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

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Summary

CD4+ T cell responses were analyzed in transgenic mice expressing a soluble form of murine CTLA-4, mCTLA4-Hy1, 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-Hy1 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-Hy1 transgenics as compared with normal mice. Although unable to deliver cognate help to B cells in vivo, T cells from mCTLA4-Hy1 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.

Stimulation 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, 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 y1 (mCTLA4-Hy1)1, which can block the interaction of CD28 and/or CTLA-4 with their costimulatory ligands (24). These mCTLA4-Hy1 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-Hy1 transgenic mice were maintained at the Basel Institute for Immunology by breeding transgenic males to C57BL/6 females (Ifla-Credo, I'Arbresle, France). Progeny were

1 Abbreviations used in this paper: mCTLA4-Hy1, mouse CTLA4-human y1.
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 μg DNP-KLH in alum adjuvant on day 0 and boosted i.p. with 100 μg 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 × 40 min; low-density cells were isolated by centrifugation over a 60% Percoll gradient (p = 1.076) and the floating 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 μg/ml KLH. The following day floating cells were collected and depleted of contaminating T cells and B cells by incubation with 10 μg/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 supplemented 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-H3'1. 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 × 10^5/well in 96-well flat bottom microtiter plates (Costar Corp., Cambridge, MA) in 200 μl total volume, with 2.5 × 10^5 irradiated allogeneic or syngeneic nu/nu spleen cells that had been preincubated for 45 min in graded concentrations of mCTLA4-H3'1, or mCD40-H40-Y1 (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 μCi [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 μg/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 × 10^5/well in 96-well flat bottom microplates with 4 × 10^4 irradiated C57BL/6 nu/nu spleen cells and various concentrations of KLH; the total volume was 200 μl. 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-D6-biotin (34); the reaction was developed with streptavidin-horseradish peroxidase (Southern Biotechnology, Birmingham, AL).

IL-4 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 μl supernatant were removed and replaced with 100 μl medium containing 10^4 IL-4–dependent CT-4S cells (35). Plates were incubated for a further 24 h and pulsed with 1 μCi [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 infected i.v. with 10^4 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 μg DNP-KLH in alum, bled 9 d after immunization, and tested for DNP-specific serum IgG by ELISA.

ELISA. Plates coated with 5 μg/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-IgG 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; Titertek, Eltabl Oy, Finland).

Results

T Cells from mCTLA4-H3'1 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-H3'1 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-H3'1 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. 1A, addition of mCTLA4-H3'1 inhibited the in vitro proliferative response to alloantigen of both mCTLA4-H3'1 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 γ1 (mCD40-H3'1) had no effect on the response.

To further confirm that T cells from mCTLA4-H3'1 transgenic mice bear a functional CD28 molecule, T cells from mCTLA4-H3'1 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
mCTLA4-Hy1 mice to generate specific antibodies was indeed due to a defect in T cell responses, mCTLA4-Hy1 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-Hy1 transgenic mice could not be ascribed to a defect in T cell responses.

T cell responses in mCTLA4-Hy1 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 KLH-specific, 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-Hy1 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-Hy1 transgenic and control mice were immunized with KLH-pulsed
dendritic cells, a procedure already described to induce efficient priming of T cell responses (38). As shown in Fig. 4, mCTLA4-Hy1 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-Hy1 inhibition in vivo, thus allowing the normal priming of antigen-specific T cell responses.

mCTLA4-Hy1 Transgenic Mice Can Generate Both IFN-γ- and IL-4-producing T Cells. The requirement for costimulation via CD28 is reported to induce efficient priming of T cell responses (38). As shown in Fig. 4, mCTLA4-Hy1 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-Hy1 inhibition in vivo, thus allowing the normal priming of antigen-specific T cell responses.

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are unable to help normal B cells when transferred into adoptive hosts (43, 44). As shown in Fig. 6, nu/nu mice injected with 10^6 purified T cells from either mCTLA4-H'y1 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.

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'y1, were generated. mCTLA4-H'y1 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'y1 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'y1 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-H'y1 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'y1 transgenic mice (30), suggesting that expression of the transgene does not impair the ability of B cells to produce antibodies. Also, mCTLA4-H'y1-mediated cross-linking of Fcγ 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'y1 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'y1 is stably expressed in the serum of transgenic mice at levels of 10-30 μg/ml; these levels are sufficient to saturate binding in vitro, and are similar...
T cell responses. The results obtained show that T cell re-
effect of blocking CD28-dependent costimulation on in vivo
sumption in the absence of CD28 costimulation.
In spite of the normal induction of T cell responses,
specific antibody production is greatly impaired in mCTLA4-
H3,1 transgenic mice (Fig. 2), suggesting that soluble CTLA-4
specific comparisons are not possible at this time.

There are several possible explanations for the observed de-
fect in antibody production together with normal T cell re-
ponses. The first is that an intrinsic difference may exist be-
tween "professional" APCs, such as dendritic cells or
Langerhans cells, and other APCs such as B cells, in the re-
quirement 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 in-
deed 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-Hy1 trans-
genics. 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 ex-
pression 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-depen-
dent antibody responses. Alternatively, as already proposed,
CD28 signaling may be required for the transition from auto-
crine to paracrine lymphokine production (20, 48). In such
an instance, T cells would be able to produce levels of ly-
mphokines which are adequate for autocrine T cell clonal ex-
pansion 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 re-
sponses. Such view might explain the defective transplant re-
jection detected in other studies, as well as the enhanced im-
munogenicity shown by tumors induced to express B7 by
DNA-mediated gene transfer (49, 50). Experiments are cur-
rently in progress to precisely define what T-dependent effector
responses are defective in mCTLA4-Hy1 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-Hy1 leads to defective B cell responses de-
spite 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 an-
tigen in mCTLA4-Hy1 transgenic mice is in contrast with a pre-
vious study (21) where in vivo treatment with CTLA4Ig
could inhibit the generation of SRBC-specific T cell prolifer-
ative responses in vitro. However, it has been previously shown
that the presence of SRBC-specific B cells is required to dem-
onstrate 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-Hy1
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 com-
parable degree of suppression of antibody responses was de-
tected 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 inter-
action, are not available to mCTLA4-Hy1 present in solu-
tion. The observation that antigen-pulsed dendritic cells are
effective in inducing T cell responses in mCTLA4-Hy1 trans-
genics 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-Hy1
in solution when dendritic cells are injected.

The mCTLA4-Hy1 transgenic mice generate normal pri-
mary 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 transgensics 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-H'y1 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'y1 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'y1 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'y1 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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