Mice Transgenic for a Soluble Form of Murine CTLA-4 Show Enhanced Expansion of Antigen-specific CD4⁺ T Cells and Defective Antibody Production In Vivo

By Franca Ronchese, Barbara Hausmann, Sabine Hubele, and Peter Lane

From the Basel Institute for Immunology, 4005 Basel, Switzerland

Summary

CD4⁺ T cell responses were analyzed in transgenic mice expressing a soluble form of murine CTLA-4, mCTLA4-H γ 1, which blocks the interaction of the T cell activation molecules CD28 and CTLA-4 with their costimulatory ligands. Consistent with previous reports (Linsley, P. S., P. M. Wallace, J. Johnson, M. G. Gibson, J. L. Greene, J. A. Ledbetter, C. Singh, and M. A. Tepper. 1992. Science (Wash. DC). 257:792), T cell-dependent antibody production was profoundly inhibited in mCTLA4-H γ 1 transgenic mice immunized with a protein antigen. Surprisingly, however, transgenic mice could generate quantitatively and qualitatively normal primary T cell responses, as measured by limiting dilution assays and lymphokine production. In addition, in vivo expansion of antigen-specific T cells after secondary or tertiary immunization was enhanced in mCTLA4-H γ 1 transgenics as compared with normal mice. Although unable to deliver cognate help to B cells in vivo, T cells from mCTLA4-H γ 1 transgenic mice were not anergic as they could help B cells to produce specific antibodies when adoptively transferred into nude hosts. Taken together, these data suggest that the engagement of CD28 and/or CTLA-4 may not be required for the induction of T cell responses, as is currently understood, but rather for the expression of T cell effector function such as the delivery of T cell help to B cells.

S timulation of T cells through the T cell receptor is by itself an insufficient signal to induce T cell proliferation. When activated in this manner, T cells enlarge and express IL-2 receptors but secrete diminished amounts of IL-2 and other lymphokines, fail to proliferate, and become unresponsive to further antigenic stimulation. To achieve full activation, T cells also require a costimulatory signal, usually provided by the APC (reviewed in 1). One accessory molecule thought to provide a costimulatory signal to T cells is B7/BB1 (2-5), which is expressed on dendritic cells and on activated B cells, T cells, and macrophages (6-10). B7/BB1 is the ligand for two T cell surface molecules, CD28 and CTLA-4. CD28 is expressed on all murine T cells and binds B7/BB1 with intermediate affinity (11, 12), while CTLA-4 is detectably expressed only after T cells have been activated and binds B7/BB1 with high affinity (13, 14). Additional ligands for CD28 and CTLA-4, which are distinct from B7/BB1 and appear to be predominantly expressed on B cells, have recently been described (15-18). Costimulation through CD28 provides a potent accessory signal to T cells that increases IL-2 production by stabilizing lymphokine mRNA (19, 20).

In vivo studies have shown that blocking of CD28-dependent signaling with a soluble form of CTLA-4 can inhibit T cell-dependent antibody production (21), allow long-term survival of xenogeneic grafts (22), and delay the rejection of allogeneic grafts (23). Although these studies are consistent with the predicted role of CD28 costimulation in enhancing T cell responses and preventing the induction of anergy, they provide little or no direct evidence that soluble CTLA-4 indeed interferes with the generation of T cell responses in vivo, or about the precise stage of the response that is affected by the treatment. To investigate this question, transgenic mice were generated that secrete a soluble form of CTLA-4, mouse CTLA4-human $\gamma 1$ (mCTLA4-H $\gamma 1$)¹, which can block the interaction of CD28 and/or CTLA-4 with their costimulatory ligands (24). These mCTLA4-H $\gamma 1$ transgenic mice were used to examine the role of CD28-dependent costimulation in the induction of T cell and B cell immune responses in vivo.

Materials and Methods

Mice. mCTLA4-H γ 1 transgenic mice were maintained at the Basel Institute for Immunology by breeding transgenic males to C57BL/6 females (Iffa-Credo, L'Arbresle, France). Progeny were

¹ Abbreviations used in this paper: mCTLA4-Hy1, mouse CTLA4-humany1.

tested at ~ 4 wk of age by determining the presence in the serum of the transgenic product. C57BL/6 *nu/nu* were obtained from Bomholtgård (Ry, Denmark), BALB/c *nu/nu* were from Iffa-Credo.

Antigens and Immunizations. KLH was obtained from Calbiochem-Behring Corp. (La Jolla, CA); coupling to DNP was performed as previously described (25). Mice were primed i.p. with 100 μ g. DNP-KLH in alum adjuvant on day 0 and boosted i.p. with 100 μ g soluble DNP-KLH at 7–14-d intervals. Serum samples were taken by tail bleeding and analyzed by ELISA.

Preparation of Dendritic Cells. A modification of the original method of Steinman et al. (26) was used. Briefly, spleen cell suspensions were prepared by digestion with a cocktail of 2.5 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) and 0.1% DNAse (Sigma Chemical Co., St. Louis, MO) in serum-free medium at 37°C for 2 \times 40 min; low-density cells were isolated by centrifugation over a 60% Percoll gradient ($\rho = 1.076$) and the floating fraction was collected. Dendritic cells were enriched by differential adherence by incubating cells in medium containing 1% normal mouse serum (NMS) on 100-mm tissue culture plates (Falcon Labware, Oxnard, CA) for 2 h at 37°C, removing the nonadherent fraction, and culturing the remaining cells overnight in medium containing 0.5% NMS and 50 μ g/ml KLH. On the next day floating cells were collected and depleted of contaminating T cells and B cells by incubation with 10 μ g/ml each of anti-Thy-1 (T24; 27) and anti-B220 (6B2; 28) mAb followed by sheep anti-rat Dynabeads (Dynal; Oslo, Norway) and magnetic adherence. The resulting population was >70-80% dendritic cells as judged by morphology and FACS® staining (Becton Dickinson & Co., Mountain View, CA) with the dendritic cell-specific mAb N418 (29).

Tissue Culture Media. All cultures were in Iscove's modified DMEM additioned with 10% FCS (Boehringer Mannheim, Mannheim, Germany), 2 mM glutamine (Gibco, Paisley, Scotland) 1% penicillin-streptomycin (Gibco), and 5×10^{-5} M 2-ME (Sigma Chemical Co.). Human rIL-2 was obtained through the courtesy of Dr. F. Sinigaglia (Hoffmann-La Roche, Basel, Switzerland).

Inhibition of In Vitro Alloresponses by mCTLA4-H γ 1. Spleen and lymph node cell suspensions from donor mice were pooled and depleted of B cells and accessory cells by incubation with sheep anti-mouse Dynabeads (Dynal), and magnetic adherence, followed by 1-2 h plastic adherence in medium containing 5% FCS. Collected cells were cultured in triplicate at 2.5 × 10⁵/well in 96-well flat bottom microtiter plates (Costar Corp., Cambridge, MA) in 200 µl total volume, with 2.5 × 10⁵ irradiated allogeneic or syngeneic nu/nu spleen cells that had been preincubated for 45 min in graded concentrations of mCTLA4-H γ 1, or mCD40-H γ 1 (30) as an isotype-matched control. After 72 h of culture cells were pulsed overnight with 1 µCi [³H]TdR/well.

Anti-CD28 Costimulation Assays. Peripheral T cells from donor mice were depleted of B cells and accessory cells as described above, and cultured in triplicate at 3×10^4 cells/well in 96-well flat bottom microtiter plates coated with graded amounts of anti-CD3 (31), in the presence or absence of soluble anti-CD28 culture supernatant (19) at 10% final concentration. After 48 h of culture cells were pulsed overnight with 1 μ Ci [³H]TdR/well.

Limiting Dilutions. 7-10 d after the last immunization, mice were killed, spleen and draining lymph nodes were pooled and depleted of B cells, and other MHC class II-expressing cells by incubation with Y-3P monoclonal antibody (32) followed by sheep anti-mouse conjugated Dynabeads (Dynal) and magnetic adherence. The numbers of KLH-specific, IL-2 or IL-3-producing T cells were determined in a limiting dilution assay as described (25) by plating different dilutions of T cells on 3×10^4 irradiated C57BL/6 nu/nu spleen cells, with or without 50 μ g/ml KLH, in complete medium in 96-well round bottom microtiter plates (Costar Corp.). IL-2 or IL-3 production was assayed by cocultivation with the appropriate indicator cell lines.

IFN- γ Assays. B-depleted spleen and lymph node cells from control and transgenic littermates were cultured at 2 × 10⁵/well in 96-well flat bottom microplates with 4 × 10⁵ irradiated C57BL/6 *nu/nu* spleen cells and various concentrations of KLH; the total volume was 200 μ l. Supernatants were harvested after 72 h of culture and tested in a sandwich ELISA using mouse recombinant IFN- γ (Genzyme, Cambridge, MA) as a standard. Capture antibody was AN18.17.24 (33), detecting antibody was XGM-D6biotin (34); the reaction was developed with streptavidin-horseradish peroxidase (Southern Biotechnology, Birmingham, AL).

IL4 Assays. Cells from transgenic and control mice were cultured as detailed for the IFN- γ assays but in the presence of 10 U/ml of recombinant human IL-2 (Roche Laboratories, Nutley, NJ); after 72 h of incubation plates were irradiated, 100 μ l supernatant were removed and replaced with 100 μ l medium containing 10⁴ IL-4-dependent CT-4S cells (35). Plates were incubated for a further 24 h and pulsed with 1 μ Ci [³H]TdR/well for 6 h. Proliferation of CT-4S was completely inhibited by an IL-4-specific monoclonal antibody (PharMingen, San Diego, CA).

Adoptive Transfer Experiments. C57BL/6 nu/nu mice were injected i.v. with 10⁶ purified T cells from sex-matched control or transgenic mice that had been immunized three times with DNP-KLH; 24 h later reconstituted or nonreconstituted nu/nu mice were immunized i.p. with 100 μ g DNP-KLH in alum, bled 9 d after immunization, and tested for DNP-specific serum IgG by ELISA.

ELISA. Plates coated with 5 μ g/well DNP-OVA were incubated with dilutions of test sera for 2 h at room temperature, washed, and incubated for a further 2 h with peroxidase-conjugated sheep anti-Ig or Sheep anti-IgG (Southern Biotechnology) as indicated. The reaction was developed by adding ABTS[®] (Boehringer Mannheim) and read on an ELISA reader (Multiskan MCC/340; Titerteck, Elfab Oy, Finland).

Results

T Cells from mCTLA4-Hy1 Transgenic Mice Are Sensitive to Anti-CD28 Costimulation In Vitro. Although immature T cells express CD28 (11), T cell development in the thymus is reportedly independent of CD28-dependent costimulation (36, 37). This would suggest that T cells in mCTLA4-H γ 1 transgenic mice should not be developmentally selected to be independent of CD28 signaling. Nonetheless, in order to test whether this could be the case, peripheral T cells from mCTLA4-H γ 1 transgenic and control mice were tested for the ability to mount in vitro primary alloresponses when in the presence of soluble CTLA-4 (14, 6). As shown in Fig. 1 A, addition of mCTLA4-H γ 1 inhibited the in vitro proliferative responses to alloantigen of both mCTLA4-H γ 1 transgenic and control T cells; the inhibition was dose dependent and followed similar dose responses in both groups of mice. A control construct also bearing the Fc portion of human $\gamma 1$ (mCD40-H $\gamma 1$) had no effect on the response.

To further confirm that T cells from mCTLA4-H γ 1 transgenic mice bear a functional CD28 molecule, T cells from mCTLA4-H γ 1 transgenic and control mice were also tested in an in vitro costimulation assay (19). Purified T cells from either group were stimulated with graded concentrations of



Figure 1. CD28-mediated signaling is functional in T cells from mCTLA4-H γ 1 transgenic mice. (A) Inhibition of primary MLR by mCTLA4-Hy1. Open symbols, control mice; closed symbols, transgenic mice. Proliferation was measured in the presence of the indicated amounts of mCTLA4-H γ 1 (circles) or of the isotype-matched control mCD40-H γ 1 (triangles); 100% response was 45,900 cpm and 52,500 cpm for control and transgenic mice, respectively. (B) Costimulation of anti-CD3 proliferative responses by anti-CD28. Open symbols, control mice; closed symbols, transgenic mice. Anti-CD3-induced proliferation was tested in the presence (triangles) or absence (circles) of anti-CD28. Values represent the mean ± SE of triplicate cultures. Peripheral T cells from control or transgenic mice were isolated and cultured as detailed in Material and Methods: (A)in a primary MLR, on irradiated allogeneic BALB/c nu/nu spleen cells in the presence of the indicated amounts of mCTLA4-Hy1 or mCD40- $H\gamma_1$; (B) on the indicated amounts of plate-bound anti-CD3, in the presence or absence of 10% supernatant from the CD28-specific hybridoma 37-51.

plate-bound anti-CD3 in the presence or absence of anti-CD28, and their proliferative response measured. T cells from either group of mice showed enhanced proliferation in the presence of anti-CD28 (Fig. 1 B), suggesting that the CD28 molecule is functional in both groups of mice.

Therefore, T cells in mCTLA4-H γ 1 transgenic mice are not CD28 independent, as their in vitro responses can be readily inhibited by the addition of mCTLA4-H γ 1, or enhanced by anti-CD28 cross-linking.

mCTLA4-Hy1 Transgenic Mice Have Reduced Antibody Re-To determine the effect of blocking costimulation sponses. via CD28 on in vivo immune responses, mCTLA4-H γ 1 transgenic and control littermates were immunized with DNP-KLH in alum and analyzed for the ability to generate DNPspecific serum antibodies. Consistent with previous reports where a soluble form of CTLA-4 was injected into mice (21), mCTLA4-H γ 1 transgenic mice showed almost undetectable primary antibody responses and significantly reduced secondary and tertiary antibody responses (Fig. 2). Further analysis of the Ig isotype of the specific antibodies revealed that in mCTLA4-H γ 1 transgenic mice most of the antibodies made were of the IgM class, with very little IgG being detected; no specific IgG subclass was found to predominate in the response (30). In contrast, control littermates generated high titers of specific IgG. Therefore, the levels of mCTLA4-H γ 1 in transgenic mice were sufficient to significantly inhibit specific antibody responses, reproducing the phenotype already described in mice injected with soluble CTLA-4 (21) and suggesting that effective in vivo blocking of CTLA-4-binding molecules had been achieved.

mCTLA4-H γ 1 Transgenic Mice Generate Normal or Enhanced T Cell Responses. To determine whether the inability of



Figure 2. DNP-specific antibody response in mCTLA4-H γ 1 transgenic mice and control littermates after immunization with DNP-KLH. Open circles, control mice; closed circles, transgenic mice. Transgenic and control mice were primed i.p. with 100 μ g DNP-KLH in alum adjuvant on day 0, and boosted i.p. at the indicated times with 100 μ g soluble DNP-KLH. Serum samples were taken by tail bleeding and analyzed by ELISA. Values represent mean \pm SE of five mice per group.

mCTLA4-H γ 1 mice to generate specific antibodies was indeed due to a defect in T cell responses, mCTLA4-H γ 1 transgenic and control littermates were immunized with DNP-KLH as described above and tested for the frequency of KLH-specific, IL-2- or IL-3-producing T cells in a limiting dilution assay. Surprisingly, as shown in Fig. 3, A and B, primary immunization with DNP-KLH induced in both groups of mice a three- to sixfold increase in the number of KLH-specific T cells with respect to unimmunized mice; no clear difference between transgenics and controls was detectable in either numbers of IL-2- or IL-3-producing T cells, or in T cell proliferation experiments (not shown). This suggests that in vivo expansion of antigen-specific T cells could occur to a comparable degree in control as well as in transgenic mice, presumably due to autocrine production of IL-2 and/or IL-4 in vivo, and that the defect in antibody production in mCTLA4-H γ 1 transgenic mice could not be ascribed to a defect in T cell responses.

T cell responses in mCTLA4-H γ 1 transgenic and control littermates were compared also after multiple antigen immunizations. After one or two boosts with DNP-KLH, control mice showed no significant increase in the numbers of KLHspecific, IL-2- or IL-3-producing T cells as compared with primary immunization. In contrast, boosting of transgenic mice further increased the numbers of antigen-specific T cells by three- to fivefold with respect to primary immunization. Thus, mCTLA4-H γ 1 transgenic mice not only have no obvious defect in the ability to generate T cell responses after immunization with a protein antigen in vivo, but in fact appear to have increased numbers of antigen-specific T cells after multiple immunizations.

To test whether the normal T cell responses detected after immunization with KLH in adjuvant could also be induced using other immunization protocols, mCTLA4-H γ 1 transgenic and control mice were immunized with KLH-pulsed



Figure 3. KLH-specific IL-2 (A) and IL-3 (B) production in mCTLA4-H γ 1 transgenic mice and control littermates after immunization with DNP-KLH. White bars, control mice; hatched bars, transgenic mice. Control and transgenic mice were left unimmunized or immunized at 7–14-d intervals as in Fig. 2, and killed 7–10 d after the last immunization. Numbers of KLH-specific, IL-2-, or IL-3-producing T cells in B-depleted spleens and draining lymph nodes were determined in a limiting dilution assay; numbers of cells producing IL-2 or IL-3 in the absence of KLH (\sim 20–50/10⁶ T cells) are subtracted. Values given represent the arithmetic mean of two mice per group; error bars show the deviation of individual values from the mean.

dendritic cells, a procedure already described to induce efficient priming of T cell responses (38). As shown in Fig. 4, mCTLA4-H γ 1 transgenic and control littermates responded equally well to immunization with KLH-pulsed dendritic cells, as comparable numbers of KLH-specific, IL-2-producing T cells were generated by both groups of mice. This suggests that the antigen presenting function of dendritic cells is not sensitive to mCTLA4-H γ 1 inhibition in vivo, thus allowing the normal priming of antigen-specific T cell responses.

mCTLA4-H γ 1 Transgenic Mice Can Generate Both IFN- γ and IL-4-producing T Cells. The requirement for costimulation via CD28 is reported to be linked to the Th phenotype of T cell clones. In vitro experiments have shown that Th1 clones, which produce IL-2, IL-3, IFN- γ , and TNF- β , require costimulation through CD28 (39). In contrast, Th2



IL-2 producing T cells / 10⁶ T cells

Figure 4. KLH-specific II-2 production in mCTLA4-H γ 1 transgenic and control littermates after immunization with KLH-pulsed dendritic cells. White bars, control mice; hatched bars, transgenic mice. Control and transgenic mice were immunized i.p. with 6 × 10⁵ KLH-pulsed dendritic cells and sacrificed 9 d after immunization. Numbers of KLH-specific, II-2producing T cells in B-depleted spleens and draining lymph nodes were determined in a limiting dilution assay; numbers of cells producing II-2 in the absence of KLH (~20-50/10⁶ T cells) are subtracted. Values given represent the arithmetic mean of two mice per group; error bars show the deviation of individual values from the mean.

clones, which produce IL-3, IL-4, IL-5, and IL-10, reportedly do not require signaling through CD28, and may be dependent on other costimulatory signals (40, 41). To evaluate the possibility that selective priming of Th2 cells was occurring in the DNP-KLH immunized mCTLA4-Hy1 transgenic mice, responding T cells were tested for the pattern of lymphokines produced. As shown in Fig. 5, A and B, after primary immunization, transgenic animals produced more IFN- γ but less IL-4 than nontransgenic controls; upon subsequent immunizations, both groups of mice produced comparable amounts of IL-4 (Fig. 5 C) but very little or no IFN- γ (not shown). No secretion of IFN- γ or IL-4 could be detected in unimmunized animals of either group under these conditions. Therefore, IL-4-producing T cells might be more sensitive than IFN- γ -producing ones to the lack of CD28mediated costimulation during priming. Although transgenic mice could produce more IFN- γ but less IL-4 upon primary immunization, these differences were not detected in secondary or tertiary responses, suggesting that both sets of Th cells were being primed in the mCTLA4-H γ 1 transgenics.

T Cells in mCTLA4-H γ 1 Transgenic Mice Are Not Anergic. In vitro and in vivo experiments have shown that T cell recognition of MHC+antigen in the absence of costimulatory signals induces a long lasting state of unresponsiveness, even if production of some lymphokines can still be detected (reviewed in 1, 42). Even if rather unlikely, it was possible that although T cells in the mCTLA4-H γ 1 transgenics were able to produce lymphokines in vitro (Figs. 3, 4, and 5), they had been anergized by in vivo recognition of antigen in the absence of costimulatory signals. To assess whether this was the case, T cells from immunized transgenic and control mice were adoptively transferred into syngeneic nu/nu mice, and tested for the ability to deliver cognate help to B cells. Previous experiments have in fact demonstrated that T cells, rendered unresponsive presumably by recognition of antigen on costimulator deficient APCs,



Figure 5. Antigen-specific IFN- γ and IL-4 production in mCTLA4-H γ 1 transgenic mice and control littermates after immunization with DNP-KLH. *Open circles*, control mice; *closed circles*, transgenic mice; the results of two mice from each group are shown. (A) In vitro IFN- γ production after primary immunization with 100 μ g DNP-KLH in alum. Units given correspond to 10⁶ input T cells. (B and C) In vitro IL-4 production after primary (B) or tertiary (C) immunization with DNP-KLH. Responding T cells were cultured at 2 × 10⁵/well.

are unable to help normal B cells when transferred into adoptive hosts (43, 44). As shown in Fig. 6, nu/nu mice injected with 10⁶ purified T cells from either mCTLA4-H γ 1 transgenic or control littermates and immunized with DNP-KLH generated significant titers of DNP-specific IgG. Low or no antibody responses were detected in nu/nu mice not injected with T cells (Fig. 6), or in nu/nu mice injected with T cells but not immunized with DNP-KLH (not shown). Therefore, T cells from transgenic donors appear not to be anergic, as they can help B cells to produce specific antibodies in vivo.



Figure 6. Specific antibody production in C57BL/6 nu/nu mice injected with T cells from mCTLA4-Hy1 transgenic or control littermates and immunized with DNP-KLH. C57BL/6 nu/nu mice were injected i.v. with 106 purified T cells from sex-matched control or transgenic mice that had been immunized three times with DNP-KLH; 24 h later reconstituted or nonreconstituted nu/nu mice were immunized i.p. with 100 μ g DNP-KLH in alum, bled 9 d after immunization, and tested for DNP-specific serum IgG by

ELISA. C57BL/6 nu/nu mice injected with T cells but not immunized did not generate detectable DNP-specific antibodies (not shown). Values given represent the geometric mean of two mice per group, error bars show deviation of the individual values from the mean.

Discussion

To study the role of the CD28/CTLA-4 T cell surface molecules in immune responses, transgenic mice that endogenously express a soluble form of mouse CTLA-4, mCTLA4-H γ 1. were generated. mCTLA4-H γ 1 binds to a number of costimulatory surface molecules expressed by various kinds of APCs, thereby blocking their ability to interact with their natural ligands CD28 and CTLA-4. mCTLA4-H γ 1 is a fusion protein between mouse CTLA-4 and human IgG1 (24); its transcription is driven from the immunoglobulin heavy chain promoter and the protein is secreted as a soluble dimer. mCTLA4-H γ 1 transgenic mice show no gross alterations in T cell development (consistent with 36, 37) or in the numbers of peripheral T cells; their T cells are sensitive to mCTLA4-Hy1 inhibition, as well as to anti-CD28 costimulation, to the same degree as T cells from normal mice (Fig. 1). T cell-independent antibody responses are normal in mCTLA4-H γ 1 transgenic mice (30), suggesting that expression of the transgene does not impair the ability of B cells to produce antibodies. Also, mCTLA4-Hy1-mediated crosslinking of $Fc\gamma$ receptors on transgenic B cells is not likely to cause defects in B cell responses, as normal and transgenic B cells respond equally well to in vitro stimulation with a soluble form of CD40 ligand (30).

mCTLA4-H γ 1 is biologically active in vivo, and its binding to B7/BB1⁺ cells such as dendritic cells in the secondary lymphoid organs of transgenic mice can be demonstrated by histological methods. mCTLA4-H γ 1 is stably expressed in the serum of transgenic mice at levels of 10–30 μ g/ml; these levels are sufficient to saturate binding in vitro, and are similar to or higher than the ones reported in studies where soluble CTLA-4 was injected into mice (21), therefore, they are presumably sufficient to achieve blocking in vivo.

mCTLA4-H γ 1 transgenic mice were used to analyze the effect of blocking CD28-dependent costimulation on in vivo T cell responses. The results obtained show that T cell responses specific for protein antigens are easily elicited in transgenic animals which express the fusion protein mCTLA4- $H\gamma 1$ (Figs. 3 and 4), although the kinetics of production of some lymphokines are somewhat altered (Fig. 5). By adoptive transfer experiments these cells are also not anergic (Fig. 6). Therefore, it appears that in vivo priming and expansion of CD4⁺ T cells may take place when CTLA-4 ligands are absent, or present in greatly reduced amounts. It should however be noted that, although antigen-specific T cells are clearly expanded in mCTLA4-H γ 1 transgenic mice, implying that T cell priming has occurred, detection of normal levels of lymphokines in vitro does not mean that such high levels are also produced in vivo in the absence of CD28 costimulation. In spite of the normal induction of T cell responses, specific antibody production is greatly impaired in mCTLA4- $H\gamma 1$ transgenic mice (Fig. 2), suggesting that soluble CTLA-4 may selectively interfere with the effector function, rather than the proliferation and expansion, of antigen-specific CD4⁺ T cells. A phenotype essentially similar to the one of mCTLA4-H γ 1 transgenics has recently been described for CD28-deficient mice: although in such mice some T cell responses are normal, antibody responses are significantly impaired (45). However, as no direct evaluation of CD4⁺ T cell function in CD28-deficient mice was presented, more specific comparisons are not possible at this time.

There are several possible explanations for the observed defect in antibody production together with normal T cell responses. The first is that an intrinsic difference may exist between "professional" APCs, such as dendritic cells or Langerhans cells, and other APCs such as B cells, in the requirement for expression of CTLA-4 ligands. Dendritic cells, which are the cells primarily responsible for the priming of naive T cells in vivo (38), may not require the expression of CTLA-4 ligands, for example, because they also express other costimulatory molecules (46, 47). That this may indeed be the case is supported by the observation reported in Fig. 4, where injection with antigen-pulsed dendritic cells is able to induce T cell responses in mCTLA4-H γ 1 transgenics. In contrast, redundant costimulatory signals may be absent or limiting on other APCs such as B cells, rendering the function of these latter APCs strictly dependent on expression of CTLA-4 ligands. Inhibition of the APC function of B cells would not be sufficient to affect T cell expansion (25), but would obviously have a drastic effect on T cell-dependent antibody responses. Alternatively, as already proposed, CD28 signaling may be required for the transition from autocrine to paracrine lymphokine production (20, 48). In such an instance, T cells would be able to produce levels of lymphokines which are adequate for autocrine T cell clonal expansion even in the absence of CD28 engagement. Such levels would however be insufficient for the expression of T helper

function for B cells, resulting in a phenotype of normal T cell responses together with defective antibody production. In either of these cases, one could hypothesize that a defect similar to the one detected in T cell-dependent B cell responses might also be detected in other T cell-dependent effector mechanisms, such as macrophage activation during delayed-type hypersensitivity, or help for the generation of CTL responses. Such view might explain the defective transplant rejection detected in other studies, as well as the enhanced immunogenicity shown by tumors induced to express B7 by DNA-mediated gene transfer (49, 50). Experiments are currently in progress to precisely define what T-dependent effector responses are defective in mCTLA4-H γ 1 transgenic mice.

One third possibility is that B7 (or other CTLA-4 ligands) is a critical signaling molecule for B cells, and that its blocking with mCTLA4-H γ 1 leads to defective B cell responses despite optimal T cell effector function in vivo. Although this possibility cannot be ruled out at the present time, it is not consistent with experimental data on the suppressive effects of soluble CTLA-4 detected in other systems (23), and is so far not supported by experimental evidence.

Our detection of normal T cell responses to a protein antigen in mCTLA4-H γ 1 transgenic mice is in contrast with a previous study (21) where in vivo treatment with CTLA4Ig could inhibit the generation of SRBC-specific T cell proliferative responses in vitro. However, it has been previously shown that the presence of SRBC-specific B cells is required to demonstrate proliferative T cell responses to SRBC in vitro (51). Because B cell responses are defective in mice treated with CTLA4Ig, the inability to demonstrate a proliferative response could be due to the lack of specific B cells in vitro, rather than to a failure to prime specific T cells in vivo.

It could be argued that the levels of serum mCTLA4-H γ 1 in these transgenic mice are not sufficient to effectively block the CTLA-4 ligands. This is unlikely to be the case, as other studies that used serum levels of soluble CTLA-4 similar or lower than the ones reported here were able to obtain long term acceptance of xenogeneic transplants (22) and induce suppression of antibody responses (21); a completely comparable degree of suppression of antibody responses was detected in the present study as well. We cannot, however, rule out the possibility that complete blocking may not be achieved by using soluble CTLA-4. For example, expression of B7/BB1 on various APCs can be upregulated after interaction with T cells (3, 52). It could then be that CTLA-4 ligands, newly expressed in the intercellular space during T cell-APC interaction, are not available to mCTLA4-H γ 1 present in solution. The observation that antigen-pulsed dendritic cells are effective in inducing T cell responses in mCTLA4-Hy1 transgenic mice makes this possibility less likely. Splenic dendritic cells, such as the ones used in the present study, express levels of CTLA-4 ligands that are significantly higher than the ones detectable on their precursors, epidermal Langerhans cells (6). Such ligands should be readily available to mCTLA4-H γ 1 in solution when dendritic cells are injected.

The mCTLA4-H γ 1 transgenic mice generate normal primary T cell responses, and paradoxically show increased numbers of antigen-specific T cells after repeated immunizations. A trivial explanation for this finding might be that impaired antibody responses in the transgenics lead to less efficient clearance of antigen, with more antigen available for the in vivo expansion of T cells. Alternatively, the regulation of T cell clonal expansion may be altered in these mice. The recent observation that activated T cells express CTLA-4 ligands (9, 10, 15, and P. Lane, unpublished observation) and efficiently act as APCs may explain this phenomenon, since T cells expressing costimulatory molecules may be able to prime regulatory CD8⁺ T cells, which then control their in vivo clonal expansion. This mechanism would be inhibited in mCTLA4-Hy1 transgenic mice, where CTLA-4 ligands are blocked. A third possibility is that, in intact mice, T cell clonal expansion is regulated by the availability of antigen on different kinds of APCs, with some APCs inducing expansion, and others maintenance of clone size. Blocking of CTLA-4 ligands with mCTLA4-H γ 1 may then selectively interfere with some (maintenance) but not others (expansion)

of these signals, leading to abnormal T cell proliferation in vivo. For example, by allowing production of higher levels of IL-2 (20, 48), CD28 signaling may indirectly cause apoptosis of antigen stimulated T cells (53) and thus control clonal expansion. Finally, it is possible that mCTLA4-H γ 1 may bind preferentially to some of its ligands, while its affinity for others may be too low to allow efficient blocking. The net effect of soluble CTLA-4 on an immune response would then depend on its relative affinity for each of these ligands, on the pattern of expression of these ligands on different cells, and on the differential signals transmitted by its receptors (CD28 vs. CTLA-4) to the responding T cells.

In conclusion, the present data show that T cell responses for a protein antigen are not impaired in mCTLA4-H γ 1 transgenic mice, and offer alternatives to explain the defect in antibody responses detected after injection of soluble CTLA-4 in vivo. This is relevant for understanding whether soluble forms of CTLA-4 could be exploited to manipulate in vivo immune responses.

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Address correspondence to Dr. Franca Ronchese, Basel Institute for Immunology, Grenzacherstrasse 487, Basel CH-4005, Switzerland.

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