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Cutting Edge: Lymphoproliferative Disease in the Absence of CTLA-4 Is Not T Cell Autonomous¹

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Mice deficient for the expression of CTLA-4 develop a lethal lymphoproliferative syndrome and multiorgan inflammation leading to death at about 4 wk of age. Here we show that RAG2-deficient mice reconstituted with CTLA-4-deficient bone marrow do not develop a lymphoproliferative syndrome despite lymphocyte infiltration mainly into pericardium and liver. Moreover, RAG2-deficient mice reconstituted with a mixture of normal and CTLA-4-deficient bone marrow remain healthy and do not develop any disease. Thus, the lethal disease observed in CTLA-4-deficient mice is not T cell autonomous and can be prevented by factors produced by normal T cells. *The Journal of Immunology*, 1999, 163: 1128–1131.

T cell activation is a complex process that is tightly regulated by TCR and costimulatory signals. Stimulation of T cells through the TCR alone is usually inefficient and/or results in clonal anergy. In contrast, concomitant triggering of the TCR and CD28, the major costimulatory molecule on T cells, leads to full activation of T cells and the generation of T cell memory (1, 2). The role of CTLA-4, a homologue of CD28 also binding to B7-1 and B7-2, is less well defined. Initially, it was characterized as a costimulatory molecule (3), but subsequent experiments established that it was more likely delivering an inhibitory signal (4–6). Despite some evidence that CTLA-4 may be dispensable for down-regulation of primary anti-viral T cell responses (7, 8) or may even act as a costimulatory molecule (9, 10), it is now generally believed that CTLA-4 turns T cells off. Biochemical studies suggesting that CTLA-4 recruits phosphatases to the TCR complex leading to its inactivation support this view (11, 12). The most impressive argument that CTLA-4 functions as a negative regulator of T cells is made by CTLA-4-deficient (CTLA-4^{-/-}) mice that die within 4 wk after birth due to a lymphopro-

liferative disorder and multiorgan inflammation (13–15). Thus, CTLA-4 expression by activated T cells seems to be required for turning these T cells off to prevent uncontrolled lymphoproliferation. Our current study indicates that this view has to be reconciled because bone marrow chimeras harboring CTLA-4^{-/-} T cells may develop infiltration of pericardium and liver but do not develop a lymphoproliferative syndrome. Moreover, mixed bone marrow chimeras producing CTLA-4^{-/-} and normal T cells are healthy and do not develop disease, demonstrating that the lymphoproliferative disorder observed in CTLA-4^{-/-} mice is not due to a T cell autonomous defect.

Materials and Methods

Mice and generation of bone marrow chimeras

CTLA-4^{-/-} (13) and RAG2^{-/-} (16) mice have been described previously. CTLA-4-deficient mice were bred heterozygously, and offspring were genotyped by PCR at the age of 2–3 wk and immediately used to generate bone marrow chimeras. A total of 10⁷ cells depleted of mature CD4⁺ and CD8⁺ T cells (by negative selection using monoclonal anti-CD4 and anti-CD8 Abs and magnetic beads (Dyna) according to the manufacturer's instructions) were transferred per RAG2^{-/-} recipient (irradiated with 3 Gy). Mixed bone marrow chimeras were reconstituted with an equal number of bone marrow cells derived from CD45.2 CTLA4^{-/-} mice and CD45.1 congenic C57BL/6 mice. Similar results were obtained if a total of 10⁷ cells or 10⁷ cells of each genotype were transferred to generate the mixed chimeras. No differences could be observed between mice reconstituted with CTLA-4^{+/+} and CTLA-4^{+/-} bone marrow. For simplicity, such bone marrow is therefore referred to as CTLA-4^{+/+}.

Analysis of T cell activation markers

Lymph node cells from the bone marrow chimeras were harvested and triple stained for the expression of CD4 (FITC)/CD8 (PE) and CD25 (biotin), CD44 (biotin), CD62L (biotin), or CD69 (biotin) followed by streptavidin coupled to APC. Lymph node cells from mixed bone marrow chimeras were triple stained for the expression of CD45.2 (biotin) followed by streptavidin coupled to APC, CD4 (FITC), and CD8 (PE) or CD45.2 (FITC), CD4 (PE) or CD8 (PE), and CD25 (biotin), CD44 (biotin), CD62L (biotin), or CD69 (biotin) followed by streptavidin coupled to APC.

Histology

Organs were isolated from chimeric mice 8 wk after reconstitution at a time point when the mice that obtained CTLA-4^{-/-} bone marrow alone had developed signs of disease. Organs were fixed in 4% buffered formaldehyde in PBS, pH 7.0, and were paraffin embedded. Sections were cut (5 μM) and stained with hematoxylin and eosin and examined by light microscopy.

Quantitative RT-PCR

Total cellular RNA was prepared from CD45.1 (CTLA-4^{+/+}) and CD45.2 (CTLA-4^{-/-}) splenocytes purified from mixed chimeras 8 wk after bone marrow transfer. Random hexamer-primed reverse transcription was performed and the level of TGF-β-, IFN-γ-, and IL-10-specific transcripts was

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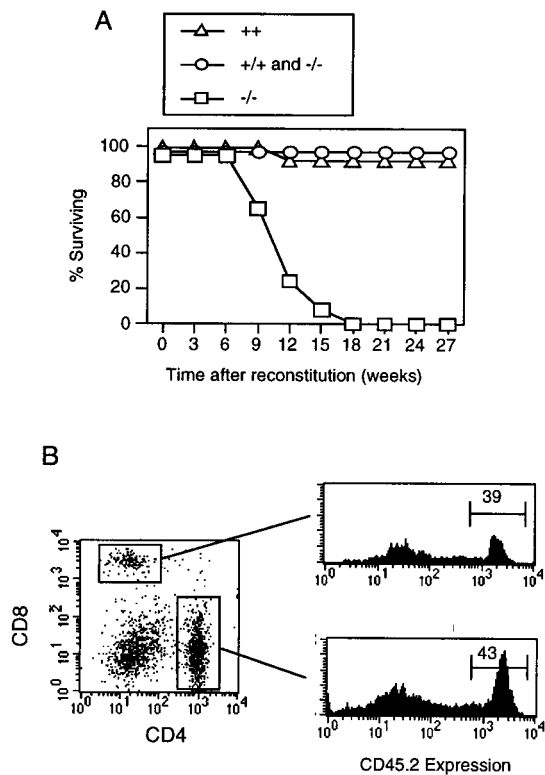


FIGURE 1. Normal survival of mixed bone marrow chimeras reconstituted with both CTLA-4^{-/-} plus control bone marrow. *A*, RAG2-deficient mice were reconstituted with T cell-depleted bone marrow derived from control (CD45.1) (Δ) or CTLA-4-deficient (CD45.2) (\square) mice or a mixture of the two (\circ) and survival of the recipient mice (12 mice per group) was monitored. *B*, CD45.2 expression (present on CTLA-4-deficient T cells) was assessed for CD8⁺ and CD4⁺ T cells 16 wk after reconstitution. Similar results were obtained between 8 and 40 wk after reconstitution.

determined by PCR coamplification of a competitor construct and cellular cDNA after standardization for the expression of β_2 -microglobulin essentially as described (17). PCR primers for TGF- β , IFN- γ , IL-10, and β_2 -microglobulin were described elsewhere (17).

Results and Discussion

To analyze whether the lymphoproliferative disorder observed in CTLA-4^{-/-} mice was T cell autonomous or could be prevented by the presence of normal T cells, various bone marrow chimeras were constructed. Thus, irradiated (3 Gy) RAG2^{-/-} mice were reconstituted with T cell-depleted bone marrow from normal mice, CTLA-4^{-/-} mice, or a 1:1 mixture of both. In case of the latter, we reconstituted CTLA-4^{-/-} bone marrow expressing CD45.2 together with bone marrow of congenic C57BL/6 mice expressing CD45.1 on all hematopoietic cells so that we could follow the fate of the individual T cell populations. While CTLA-4^{-/-} mice die shortly (3–5 wk) after birth (13–15), RAG2^{-/-} chimeras receiving CTLA-4-deficient bone marrow alone died around week 10 after reconstitution. In marked contrast, the mixed bone marrow chimeras remained healthy for at least 6 mo (Fig. 1*A*). Mixed chimerism was confirmed by assessing CD45.2 expression of CD4⁺ and CD8⁺ T cells (Fig. 1*B*). Similar results were obtained between 8 and 40 wk after reconstitution and was comparable in the T and B cell compartment (not shown). Histological analysis of organs derived from diseased CTLA-4^{-/-} chimeras revealed inflammatory lymphocyte infiltrates mainly in pericardium with slight myocardial involvement (Fig. 2*C*) and liver (not shown). However, inflammation was clearly less extensive than in 4-wk-old CTLA-

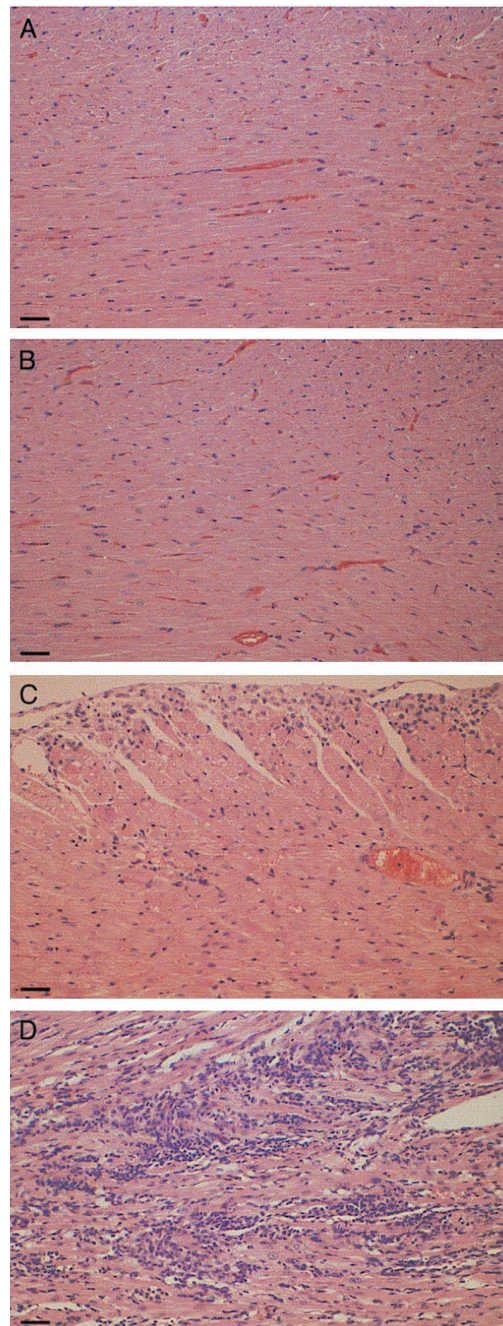


FIGURE 2. Mixed bone marrow chimeras do not develop multifocal organ infiltration. RAG-2-deficient mice were reconstituted with T cell-depleted bone marrow derived from control mice alone (*A*), from both control plus CTLA-4-deficient mice (*B*), or from CTLA-4 deficient mice alone (*C*) and the heart was examined by histology. For comparison, the heart of a 4-wk-old CTLA-4-deficient mouse was included (*D*). Bar represents 100 μ m.

4^{-/-} mice (Fig. 2*D*) and was not present in all CTLA-4^{-/-} chimeras (not shown). In contrast, mixed bone marrow chimeras as well as control chimeras showed no signs of organ inflammation (Fig. 2, *A* and *B*). Thus, chimeras receiving CTLA-4^{-/-} bone marrow alone developed lethal inflammation of multiple organs. However, this disease could be prevented by the presence of normal T cells, demonstrating that disease does not develop because T cells stimulated through the TCR have to be down-regulated by a signal mediated by CTLA-4. Instead, normal T cells appear to produce a factor (or factors) upon ligation of CTLA-4 that is able to prevent

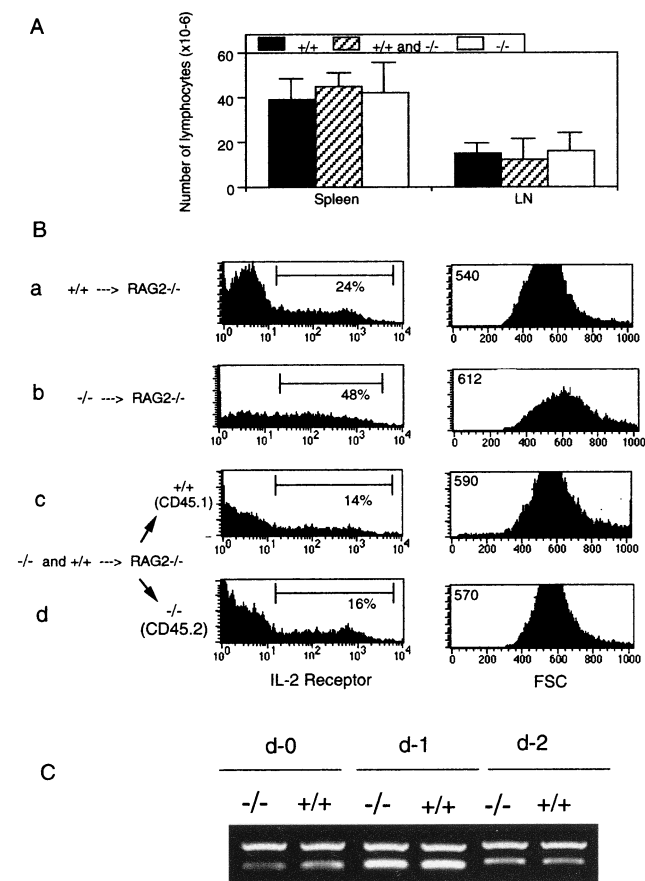


FIGURE 3. CTLA-4-deficient T cells fail to induce lymphoproliferative disease in chimeric mice. RAG2-deficient mice were reconstituted with T cell-depleted bone marrow derived from control (CD45.1) or CTLA-4-deficient (CD45.2) mice or a mixture of the two. **A**, Lymphocyte numbers in spleens and pooled mesenteric lymph nodes were assessed 8 wk later. **B**, Expression of IL-2R α -chain and forward scatter (FSC) is shown for CD4⁺ T cells derived from the various chimeras. **a**, RAG2^{-/-} mice receiving control bone marrow. **b**, RAG2^{-/-} mice receiving CTLA-4^{-/-} bone marrow. **c** and **d**, RAG2^{-/-} mice receiving control (CD45.1) and CTLA-4-deficient (CD45.2) bone marrow. Cells were triple stained for the expression of CD45.1/CD45.2, IL-2R α -chain, and CD4. The percent IL-2R-positive cells and average FSC are indicated in the figures. One representative experiment of three is shown. **C**, Spleen cells were isolated from mixed chimeras and sorted for the expression of CD45.2 and CD45.1. After standardization for expression of β_2 -microglobulin, the presence of TGF- β mRNA was assessed by quantitative RT-PCR at 0, 1, and 2 days after *in vitro* stimulation with anti-CD3 Abs. The upper band represent the competitor (19,500 molecules).

organ inflammation *in trans*. Interestingly, assessing lymphocyte numbers in spleen and lymph nodes in the various chimeras revealed normal cell numbers, indicating that CTLA-4^{-/-} T cells fail to cause lymphoproliferative disease even in the absence of normal T cells (Fig. 3A). Moreover, the architecture of the lymph nodes was normal in all types of chimeric mice, while it was grossly distorted in CTLA-4^{-/-} mice (not shown).

CD4⁺ T cells are the first cells to become activated in CTLA-4^{-/-} mice (15). In contrast, analysis of various activation markers expressed by the CD4⁺ T cells derived from the mixed chimeras did not reveal any uncontrolled activation of CTLA-4-deficient T cells. Specifically, both CD45.1⁺ (control) and CD45.2⁺ (CTLA-4^{-/-}) CD4⁺ T cells exhibited comparable expression of the IL-2R α -chain and forward scatters as measures for activation (Fig. 3B). Interestingly, CD4⁺ T cells in chimeras that received bone

marrow from CTLA-4^{-/-} mice alone exhibited up-regulated expression of IL-2R α and were blasted, indicating that they were activated (Fig. 3B). Similar observations were made for CD8⁺ T cells and for the expression of CD69, CD44, and CD62L (not shown). T cells derived from CTLA-4^{-/-} mice that develop lymphoproliferative disorder show hyperphosphorylation (11). By contrast, T cells isolated from the chimeras were not hyperphosphorylated, again indicating that these CTLA-4^{-/-} T cells are not activated in such an uncontrolled manner, as observed in the knockouts (not shown).

Thus, our results demonstrate that T cells devoid of CTLA-4 expression do not develop lymphoproliferative disorder, but may cause multiorgan inflammation. However, this disease, can be prevented by a factor (or factors) produced by normal T cells. The recent finding that CTLA-4 triggers the production TGF- β (18) together with the observation that TGF- β ^{-/-} mice develop multifocal inflammation in various organs (19, 20) may suggest that the principle reason for disease in CTLA-4^{-/-} mice is a failure to produce TGF- β or related factors rather than defective down-regulation of T cell activation in CTLA-4-deficient T cells. However, CTLA-4^{-/-} T cells isolated from mixed chimeras expressed normal levels of TGF- β (Fig. 3C), IL-10, and IFN- γ (not shown) mRNA upon freshly after isolation and after stimulation with anti-CD3. Although this result demonstrates that CTLA-4^{-/-} T cells can produce antiinflammatory cytokines such as TGF- β and IL-10, it remains possible that the CTLA-4^{-/-} T cells have been instructed *in vivo* by normal TGF- β - and IL-10-producing T cells to themselves be committed to the generation of these cytokines. This interpretation is compatible with the recent observation that exogenous TGF- β and IL-10 can trigger their own production in Th cells, in this way replacing the CTLA-4 signal. (21, 22).

Our data demonstrate that the disease observed in CTLA-4^{-/-} mice is not due to a T cell autonomous defect. By contrast, CTLA-4 triggering on normal T cells stimulates the production of a factor (or factors) that are sufficient to inhibit disease induced by CTLA-4^{-/-} T cells. Thus, our results suggest that the CTLA-4-recruited phosphatases that dephosphorylate molecules of the CD3 complex (11, 12) may not lead to complete inactivation of T cells but may reduce TCR-mediated signals to the extent that the cytokine production is shifted to the production of TGF- β . The here-described healthy chimeras containing both normal and CTLA-4-deficient T cells will allow us to definitively assess the *in vivo* role of CTLA-4 in regulating specific T cell responses.

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