleen cells (not shown) from all three groups of BiAb-treated mice, relative to untreated mice, produced significantly lower amounts of Th1 cytokines, IL-2 ($p < 0.001$), IFN-γ ($p < 0.001$) and TNF-α ($p < 0.005$); but IL-4 (Th2 cytokine) levels were comparable to the levels seen in the control group (Fig. 4b).

CTLA-4 engagement induces expansion of CD4+CD25+ T cells

To see differences in T cell phenotypes, we tested spleen and lymph node cells from treated and untreated mice for different cell-surface markers by FACS analysis. Most interestingly, we noted an increase in the number of CD4+CD25+ T cells (not shown) from all three groups of BiAb-treated mice, compared to untreated mice, produced significantly lower amounts of Th1 cytokines, IL-2 ($p < 0.001$), IFN-γ ($p < 0.001$) and TNF-α ($p < 0.005$); but IL-4 (Th2 cytokine) levels were comparable to the levels seen in the control group (Fig. 4b).
draining lymph nodes (not shown), suggesting that these cells might have an important regulatory function in BiAb-treated mice.

**TGF-β1 and IL-10 may have a significant role in the down-regulation of EAT**

To see if there is an association between the BiAb-induced immunomodulation and immunoregulatory cytokines, we tested for the production of certain cytokines by spleen and lymph node cells upon mTg re-stimulation. As shown in Fig. 8, upon mTg stimulation, spleen cells from BiAb-treated mice produced higher levels of TGF-β1 (p < 0.001) compared to cells from untreated mice. The levels of IL-10 produced by cells from BiAb-treated mice were not different from the levels produced by cells from untreated mice. These results suggested that enhanced levels of TGF-β1 coupled with lower levels of IL-2, IFN-γ and TNF-α might be responsible for decreased T cell responses and disease severity in BiAb-treated mice. Lymph node cells from BiAb-treated and untreated animals also demonstrated the same trends in TGF-β1 and IL-10 responses (results not shown).

To test if TGF-β1, IL-10 and IL-4 could suppress responses against mTg, we carried out T cell proliferation and cytokine production assays in the presence or absence of neutralizing antibodies against TGF-β1, IL-10 and IL-4. As shown in Fig. 9, there was some increase in the proliferation and IL-2 production in the presence of saturating concentrations of anti-IL-10 antibody, but the increases in both proliferation and IL-2 production were more profound in the presence of anti-TGF-β1 alone, or a combination of anti-TGF-β1 and IL-10, compared to cultures receiving isotype-matched control antibodies. However, there was no additive effect when both anti-IL-10 and anti-TGF-β1 were used together. Anti-IL-4 antibody showed no effect on the T cell proliferative response to mTg (results not shown). These data showed that TGF-β1, and to a lesser extent IL-10, suppressed Tg-specific T cell responses in BiAb-treated mice.

**Role of CD4+CD25+ T cells in BiAb-induced suppression of mTg-specific T cells**

CD4+ cells from BiAb-treated mice showed a marked reduction in the proliferative response to mTg relative to cells from the untreated group. Since we had seen an increase in the number of CD4+CD25+ T cells in BiAb-treated mice, we tested to see whether these cells could play a role in down-regulating mTg-specific T cell responses. CD4+CD25+-depleted CD4+ cells from BiAb-treated mice, but not untreated mice, showed an enhanced proliferative response to mTg (Fig. 10a). When
we co-cultured CD4+CD25+ cells from mice treated with BiAb and CD4+CD25− spleen cells from untreated mice, we noted that the response to mTg was significantly suppressed (Fig. 10b). However, addition of CD4+CD25+ T cells from untreated mice to CD4+CD25− cells from BiAb-treated mice failed to suppress the T cell response to mTg (Fig. 10b).

**Discussion**

In previous work, we generated a BiAb that can simultaneously bind to both the TSHR and cross-link CTLA-4, and used it to down-modulate the immune response, both *in vitro* and *in vivo*, against allogeneic cells expressing TSHR (27). In the present study, we demonstrate that this BiAb can prevent EAT by suppressing Tg-specific T cell responses. Further, we

**Table 1. Modulation of thyroiditis in CBA/j mice using BiAb**

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<thead>
<tr>
<th>Group*</th>
<th>Number of mice with different degrees of EATb</th>
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<tr>
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<td>0</td>
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<tr>
<td>Control</td>
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<td>BiAb group 2</td>
<td>3</td>
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*aMice were treated with BiAb as described in Methods.

*bThyroids collected upon termination of experiments on day 45 were evaluated for pathology. 0–4+ indicates relative degree of lymphocyte infiltration and follicular damage.
show that BiAb-treated mice had a higher frequency of CD4+CD25+ regulatory T cells, and lymphocytes from these mice upon re-stimulation produced significantly higher levels of TGF-β1, and lower levels of IFN-γ and TNF-α. These results indicated that an increase in the frequency of CD4+CD25+ cells and a shift in cytokine balance might be responsible for disease suppression.

Earlier studies demonstrated that either the whole antibody or the Fab2 fragment with specificity for CTLA-4 can elicit CTLA-4-mediated signaling and down-regulate T cell function (25-28). Recently, it has been shown that allogeneic cells either coated with a bivalent anti-CTLA-4 antibody (27) or genetically modified to express single-chain anti-CTLA-4 antibody (28) can survive longer in vivo. This enhanced...
survival is most likely due to the ability of the anti-CTLA-4 to cross-link CTLA-4 expressed on alloreactive T cells in vivo and inhibit T cell function. However, it may be more difficult, if not impossible, to genetically modify terminally differentiated tissues to express anti-CTLA-4 antibody prior to their use for transplantation and thus might limit the potential use of this approach for human treatment. In contrast, antibodies that can cross-link CTLA-4 on activated autoreactive T cells in close proximity to the autoimmune target tissue antigen might not only down-regulate their function, but could also provide tissue/antigen specificity.

We found that T cell activation against mTg was down-regulated significantly only when mice were treated with BiAb. Administration of an identical dose of either anti-TSHR or anti-CTLA-4 alone, or as a mixture, or an isotype control BiAb, had no effect on T cell responses against mTg. More importantly, the immune response to ovalbumin was unaffected even though the BiAb-treated mice received both mTg and ovalbumin simultaneously. Based on these observations and the ability of BiAb to accumulate in the thyroid, we speculate that engagement of CTLA-4 likely occurred in the thyroid where mTg-specific T cells, activated in the periphery by inoculated mTg, could encounter the BiAb. In this context, it is interesting to note that thyrocytes can act as APC by expressing high levels of MHC class II molecules (46,47) and thus allow direct recognition of thyrocytes by specific T cells. Another plausible mechanism is that the thyrocyte-bound BiAb could cross-link CTLA-4 on T cells while it engages antigenic peptide on thyroid-resident conventional APC. Collectively, our results support two previously reported observations, i.e. (i) transient blockade of CTLA-4 does not induce hyper-activation of T cells (48,49) and (ii) cross-linking CTLA-4 at close proximity to antigen presentation can inhibit T cell responses (25).

As expected, the suppression of the mTg-specific response was more profound in mice that received the treatment earlier. However, the effect of treatment with BiAb was evident even in mice that received treatment starting as late as day 28.

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**Fig. 7.** Increase in CD4+CD25+ T cells in BiAb-treated mice: mice were treated as described in Methods. On day 45, spleen cells were collected, stained with FITC-conjugated mouse CD4 antibody and PE-labeled anti-mouse CD25 antibody, and analyzed in a FACS analyzer using CellQuest software. 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 in each group is indicated in the main rectangle.

**Fig. 8.** TGF-β1 and IL-10 responses in BiAb-treated mice. Mice were treated as described in Methods. On day 45 post-immunization, spleen cells were collected and stimulated with mTg for 36 h at 37°C. Spent medium was collected and tested for TGF-β1 and IL-10 by ELISA. Results are expressed as mean ± SD of triplicate values from five mice tested individually. Statistical significance (p value) was calculated by comparing each value with the corresponding values of the Tg control group. An asterisk represents a statistically significant value.
Although mTg-specific T cells were persistently exposed to endogenous thyroid antigens in both untreated and treated mice, CD4+CD25+ T cell numbers and TGF-β1 levels were increased only in BiAb-treated mice irrespective of the time of administration of the BiAb. This is consistent with earlier reports of a persistent increase in the number of CD4+CD25+ T cells upon induction of oral tolerance in mice against ovalbumin (50,51). Furthermore, CD4+CD25+ cells from only BiAb-treated mice could suppress the anti-mTg response of CD4+CD25- T cells from untreated mTg-primed control mice. Our results suggest that CTLA-4 engagement upon antigen presentation, either directly or through cytokine production, may play an important role in selective expansion of antigen-specific CD4+CD25+ regulatory T cells. In an alloresponse model, we have observed a similar increase in the percentage of CD4+CD25+ T cells in BiAb-treated tolerant mice. These cells can suppress the proliferation of CD4+CD25- T cells in vitro more potently than CD4+CD25+ T cells isolated from untreated mice (unpublished data). Although a critical role for dendritic cells in the induction of antigen-specific regulatory T cells has been proposed (52), at this time the mechanism through which BiAb can induce regulatory T cells is not apparent.

Regulatory T cell-mediated tolerance induction could be mediated through suppression of inflammatory cytokines, like IFN-γ and TNF-α, with a concomitant increase in the production of suppressor cytokines like TGF-β1, IL-10 and IL-4. Earlier studies have shown that persistent high levels of IL-4 and IL-10 can prevent thyroid apoptosis through enhanced expression of Bcl-xl and c-flip by thyrocytes (53–55). However, levels of these cytokines were unaffected in BiAb-treated mice, relative to controls, indicating that they might not

Fig. 9. Role of IL-10 and TGF-β1 in BiAb-induced down-regulation of the T cell response. Proliferative (a) and IL-2 (b) responses of T cells from BiAb-treated mice against mTg in the presence of saturating concentrations of anti-mouse IL-10 or TGF-β1 antibodies added individually or in combination. Each value represents the mean ± SD of three separate experiments carried out in triplicate. Statistical significance (p value) was calculated by comparing each value with the corresponding values of isotype antibody control. An asterisk represents a statistically significant value.

Fig. 10. Role of CD25+ cells from BiAb-treated mice in suppressing the T cell response. Control and BiAb-pretreated mice were immunized with mTg. CD4+CD25+ and CD4+CD25- T cells from both treated and untreated mice were purified from pooled spleen and lymph node cells using magnetic separation methods. Spleen cells from naive mice depleted of CD3+ cells using anti-mouse CD3 antibody-labeled magnetic beads were used as APC. (a) Proliferation in response to mTg was measured either individually for CD4+CD25+ and CD4+CD25- cells from both treated and untreated mTg-immunized mice or using a mixture of the two populations (CD4+CD25+ and CD4+CD25-) from the same group of mice. (b) CD4+CD25+ T cells from BiAb-treated mTg-primed mice suppressed the response of CD4+CD25- T cells from mTg-primed, but untreated mice. However, the reciprocal combination had no suppressive effect.
be responsible for the protection. In contrast, other studies have suggested that pro-inflammatory cytokines, like IFN-γ and TNF-α produced in the thyroid, can play a critical role in the induction of thyrocyte apoptosis (56-58). In this study, we observed an increase only in the production of TGF-β1, without significant changes in either IL-4 or IL-10 levels, in BiAb-treated mice. Neutralization of TGF-β1 significantly enhanced mTg-specific T cell responses and suggested that this cytokine might have an important suppressive role. TGF-β1 is a major immunoregulatory cytokine (59-61), and its effects depend on the stage of differentiation of cells and presence of other cytokines (62-63). For example, TGF-β1 alone can inhibit proliferation and cytokine secretion by resting CD4+ T cells, but its inhibitory effect on activated T cells is seen only in the presence of IL-10 (64). This is likely due to IL-10-mediated up-regulation of TGF-β1 receptors on activated T cells that results in enhanced TGF-β1 binding. However, in the present study, there was no significant increase in IL-10 levels in BiAb-treated mice relative to untreated animals. This would suggest that although higher levels of IL-10 might be required to overcome the effects of proinflammatory cytokines, levels of IL-10 found in BiAb-treated mice might be sufficient to promote the suppressive effects of TGF-β1 in the presence of reduced levels of IFN-γ and TNF-α found in these mice.

In summary, our results show that engagement of CTLA-4 on activated T cells in close proximity to the thyroid can induce tissue or antigen-specific immune tolerance even when the BiAb is given at different times after mTg inoculation. The disease suppression was associated with increased numbers of CD4+CD25+ T cells and an increased production of TGF-β1 concomitant with suppressed levels of IFN-γ and TNF-α. Further studies are needed to fully delineate the phenotype and antigen specificity of these CD4+CD25+ T cells to determine the mechanism of tolerance induction and disease suppression upon tissue-targeted CTLA-4 engagement.

Acknowledgements

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>BiAb</td>
<td>bispecific antibody</td>
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<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
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<tr>
<td>EAT</td>
<td>experimental autoimmune thyroiditis</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>transforming growth factor</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>TSHR</td>
<td>thyrotropin receptor</td>
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References


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