

Induction of Interleukin 12 Responsiveness Is Impaired in Anergic T Lymphocytes

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Summary

The cytokine, interleukin 12 (IL-12), stimulates both natural killer cells and T cells to proliferate and to secrete interferon γ (IFN- γ). The T cell proliferative response to IL-12 must be induced and is evident after T cell receptor-mediated stimulation. As reported here, tolerant CD4⁺ T cells and clones, that are anergic for IL-2 production, are also anergic for induction of the proliferative response to IL-12. Murine T helper 1 clones tolerized in vitro, as well as anergic CD4⁺ T cells isolated from mice tolerized to the Mls-1^a antigen (Ag) in vivo, demonstrated defective induction of proliferation to IL-12 upon restimulation with Ag. IL-12-enhanced production of IFN- γ was observed in both control and anergic cells after Ag/antigen-presenting cell (APC) activation, although total IFN- γ secretion by anergic cells was less than that produced by control cells, even in the presence of IL-12. These data indicate that T cell clonal anergy results in profound inhibition of proliferative responses, since the autocrine growth factor, IL-2, is not produced, and the APC-derived cytokine, IL-12, is not an effective stimulus for anergic T cell proliferation.

T cell anergy can be induced in Th1 clones in vitro by ligation of the TCR in the absence of costimulatory signals (1–3), and can also be induced in vivo in mature CD4⁺ T cells by the injection of superantigens (4–6). Such anergy is characterized by a block in IL-2 production, resulting in proliferative nonresponsiveness to Ag (7). In addition, anergic Th1 and Th0 clones are nonresponsive for the induction of proliferation to IL-4 (8, 9). These data suggest that the focus of functional anergy is to prevent expansion of tolerant Ag-reactive clones upon Ag challenge. It was recently determined that the cytokine, IL-12, produced by macrophages and B cells (10), stimulates the proliferation of T cells after TCR-induced activation and induces the production of IFN- γ (11–13). Furthermore, IL-12 plays an important role in directing a predominantly Th1-like response in T cell populations (14, 15). The work reported here demonstrates that anergic T cells are impaired for induction of proliferation to IL-12, as they are for induction of proliferation to IL-4 (8, 9) and for induction of IL-2 synthesis (1–3, 7).

Materials and Methods

Mice. Female B10.BR/SgSnJ or B10.A/SgSnJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used at 6–12 wk of age as the source of splenocyte APC for the maintenance and stimulation of T cell clones. Female CBA/J (Mls-1^a) or CBA/Ca (Mls-1^b) mice were obtained from the National Cancer Institute (National Institutes of Health, Bethesda, MD)

and were used as sources of spleen cells for in vivo tolerance experiments. Homozygous TCR V β 8.1, Mls-1^b transgenic mice (6, 16) were bred at the University of Pennsylvania.

Anergy in Th1 Clones. The pigeon cytochrome *c* (PCC):H-2^k-reactive Th1 clones, A.E7 and AC24, were maintained by cycles of Ag stimulation and rest, as described (17). Anergy was induced in rested cells by culture with immobilized anti-CD3 Ab in the absence of APC (17), followed by rest in medium for 3–12 d. Rested anergic or control cells (1–2 \times 10⁴/well) were then restimulated by culture with Ag plus irradiated splenic APC (5 \times 10⁵/well) in the presence or absence of murine rIL-12 (18; kindly provided by Dr. Stanley Wolf, Genetics Institute, Cambridge, MA). The Ag used were PCC (Sigma Chemical Co., St. Louis, MO) or a PCC splice peptide corresponding to amino acids 86–89; 93–103(93E) (19), which was synthesized and HPLC purified in the Department of Pathology and Laboratory Medicine Protein Facility, University of Pennsylvania. Aliquots of supernatant were removed from restimulation cultures on day 2 to assay IFN- γ production, and cells were then pulsed with [³H-methyl]TdR (1 μ Ci/well) for 18 h before harvesting (PHD Cell Harvester; Cambridge Technology, Inc., Watertown, MA). Triplicate values for each assay were generally within 15% of the SEM.

T Cell Anergy In Vivo. Adult TCR V β 8.1 transgenic mice were injected intravenously with 1.5 \times 10⁷ Mls-1^a-positive T-depleted CBA/J spleen cells to induce anergy, or were injected with PBS for control mice. On day 14 after injection, the inguinal, popliteal, axillary, brachial, and cervical LN were removed and CD4⁺ T cells were purified and restimulated (5 \times 10⁴/well) for 84 h with Mls-1^a-positive stimulator cells (5 \times 10⁵/well), as described (6), in the presence or absence of murine rIL-12.

IFN- γ Production. IFN- γ concentrations were determined using an ELISA assay that employed the R4-6A2 mAb (20) for capture, and a polyclonal rabbit anti-mouse IFN- γ serum for detection with horseradish peroxidase-coupled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and the substrate, 2,2'-azino-di[3-ethyl-benzthiazoline sulfonate(6)] (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Dilutions of culture supernatants were assayed in the linear portion of the dose-response curve. OD₄₀₅ values were converted to units based on a standard IFN- γ preparation obtained from GIBCO BRL (Gaithersburg, MD).

Results and Discussion

Induction of IL-12 Responsiveness for Proliferation. The CD4⁺ Th1 clone, A.E7, was cultured with APC and suboptimal concentrations of specific Ag, in the presence or absence of exogenous murine rIL-12 (Fig. 1). A low level of proliferation in response to IL-12 was noted in the absence of Ag, but addition of Ag to the culture resulted in greatly increased proliferation to IL-12, even at Ag concentrations that alone stimulated little proliferation. Optimal responses were found with IL-12 at 1–3 ng/ml, and were always synergistic with Ag stimulation. Similar results were found with two additional Th1 clones (Fig. 2 A and data not shown). Thus, Ag/APC activation can stimulate the Th1 clones used in this study for a proliferative response to IL-12, as has been reported for other T cells (11).

Impaired Induction of IL-12-mediated Proliferation in Anergic Th1 Cells. As previously reported, activation of Th1 cells by immobilized anti-CD3 Ab in the absence of APC results in the induction of anergy, which is characterized by the long-lived inability to produce IL-2 or to proliferate to subsequent stimulation by Ag plus competent APC (17, 21). Th1 cells constitutively express IL-2R, and, after induction of anergy, rested anergic cells continue to express IL-2R and to maintain proliferative responses to exogenous IL-2 (1–3). In contrast, the ability to proliferate in response to IL-4 must be induced in Th1 cells, and anergic cells are defective for this induction by Ag (8). Similar to the IL-4 situation, optimal proliferation in response to IL-12 must also be induced in Th1 cells. Therefore, to determine whether anergic Th1 cells can be induced by Ag to proliferate to IL-12, the A.E7 or

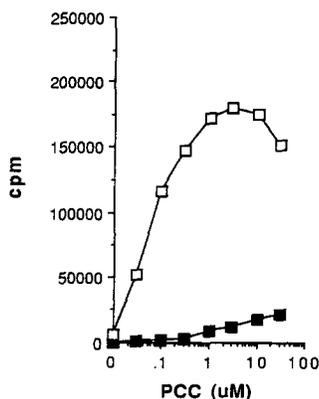


Figure 1. Synergistic activation of Th1 cell proliferation by Ag and IL-12. A.E7 cells were cultured with APC plus Ag (PCC) in the absence (closed symbols) or presence (open symbols) of murine rIL-12 (0.2 ng/ml). Proliferation was assessed by incorporation of [³H]TdR (cpm).

AC24 Th1 clones were first precultured with immobilized anti-CD3 Ab to induce anergy, and then were rested before restimulation with Ag/APC in the presence or absence of murine rIL-12.

As shown in Fig. 2, proliferative anergy to Ag/APC alone was evident in cells that had been precultured with immobilized anti-CD3 Ab (Fig. 2, open symbols, 0 ng/ml IL-12). Proliferation to IL-12 in the absence of Ag was low for both control and anergic cells (Fig. 2, closed symbols). The striking observation was that the Ag-mediated induction of responsiveness to IL-12 was considerably decreased in anergic vs control cells (Fig. 2, open boxes vs open triangles, respectively), indicating that anergic cells are severely impaired for induction of IL-12 responsiveness. This result was confirmed with three Th1 clones and was observed at all Ag concentrations tested (data not shown). Decreased proliferation to IL-12 was apparent both in anergic cells rested for a relatively short time, such as 4–5 d (Fig. 2, A and B) and in those cells rested for 12 d after induction of anergy (Fig. 2 C). Decreases ranged from 50–85% when IL-12 was present at maximal concentrations, and anergic cells were 10–1,000-fold less sensitive to IL-12 than control cells. The minimal IL-12-mediated enhancement of proliferation still evident in anergic cells cultured with Ag may reflect a small number of cells in the population that escape anergy induction, or may indicate that anergic cells exhibit a greatly decreased, but not completely abolished response to IL-12. These results thus demonstrate that, in addition to a block in IL-2 production (1–3, 7) and a block in the induction of competency to proliferate to IL-4 (8), long-term anergic Th1 cells are also impaired for the TCR-mediated induction of IL-12 responsiveness. All three defects result in T cells that are compromised for proliferation, since anergic cells cannot produce IL-2 and cannot respond to growth factors secreted by other cell types.

Role of IL-2 in the Induction of IL-12 Responsiveness. To determine whether the well-documented defect in IL-2 production in anergic cells contributes to the additional defect in IL-12 responsiveness, we first tested whether ongoing IL-2 synthesis was required for proliferation to IL-12 after Ag stimulation. Thus, rested control or anergic A.E7 cells were activated overnight with APC and Ag at the concentrations shown in Fig. 3 A, in the absence of IL-12. APC were then removed, and washed T cells were cultured with IL-12 in the presence or absence of Cyclosporin A (CsA) to inhibit any residual IL-2 production in control cell samples. CsA had little effect on the IL-12-mediated proliferation of Ag-preactivated control or anergic cells, indicating that the anergic cell defect cannot be explained by ongoing IL-2 production in control, but not anergic cells.

However, a requirement for IL-2 during the Ag activation step was still possible. This question was addressed using two approaches. First, control A.E7 cells were activated by Ag/APC in the presence of CsA to block IL-2 synthesis. T cells were then recovered, washed, and cultured with IL-12 alone. As seen in Fig. 3 B, the presence of CsA during the Ag activation step did, in fact, partially inhibit the subsequent response to IL-12, suggesting that IL-2 production, or another CsA-

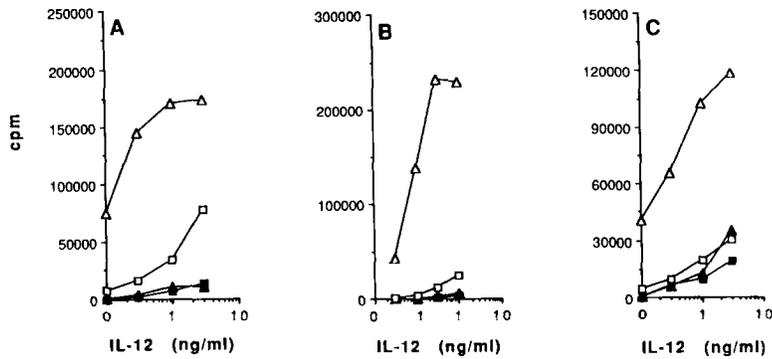


Figure 2. Anergic Th1 cells are defective for induction of IL-12-mediated proliferation. (A) Control or anergic AC24 cells were rested for 4 d before restimulation by APC plus PCC at 0 or 0.25 μ M, in the presence of increasing concentrations of IL-12. (B) Control or anergic A.E7 cells were rested for 5 d before restimulation with APC plus PCC at 0 or 20 μ M, in the presence of IL-12. (C) Control or anergic A.E7 cells were rested for 12 d before restimulation with APC plus PCC at 0 or 25 μ M, in the presence of IL-12. Control cells (triangles); anergic cells (boxes). Plus PCC (open symbols); minus PCC (closed symbols).

sensitive signaling pathway, is required for the induction of IL-12 responsiveness. We therefore employed a second approach, in which control or anergic A.E7 cells were activated by Ag/APC in the presence of exogenous IL-2, to determine whether IL-2 reconstitution of anergic cell activation cultures would allow subsequent proliferation to IL-12 at control cell levels.

Fig. 3 C demonstrates that the IL-12-mediated proliferation of both anergic and control cells was enhanced by prior exposure to IL-2, even in the absence of Ag. It is interesting to note that control cell proliferation to IL-12 was also enhanced by exposure to IL-2 during Ag activation, suggesting suboptimal Ag-mediated IL-2 production for this response by control cells, and supporting a role for IL-2 during induction of IL-12 responsiveness. It is difficult to evaluate the IL-2 concentration needed during activation for optimal subsequent IL-12-mediated proliferation, due to IL-2 use during the activation step. Complementary experiments that employed anti-IL-2/IL-2R Ab to block endogenous IL-2 use by

control cells were inconclusive because complete inhibition was not achieved (data not shown). However, the data shown in Fig. 3 C indicate that IL-2 reconstitution of anergic cell Ag activation cultures resulted in only a marginal increase in subsequent proliferation to IL-12, and was insufficient to allow IL-12-mediated proliferation at control cell levels. The IL-12 response of anergic cells activated with an optimal Ag concentration plus IL-2 was only 60% of the response found with control cells activated minus IL-2, and was only 20% of the response of control cells activated plus IL-2. These results indicate that defective IL-2 production by anergic cells is not solely responsible for the defect in induction of IL-12 responsiveness. Similar results were found when the anergic cell defect for induction of IL-4 responsiveness was analyzed (8).

Impaired Induction of IL-12-mediated Proliferation Is Observed in Anergic CD4⁺ T Cells Tolerized In Vivo. We tested whether normal T cells that are tolerized in vivo are also resistant for the induction of IL-12 responsiveness. Anergic CD4⁺ T cells were obtained from TCR V β 8.1 transgenic

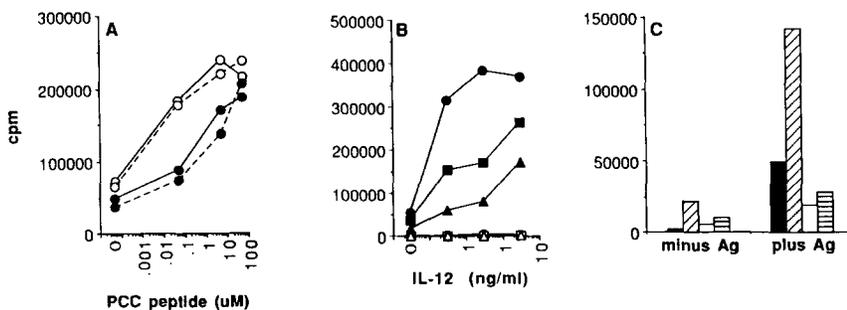


Figure 3. Effects of CsA and IL-2 on IL-12-mediated proliferation. (A) CsA does not inhibit the IL-12-mediated proliferation of control or anergic Th1 cells after activation by Ag:APC. Control (open circles) or anergic (closed circles) A.E7 cells were rested for 8 d and then were activated for 18 h by culture with APC plus Ag at the concentrations indicated. Viable cells were then collected, washed, and cultured with IL-12 (1 ng/ml) in the presence (broken lines) or absence (solid lines) of CsA (200 ng/ml) for 2 d. Note that the anergic cells required a 100-fold greater Ag concentration than control cells to achieve a comparable subsequent response to IL-12. (B) CsA does inhibit the Ag-

mediated induction of IL-12 responsiveness. Control A.E7 cells were cultured for 18 h without Ag (open symbols) or with 50 μ M PCC peptide plus APC (solid symbols), in the presence of CsA at 0 ng/ml (circles), 100 ng/ml (boxes), or 500 ng/ml (triangles). Viable cells were then recovered, washed, and incubated with IL-12 for proliferation (2×10^4 /well). Cells precultured with or without CsA had comparable recoveries and subsequent responses to exogenous IL-12 (data not shown), demonstrating that CsA was not toxic in the activation step. (C) IL-2 addition during Ag activation does not fully reconstitute the subsequent response of anergic cells to IL-12. Control or anergic A.E7 cells were rested for 3 d and then were cultured for 18 h with APC in the absence or presence of 5 μ M Ag, plus or minus exogenous IL-2 (0.5 ng/ml). After culture with Ag/IL-2, viable T cells were recovered, washed, and rested for 3 d in medium alone. Finally, viable cells were collected for stimulation of proliferation in response to IL-12 alone (1 ng/ml) at 10^4 cells/well. Cells cultured without IL-12 incorporated only background levels of [3 H]TdR (180–480 cpm; data not shown). Control cells (–) IL-2 (solid bars); control cell (+) IL-2 (hatched bars); anergic cells (–) IL-2 (open bars); and anergic cells (+) IL-2 (horizontal stripes). Although the growth of anergic cells in exogenous IL-2 can result in the reversal of anergy (17, 24), anergy was not lost in these experiments, perhaps because cell division was limited after a single exposure to IL-2. Anergic cell recoveries from cultures containing IL-2 were increased by twofold over those from cultures without IL-2, but we have found that a fivefold increase is needed to demonstrate reversal of anergy (Quill, H., unpublished observations).

Mls-1^b mice after injection of Mls-1^a-positive spleen cells. These transgenic mice express V β 8.1 on >95% of peripheral T cells and become tolerant for TCR-mediated proliferative responses after intravenous exposure to the Mls-1^a superantigen that is recognized by V β 8.1 (6). The persistence of a large number of anergic V β 8.1 cells is evident in the periphery of these mice, and these anergic cells are unable to produce IL-2 or to proliferate when stimulated in vitro with Mls-1^a-positive APC (6, and Table 1). In the presence of IL-12, both control and anergic cells showed enhanced proliferation over that seen with Ag alone, but the anergic cell response was considerably lower than that found with control cells (Table 1). Therefore, anergic CD4⁺ T cells tolerized in vivo also exhibit impaired IL-12 responsiveness, similar to our findings with Th1 clones that are tolerized in vitro.

Effect of IL-12 on the Production of IFN- γ by Anergic Th1 Cells. In addition to stimulating T cell proliferation, IL-12 is known to induce IFN- γ production by T cells and to synergize with TCR-mediated stimulation, IL-2 and other stimuli for IFN- γ production (12, 13). As an independent assay for induction of IL-12 responsiveness in anergic Th1 clones, we also measured IFN- γ secretion during culture with Ag/APC plus IL-12. In contrast to the nearly complete block in induction of proliferation to Ag/APC, anergic Th1 cells can be stimulated to produce IFN- γ , but they are less sensitive to Ag for this response (2). As shown in Fig. 4 B, anergic AC24 cells stimulated by Ag/APC, in the absence of IL-12, required a 100-fold higher Ag concentration than control cells for equivalent IFN- γ production. In the absence of Ag, IL-12 did not stimulate IFN- γ secretion in either control or anergic

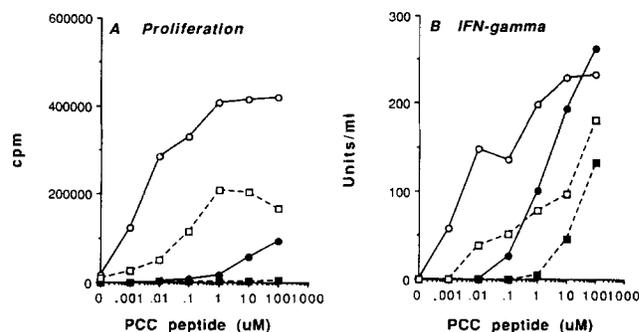


Figure 4. IL-12-mediated enhancement of IFN- γ production by Ag-activated anergic vs control Th1 cells. Control (circles, solid lines) or anergic (boxes, broken lines) AC24 cells were rested for 13 d before restimulation with Ag/APC in the absence (closed symbols) or presence (open symbols) of rIL-12 (5 ng/ml). Aliquots of culture supernatants were removed for IFN- γ assay (B) and the cells were then pulsed with [³H]TdR to assess proliferative responses (A). Similar results were found with anergic A.E7 cells, although A.E7 clone was generally less sensitive than the AC24 clone for IL-12-mediated enhancement of IFN- γ production.

cells. When IL-12 was added with Ag, the response of both control and anergic cells was enhanced, such that a 100-fold lower Ag concentration could be used to stimulate IFN- γ production. However, even in the presence of supraoptimal IL-12 concentrations, IFN- γ production by anergic cells was less than that found for control cells. Addition of exogenous IL-2 together with IL-12 during Ag stimulation (data not shown) could not fully restore the IFN- γ response, confirming the results obtained in proliferation assays and indicating that

Table 1. Impaired Induction of IL-12-mediated Proliferation in Anergic CD4⁺ T Cells Tolerized to Mls-1^a In Vivo

T cell source*	IL-12 (3 ng/ml)	Stimulator cells [†]	
		CBA/Ca (Mls-1 ^b)	CBA/J (Mls-1 ^a)
<i>cpm</i>			
Experiment 1			
Control mice	-	325 (109) [§]	48,316 (2,741)
	+	374 (80)	84,207 (4,119)
Mls-1 ^a -inoculated mice	-	270 (96)	736 (110)
	+	637 (200)	3,731 (869)
Experiment 2			
Control mice	-	210 (17)	20,908 (1,245)
	+	294 (85)	36,109 (1,798)
Mls-1 ^a -inoculated mice	-	439 (132)	1,970 (270)
	+	364 (66)	3,945 (1,476)

* CD4⁺ LN T cells were isolated from Mls-1^b, V β 8.1 transgenic mice inoculated with Mls-1^a spleen cells 14 d previously, or were isolated from control V β 8.1 mice injected only with PBS.

[†] T-depleted spleen cells from Mls-1^b or Mls-1^a normal mice were irradiated and cultured with V β 8.1 T cells to assess T cell proliferative responses.

[§] SEM of triplicate samples.

impaired induction of IL-12 responsiveness cannot be explained solely by the failure of anergic cells to produce IL-2.

Although the clear functional consequence of anergy is decreased Ag-dependent IFN- γ production even in the presence of IL-12, it is not yet known why IL-12 cannot rescue the IFN- γ response. Our data suggest the possibility that a primary IL-12-independent defect in Ag-mediated IFN- γ production may explain the decrease in total IFN- γ secretion by anergic cells cultured with Ag plus IL-12. As seen in Fig. 4 B, Ag does activate IL-12-dependent IFN- γ secretion in anergic cells, but only to a suboptimal level vs control cells. The difference between control and anergic cells seen in the absence of IL-12 is maintained in the presence of IL-12. Thus, although IL-12 cannot override the anergic cell defect in IFN- γ production, the relative activity of IL-12 is similar with control and anergic cells, suggesting that the limited IFN- γ response induced by Ag can be enhanced normally by IL-12 in anergic cells. It is possible, therefore, that decreased synergy between IL-12 and Ag/APC-mediated signals reflects an impaired contribution by TCR-mediated events, and not an intrinsic defect in the induction of the response to IL-12.

Although other explanations are possible, this interpretation would suggest a dissociation of IL-12 responses in anergic cells, such that IL-12-mediated proliferation is severely blocked, but that the IL-12-mediated enhancement of IFN- γ production can still occur. Dissociation of IL-12 responses might reflect different signaling pathways used for proliferation vs IFN- γ production, or might result from heterogeneity in the IL-12R, which has not yet been well characterized (22).

IL-12 is thought to play a major role in generating and, perhaps, in perpetuating Th1-type responses *in vivo* (23), and it is of interest that anergy in both Th1 clones and in normal CD4⁺ T cells includes tolerance for IL-12-mediated proliferation. Once reagents specific for the murine IL-12R are available, it will be important to determine if the defect in anergic cells reflects an inability to express one or more of the putative subunits of the IL-12R (22), or alternatively, reflects a block in signaling pathways mediated by this receptor. The current results extend our understanding of anergic CD4⁺ T cells by demonstrating an additional functional defect that may contribute to the maintenance of tolerance for Th1-type responses.

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