Antigen-specific Activation, Tolerization, and Reactivation of the Interleukin 4 Pathway In Vivo

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Summary

The outcome of immune responses critically depends on the pattern of lymphokines secreted by CD4+ T cells. CD4+ T cells may differentiate into interleukin 2 (IL-2) and interferon γ secreting T helper 1 (Th1)-like cells or IL-4/IL-5/IL-10 secreting Th2-like cells. However, the mechanisms that regulate production of IL-4 or other T cell lymphokines in vivo remain unknown. We use the superantigen, Staphylococcus enterotoxin A (SEA), as a model antigen to characterize the signals that regulate the production of IL-4 in vivo. Induction of IL-4 in normal CD4+ T cells required stimulation with both antigen and IL-4. SEA-specific CD4+ T cells produced large amounts of IL-4 when restimulated within 10 d after in vivo priming. Repetitive application of both signals was required to prevent downregulation of IL-4 production. Although controversy exists regarding the susceptibility of Th2-like cells to tolerogenic signals, high doses of superantigen readily abolished the capacity to produce IL-4 in both naive T cells and in T cells already primed for IL-4 production. Infection with the nematode, *Nippostrongylus brasiliensis*, reversed the established T cell tolerance, whereas the signals which induced IL-4 production in normal T cells, antigen and IL-4, were not capable of reversing superantigen-specific tolerance in vivo. The major parameter that correlated with the capacity of parasitic infection to break tolerance was the magnitude of the lymphoproliferation seen during the course of the infection. The capacity to activate or tolerize the IL-4 pathway in an antigen-specific fashion should prove useful in the design of antigen-specific therapies for autoimmune and allergic diseases.

Activation of CD4+ T cells by their target antigens and APC induces a cascade of differentiation events that result primarily in the secretion of IL-2. During the subsequent course of the immune response, CD4+ T cells may differentiate into IL-2 and IFN-γ secreting Th1-like cells or IL-4/IL-5/IL-10 secreting Th-2 like cells (1, 2). Thus, T cell activation by antigen and APC initiates not only effector T cell functions, but also programs the lymphokine pattern that primed T cells secrete during subsequent interactions with their target antigens. Although a number of factors have been proposed (3–13) to regulate the type of lymphokine produced, it appears that lymphokines themselves may play the most dominant role in the regulation of the capacity of naive T cells to differentiate into Th1- or Th2-like populations (9–12).

The requirements for the induction of the IL-4 pathway have been extensively characterized in vitro (8–12). The capacity to produce IL-4 is not a predetermined characteristic of a subpopulation of CD4+ T cells, but reflects a differentiation event during the process of T cell activation (2). The presence of IL-4 at the time of stimulation of T cells with either polyclonal activators or specific antigen results in the priming of T cell populations which, when restimulated, produce high levels of IL-4, but little or no IL-2 or IFN-γ. Conversely, activation of resting T cells in the absence of IL-4 results in the priming for IFN-γ production and this priming can be blocked by the presence of IL-4. Although IL-4 can readily be induced in vivo with polyclonal stimulators (8–12) such as injection of anti-IgD or parasite infection, the specific signals needed to induce IL-4 could not be readily analyzed. Induction of IL-4 can be diminished by treatment of animals with anti-IL-4, suggesting that IL-4 is also required for its own induction in vivo (4, 14–17).

Although progress has been made in understanding the regulation of the induction of cytokine production, much less is known and considerable controversy exists regarding the requirements for the induction of nonresponsiveness or anergy in Th1- and Th2-like CD4+ T cells. CD4+ Th1 clones can be easily rendered anergic by culture with antigen-pulsed, fixed APC or plate-bound anti-CD3, whereas similar treatment of Th2 clones results in no diminution of their capacity to produce IL-4 (18–23). It is difficult to generalize from these studies of T cell clones as to the requirements

for the tolerization of naive CD4+ T cells in vitro because the protocols developed for use with clones are relatively ineffective in the induction of anergy in naive or short-term stimulated T cells. Studies in vivo have similarly demonstrated that those strategies that permit the induction of tolerance, such as the injection of deaggregated human IgG or high doses of hapten–protein conjugates, result in a selective deficiency of antigen-specific proliferation, IL-2, and IFN-γ production, but prime rather than tolerize IL-4–producing cells (24–26). Taken together, these as well as other studies (27–29) strongly suggest that the IL-4 pathway is profoundly resistant to tolerance induction both in vitro and in vivo, or alternatively, that the conditions for the induction of tolerance in Th2-like cells have not yet been defined.

Here we use the superantigen, Staphylococcus enterotoxin A (SEA),[1] as a model antigen to characterize the signals that regulate the IL-4 pathway in vivo. In BALB/c mice, SEA selectively activates TCR Vβ1+ and Vβ10+ T cells (30), without detectable priming for IL-4. First, we demonstrated that administration of IL-4 during the period of T cell activation with SEA was required to prime T cells in vivo for IL-4 production. We then tested the responsiveness of the IL-4 pathway to tolerogenic signals in vivo. It is surprising that the IL-4 pathways of both naive and primed T cells were highly susceptible to tolerance induction. Lastly, the restoration of the IL-4 pathway in tolerant T cells required polyclonal activation and expansion of the tolerant population, whereas those stimuli which were effective in priming naive T cells for IL-4 production failed to induce IL-4 in tolerant T cells.

Materials and Methods

Animals. Virus-free female BALB/c mice were purchased from Frederick Animal Facility (National Cancer Institute, National Institutes of Health [NIH], Frederick, MD) and maintained in the animal facility of the Laboratory of Immunology, National Institutes of Allergy and Infectious Diseases [LI, NIAID], NIH. All mice were used between 6 and 15 wk of age and were cared for according to NIH guidelines.

Antibodies, Cytokines, and Superantigens. PE-labeled anti–mouse CD4 mAb, GK 1.5, was purchased from Becton Dickinson & Co. (Mountain View, CA); FITC-labeled anti-TCR Vβ10 and anti-TCR Vβ6 mAb were purchased from PharMingen (San Diego, CA); and purified rat anti–mouse IL-4 mAb 11B11 prepared by Verax Corp. (Hanover, NH) was a generous gift from Dr. W. E. Paul (LI, NIAID, NIH). Rat anti–mouse FcR mAb (24G2; American Type Culture Collection [ATCC], Rockville, MD) was used as diluted ascites. Highly purified, murine rIL-4 was the generous gift of Dr. A. Levine (Monsanto Corp., St. Louis, MO). 1 μg of IL-4 had the biological activity of 2 × 10⁶ U. Human rIL-2 was a gift from Cetus Corp. (Emeryville, CA). SEA was purchased from Toxin Technologies (Sarasota, FL) and SEB from Sigma Chemical Co. (St. Louis, MO). For cell separations, we used anti-CD8 mAb 3.155, anti-CD4 mAb RL172, and anti-Thy 1.2 mAb HO 13.4 as hybridoma supernatants (ATCC).

In Vivo Treatment. Mice were primed by a single subcutaneous injection of 50 μg SEA. Immediately after the first injection and subsequently every 8 h, the mice received the indicated dose of IL-4 intraperitoneally. SEA-specific tolerance was induced by a single subcutaneous injection of 100 μg SEA. In selected experiments, animals were infected subcutaneously at the indicated time with 700 third stage larvae of *N. brasiliensis* as previously described (14, 29).

Preparation of T Cells and Accessory Cells. Mesenteric lymph nodes and spleens were isolated and depleted of erythrocytes by lysis with ACK buffer. The cells were then incubated with anti-CD8, washed, and CD4+-enriched cells isolated by passage over mouse T cell columns (Biotex, Edmonton, Alberta, Canada). The resultant preparations were highly enriched in CD4+ cells and contained no detectable CD8+ cells. Accessory cells were prepared from the spleen of untreated, syngenic mice. After lysis with ACK lysing buffer, the cells were incubated with anti-CD8, anti-CD4 mAb, and anti-Thy 1.2, washed, and subsequently incubated for 45 min at 37°C with rabbit C (Cedarlane, Westbury, NY). These preparations contained no detectable CD4+ or CD8+ cells. Before use, accessory cells were irradiated with 3,000 rad from a Cesium source.

In Vitro Stimulation. CD4+-enriched cells were seeded in 96-well plates at three different cell numbers (0.25, 0.5, and 10⁶ cells) together with 10⁶ accessory cells in 250 μl of DMEM medium supplemented with 10% heat inactivated FCS (Biofluids, Inc., Rockville, MD), L-glutamine (2 mM), 2 β-ME (0.05 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells from each experimental group were cultured with media alone, with SEA (10 μg/ml), or with SEB (10 μg/ml) in the presence of rIL-2 (5 U/ml). After 2 d, supernatants were immediately used for lymphokine analysis or stored at −20°C.

Lymphokine Analysis. The IL-2 and IL-4 content of the culture supernatants was determined by assay on the IL-2–dependent CTLL cell line and on the IL-4–dependent CT.4S cell line (31), respectively. Serial dilutions of 2-d culture supernatants were incubated with CTLL (5 × 10³) in the presence of anti-IL-4 mAb 11B11 (32) or with CT.4S cells (5 × 10³). All assays also contained controls in which the cell lines were incubated with known amounts of rIL-2 and rIL-4. [³H]TdR incorporation was assayed after 20 h of culture (CTLL) or after 44 h of culture (CT.4S). 1 U of IL-2 was defined as half-maximal stimulation of CTLL cells; we could regularly detect 0.01 U/ml of IL-2. Lymphokine production is shown as units of IL-2 or IL-4 produced at a cell density of 10⁶ cells/ml.

FACS® Analysis. For FACS® analysis (Becton Dickinson & Co.) freshly prepared lymph node or spleen cells (10⁶) were first incubated with mAb 24G2 and subsequently stained for 30 min with 1–3 μg/ml of directly labeled anti-CD4 and anti-TCR Vβ10 or anti-CD4 and anti-TCR Vβ6 at 4°C. The cells were washed and the relative number of each cell population was determined by two color flowcytometry using a FACScan® (Becton Dickinson & Co.).

Results

Induction of Antigen-specific IL-4 Production In Vivo. Based on in vitro studies which demonstrate that induction of IL-4 production in CD4+ T cells requires both a TCR-mediated stimulus and IL-4, we speculated that the combined administration of nontolerogenic doses of SEA and IL-4 should upregulate the IL-4 pathway in vivo. Animals were primed with a single injection of SEA (50 μg) and treated on days 0–2 with IL-4 (1 μg every 8 h). Control animals received SEA alone, IL-4 alone, or no treatment. On day 6 after priming, CD4+ T cells were isolated from both lymph nodes and spleen and evaluated for lymphokine production in vitro after stimulation with either SEA or SEB. CD4+ T cells from

[1]*Abbreviation used in this paper:* SEA, Staphylococcus enterotoxin A.

untreated animals or animals treated with SEA alone or IL-4 alone produced no detectable IL-4 when stimulated in vitro (Fig. 1, *A* and *B*). In sharp contrast, CD4+ T cells isolated from animals primed with SEA and IL-4 produced large amounts of IL-4 when restimulated in vitro with SEA. In 10 independent studies, IL-4 production was upregulated between 100- and 1,000-fold. IL-4 was induced selectively in SEA-responsive T cells since SEB-responsive T cells from the same animals produced no detectable IL-4 when challenged in vitro (Fig. 1, *A* and *B*). Induction of IL-4 production was not observed at doses of IL-4 <1 μg every 8 h (Fig. 1 *B*).

The absolute amount of IL-4 produced by SEA-responsive CD4+ T cells by this priming protocol varied in different experiments by a factor of 10. To compare the efficiency of priming for IL-4 production by injection of SEA and IL-4 with a well-characterized stimulus for the induction of IL-4 in vivo, we compared the IL-4–producing capacity of T cells primed with SEA and IL-4 with the IL-4 producing capacity of SEA-responsive T cells derived from *N. brasiliensis*–infected

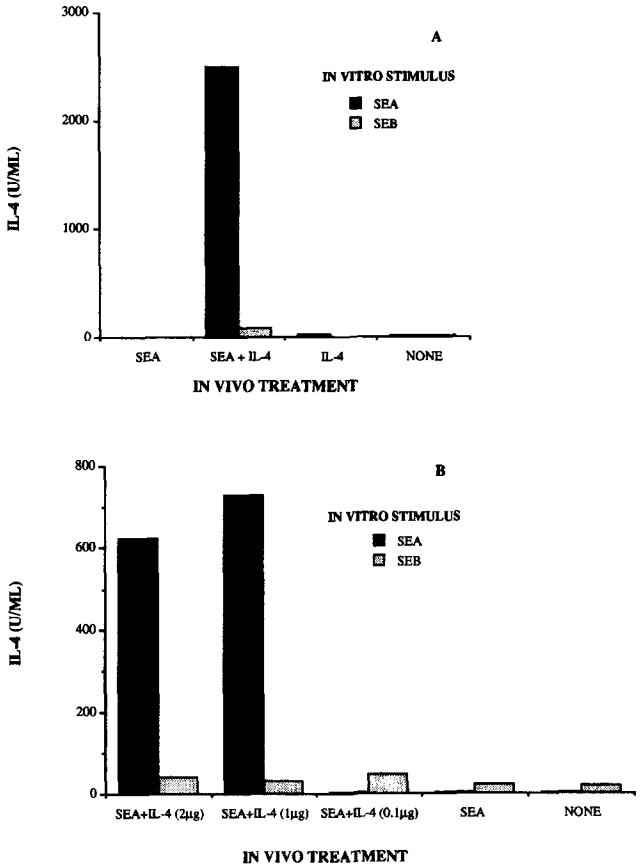

Figure 1. Induction of SEA-specific IL-4 production in CD4+ T cells. BALB/c mice were primed with a single subcutaneous injection of SEA (50 μg), with SEA and IL-4, with IL-4 alone, or received no treatment. Immediately with the first injection of SEA and subsequently every 8 h, the mice received nine injections of IL-4 (1 μg every 8 h, i.p., [*A*] or 0.1–2 μg [*B*]). CD4+ lymph node and spleen cells were isolated on day 6 after priming with SEA and IL-4 production was measured after stimulation with syngeneic APC and either SEA (10 μg/ml) or SEB (10 μg/ml) in the presence of rIL-2 (5 U/ml). (*A*) 1 of 10 similar experiments.

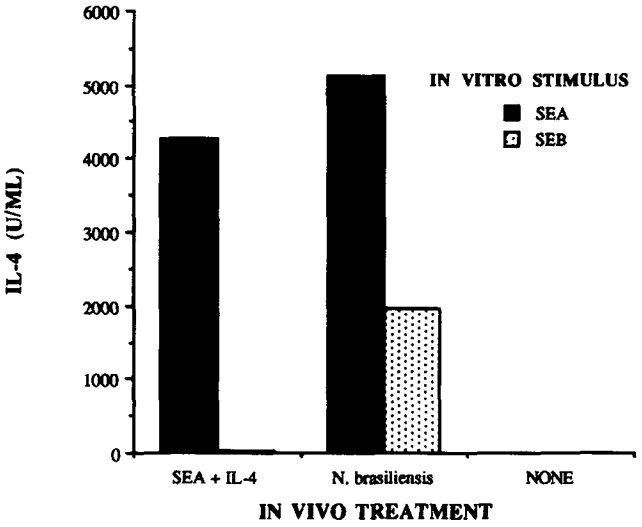

Figure 2. CD4+ T cells from animals primed with SEA and IL-4 or from animals infected with *N. brasiliensis* produce equivalent amounts of IL-4. BALB/c mice were infected with 700 third stage larvae of *N. brasiliensis*, primed with SEA and IL-4 (1 μg every 8 h for 3 d), or received no treatment. IL-4 production by CD4+-enriched lymph node and spleen cells was assayed on day 8 after infection or priming with SEA and IL-4 as described in Fig. 1.

animals. *N. brasiliensis* is one of the most powerful in vivo stimuli for induction of IL-4 production in CD4+ T cells. Repeated studies demonstrated that CD4+ T cells from animals primed with SEA and IL-4 and animals infected with *N. brasiliensis* produced a very similar quantity of IL-4 in response to SEA in vitro (Fig. 2).

Previous studies have shown that the induction of IL-4 production in vitro or in vivo requires 3–6 d of stimulation. After priming with SEA and IL-4, SEA-specific IL-4–producing CD4+ T cells were first detectable after 3 d and peak levels of IL-4 production were observed between days 6 and 10 (Fig. 3 *A*). In most experiments, the capacity to produce IL-4 declined sharply between days 10 and 14. This sharp decline in CD4+, SEA-responsive, IL-4–producing T cells could either be due to a downregulation of the IL-4 pathway or could be secondary to a deletion of the CD4+, SEA responsive (Vβ10) population. However, as shown in Table 1, the sharp decline in SEA-specific IL-4–producing T cells was never associated with a detectable decrease in CD4+Vβ10+ T cells. These kinetic studies suggested that the capacity of SEA and IL-4 to induce IL-4–producing T cells is only transient, and that the continuous presence of the inducing stimuli is required for maintenance of the pathway. To directly test this possibility, we injected animals a second time with the stimuli originally required to activate the IL-4 pathway in CD4+ T cells. Repriming of the animals with SEA and IL-4 prevented downregulation of the IL-4 pathway (Fig. 3 *B*).

Effect of Induction of SEA-specific IL-4 Production on SEA-specific IL-2 Production. Induction of IL-4–producing T cells in vitro and in certain situations in vivo has been associated

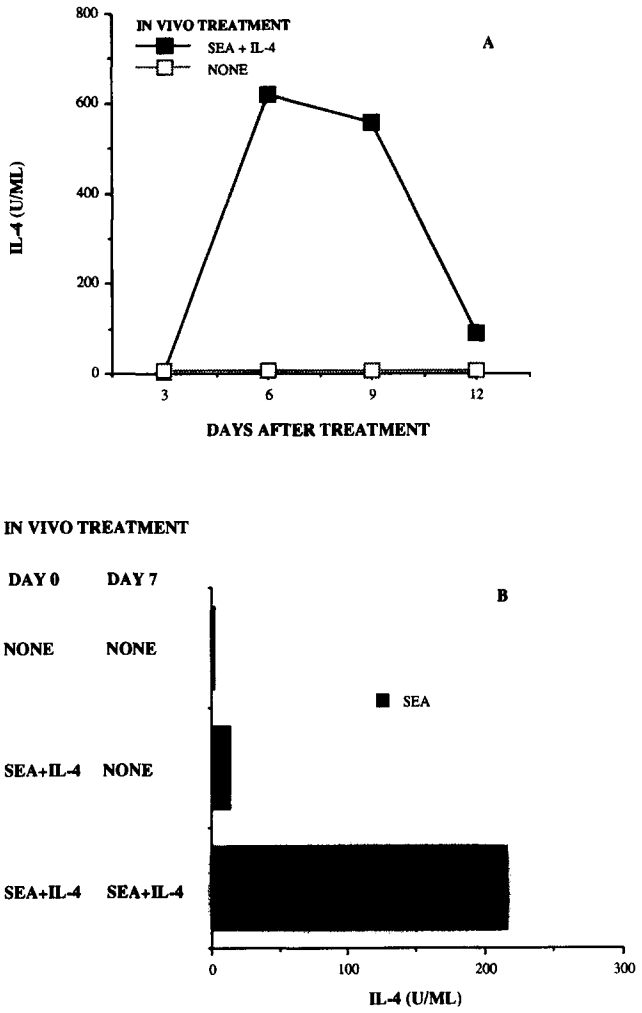

Figure 3. Repetitive treatment with SEA and IL-4 prevents downregulation of IL-4 production. (*A*) BALB/c mice were either primed with a single subcutaneous injection of 50 μg SEA and IL-4 (1 μg every 8 h for 3 d) or received no treatment. CD4+ T cells were assayed for IL-4 production in response to SEA on the day indicated as described in the legend to Fig. 1. (*B*) BALB/c mice were primed with SEA and IL-4 as in *A* or received no treatment. One group of the animals that had been primed with SEA and IL-4 received a second treatment with SEA (50 μg) and IL-4 (1 μg every 8 h for 3 d), starting on day 7. On day 13, CD4+ cells were isolated and assayed for SEA-specific IL-4 production.

with a downregulation of IL-2 production (2–4, 8, 11). To determine whether the priming regimen of superantigen and IL-4 activated IL-4 production independently of suppression of the IL-2 pathway, we measured IL-2 production by CD4+ T cells from animals primed with SEA alone or primed with SEA and IL-4. Injection of mice with 50 μg of SEA alone resulted in a 10-fold reduction of IL-2 production in response to SEA. A decline of similar magnitude was seen in the production of IFN-γ and IL-3 (data not shown). However, SEA-specific IL-2 production was still 10 times higher than background, and injection of animals with the combination of SEA and IL-4 did not further diminish the capacity to produce IL-2 (Fig. 4). The two concentrations of IL-4 used in this study resulted in an increase of IL-4 production of two to three orders of magnitude (Fig. 1 *B*).

Tolerization of the IL-4 Pathway. The ability to generate a potent SEA-specific IL-4 response in vivo allowed us to directly investigate whether superantigen-induced tolerance only affects the IL-2 pathway or whether superantigens also tolerize the IL-4 pathway. We first injected BALB/c mice with a tolerogenic dose of SEA (100 μg) and 5 d later treated the animals with the priming regimen of SEA and IL-4. Pretreatment of mice with tolerogenic doses of SEA completely abolished their response to a subsequent treatment with SEA and IL-4 (Fig. 5 *A*). To determine if the IL-4 pathway is also sensitive to tolerizing signals applied during the induction phase, mice were primed with SEA (50 μg) and IL-4, but then received two additional injections of SEA (50 μg) on days 3 and 6. When CD4+ T cells from these animals were restimulated in vitro, they produced no detectable IL-4 in response to SEA (Fig. 5 *B*). This downregulation of the IL-4

Table 1. *Silencing of the IL-4 Pathway Is Not Associated with a Decrease in CD4+ Vβ10+ T Cells*

In vivo treatment			Vβ10+/CD4+ cells	
Day 0	Day 3	Day 6	Lymph nodes	Spleen
			%	
None	None	None	7.9	6.4
SEA + IL-4	None	None	5.8	5.3
SEA + IL-4	None	SEA	4.5	5.1
SEA + IL-4	SEA	SEA	6.8	7.0

Animals received either no treatment or were primed with SEA (50 μg) and IL-4 (1 μg every 8 h for 3 d). Some of the animals received one or two additional injections of SEA (50 μg) on the indicated days. On day 12, lymph node and spleen cells were isolated and stained with anti-CD4 and anti-Vβ10 mAb. The relative numbers of CD4+ Vβ10+ T cells were determined by FACS analysis. The data, derived from the experiments in Fig. 5, are representative of two to three independent experiments.

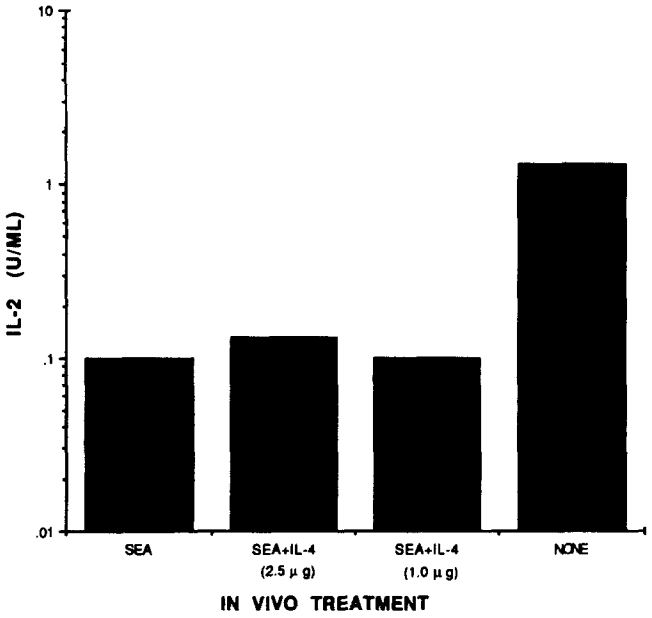

Figure 4. Effect of induction of IL-4 in CD4+ T cells on their capacity to produce IL-2. BALB/c mice were primed with a single subcutaneous injection of SEA (50 μg), with SEA and IL-4 (2.5 or 1 μg every 8 h for 3 d), or received no treatment. SEA-specific IL-2 production by CD4+ cells was assayed on day 6 after priming. The results are representative of 10 similar experiments.

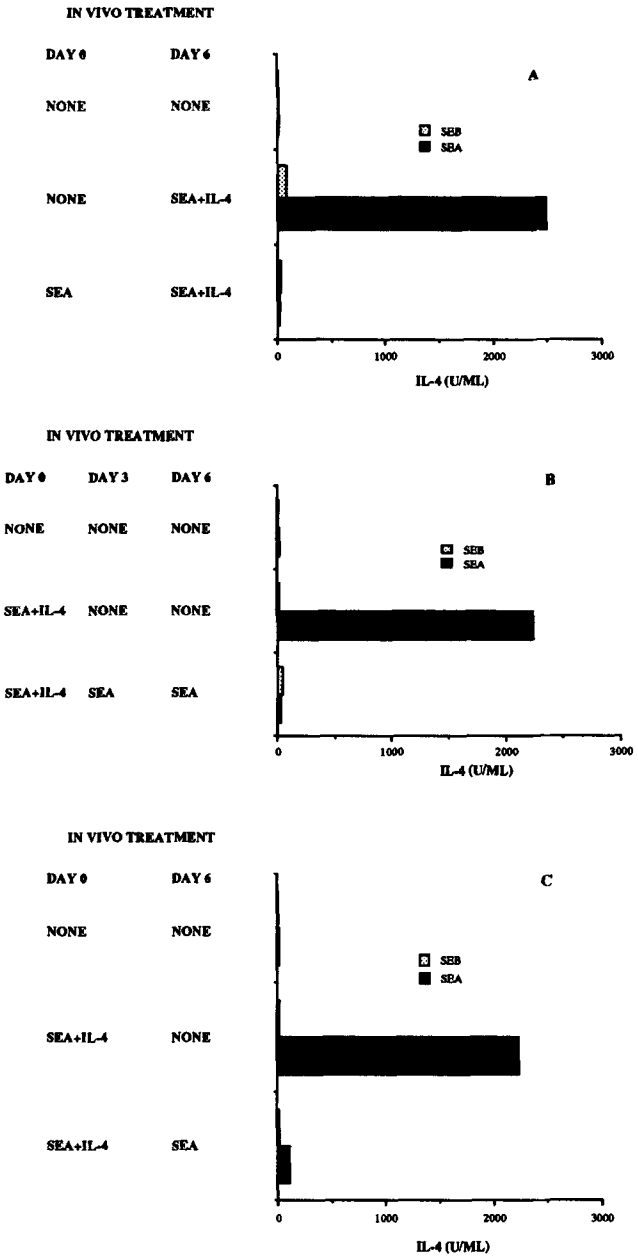

Figure 5. The IL-4 pathway is susceptible to tolerance induction in both naive T cells and T cells primed for IL-4 production. (*A*) Induction of tolerance in naive T cells. Animals were not treated or primed with SEA (100 μg, group III). On day 6, animals in groups II and III were injected with SEA (50 μg) and IL-4 (1 μg every 8 h for 3 d). On day 12, CD4+ T cells were isolated and assayed for SEA-specific IL-4 production. (*B*) Induction of tolerance during priming for IL-4 production. Animals were not treated or primed with SEA (50 μg) and IL-4 (1 μg every 8 h for 3 d). Animals in group III received two additional injections of SEA (50 μg) on days 3 and 6. On day 12, CD4+ T cells were isolated and assayed for SEA-specific IL-4 production. (*C*) Induction of T cell tolerance in primed T cells. Animals were not treated or primed with SEA (50 μg) and IL-4 (1 μg every 8 h for 3 d). Animals in group III received one additional injection of SEA (50 μg) on day 6. On day 12, CD4+ cells were isolated and assayed for SEA-specific IL-4 production. The percentage of Vβ10+ cells among CD4+ T cells is shown in Table 1. Similar results were obtained in two to three other experiments.

pathway was not prevented by additional treatment with IL-4 (until day 6; data not shown). The downregulation of IL-4 production was also not secondary to deletion of SEA-responsive CD4+ T cells, since animals that received one single or repetitive injections of SEA had similar percentages of SEA-responsive CD4+Vβ10+ T cells in lymph nodes or spleen between days 6 and 12 after injection of SEA (Table 1 and data not shown).

As the ability of Th2 or Th0 T cell clones to produce IL-4 is resistant to tolerogenic signals in vitro (20, 22, 23), it was of interest to examine in vivo the susceptibility of CD4+ T cells that had been primed for IL-4 production to tolerogenic signals. Mice were therefore primed with SEA and IL-4 and on day 6 after treatment, when IL-4 induction is maximal (Fig. 3 *A*), mice received a second injection of SEA (50 μg). It is surprising that CD4+ T cells from mice that received a second injection of SEA on day 6 produced <1% of the IL-4 produced by T cells from control animals that were primed in identical fashion, but that did not receive the second injection of SEA (Fig. 5 *C*).

Reactivation of the IL-4 Pathway. The failure to induce IL-4 in SEA-tolerant CD4+ T cells by treatment with SEA and IL-4 appears to conflict with our previous study which showed that *N. brasiliensis* infection induced SEB-specific IL-4 production in SEB-tolerant CD4+ T cells (29).

To study more closely the requirements to induce IL-4 in tolerant CD4+ T cells, CD4+ T cells from SEA-tolerant and control mice were isolated at different times after infection with *N. brasiliensis*. On day 7 after infection, CD4+ T

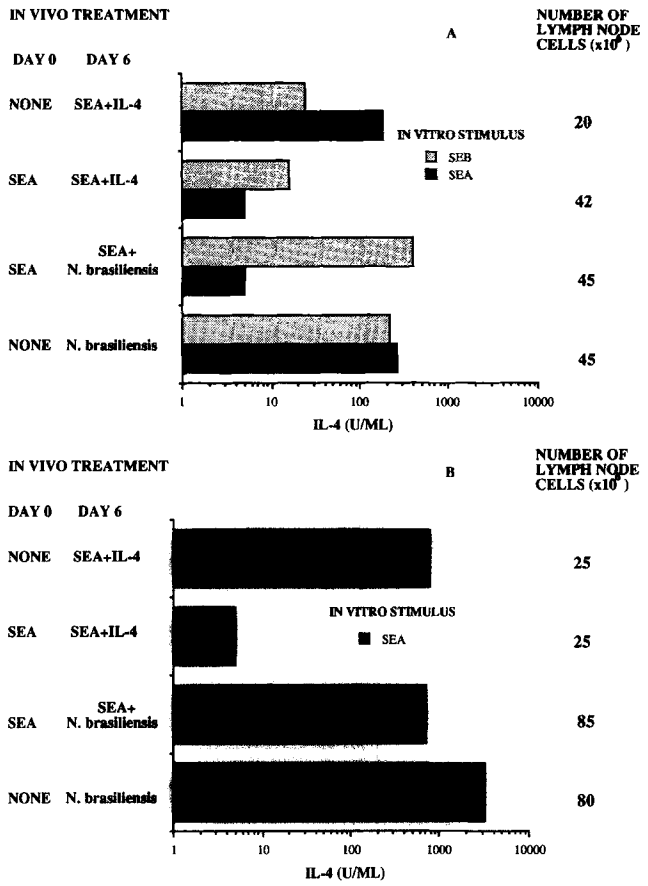

Figure 6. Reactivation of the tolerant IL-4 pathway requires reprogramming during parasite-mediated cell division. Mice were either primed with SEA (100 μg) or received no treatment. On day 6, animals were treated with SEA (50 μg) and IL-4 (1 μg every 8 h for 3 d), with SEA (50 μg) and infection with *N. brasiliensis*, or only infected with *N. brasiliensis*. On day 13 (*A*) or on day 15 (*B*), the number of mesenteric lymph node cells was determined, and $CD4^+$ T cells were assayed for IL-4 production in response to SEA or SEB.

cells from SEA-tolerant mice were not capable of producing IL-4 in response to SEA, whereas IL-4 was readily detected in SEB-responsive control populations (Fig. 6 *A*). However, on day 9 after infection, $CD4^+$ T cells from SEA-tolerant mice produced large amounts of IL-4 when challenged with SEA (Fig. 6 *B*). Although the absolute amount of IL-4 produced by $CD4^+$ cells from tolerant animals was still about fivefold lower than the amount of IL-4 produced by $CD4^+$ cells from control animals, *N. brasiliensis* infection induced more than a 100-fold increase in SEA-specific IL-4 production in the tolerant animals (Fig. 6 *B*). There was a close correlation between the magnitude of parasite-induced lymphoproliferation and the reversion of SEA-specific tolerance. At day 7 after the infection, the number of lymph node cells was only moderately increased, whereas the number of lymph node cells increased 3.5-fold during the next 2 d of infection (Fig. 6, *A* and *B*). However, the percentage of $CD4^+V\beta10^+$ T cells was constant (data not shown) during the entire period of infection with *N. brasiliensis*, indicating in vivo expansion of the SEA-responsive T cells. As previously reported (29), induction of IL-4 production was not accompanied by detectable SEA-specific IL-2 production (data not shown).

Discussion

Previous studies in vitro have strongly implied that one of the most critical factors that determines the differentiation of resting T cells into Th2-like IL-4–producing cells is IL-4 itself (8–12). We have analyzed the immune response to the superantigen, SEA, to determine the requirements for the induction of a Th2-like response in vivo. Priming of animals with SEA together with IL-4 resulted in a marked upregulation of SEA-specific Th2-like cells. Administration of either antigen alone or IL-4 alone failed to prime for IL-4 production. The induction of IL-4 was completely specific for the superantigen injected as no IL-4 production was seen in response to the control superantigen, SEB. When mice were injected with IL-4 for 3 d after priming with SEA, our ability to detect IL-4–producing T cells in vitro persisted until days 10–12, but not longer. It is unlikely that the waning of the IL-4–producing population was secondary to deletion of the superantigen-specific T cells, as no deletion of the SEA-responsive $V\beta$ populations was observed. Continued administration of IL-4 and SEA was required to maintain the Th2-like cells in vivo.

The requirement of IL-4 for the priming of Th2 cells in vivo is quite consistent with the results of studies that have used polyclonal inducers of IL-4 production and anti-IL-4 mAbs to define the role of IL-4 in priming for Th2-like responses (15, 17, 33). However, very few studies have attempted to characterize in vivo the signals required for the induction of IL-4 in normal $CD4^+$ T cells. Chatelain et al. (34) have shown that injection of healer C3H mice with IL-4 locally into *Leishmania*-infected footpads results in an increased production of IL-4 and IL-5, but a decreased production of IFN-γ, by draining lymph node cells harvested 48 h later. However, this shift in cytokine production was only transient, and all animals were ultimately able to heal their infections and produced Th1-like responses upon in vitro challenge.

Although our studies define the minimal requirements for the induction of IL-4 in normal T cells, it should be emphasized that it is very difficult to rule out the involvement of other cytokines particularly, IL-2, in the differentiation of SEA-specific Th2-like cells in vivo. A number of in vitro studies have suggested that IL-2 is required both for the induction and production of IL-4 (10, 12, 29, 35). The concentration of SEA we used for the induction of IL-4 was only partially tolerogenic, and SEA-responsive T cells were still capable of producing measurable amounts of IL-2 when stimulated in vitro with SEA. Thus, sufficient IL-2 may have been generated after injection of SEA and IL-4 to facilitate induction of IL-4 in $CD4^+$ T cells. Alternatively, it is possible that in vivo, other cytokines can substitute for IL-2, as recently suggested by studies with mice with targeted disruption of the IL-2 gene (36). In preliminary experiments, we have observed that treatment of mice with anti-IL-2 or with cyclosporin A at the time of priming with SEA and IL-4 did

not inhibit the generation of SEA-specific IL-4–producing T cells.

Our ability to specifically prime superantigen-specific IL-4–producing T cells in vivo allowed us to directly examine if IL-4–producing T cells could be tolerized in vivo. When SEA was first administered in a tolerogenic dose, our ability to prime for IL-4–producing T cells with SEA and IL-4 was completely abrogated. It is surprising that tolerance could also be induced in primed IL-4–producing cells. This dramatic susceptibility of Th2-like cells to tolerance induction is at marked variance with studies on Th2 and Th0 T cell clones in vitro in which the production of IL-4 is resistant to tolerogenic signals (22). It should be emphasized that our studies do not allow any conclusions to be drawn about the mechanism of induction of Th2-like T cell tolerance. As noted above, induction and expansion of Th2-like T cells in vivo may require IL-2, and the tolerization protocols used in our studies may tolerize the IL-2 pathway and thus inhibit the development or continued expansion of Th2-like T cells. IL-4 production by Th2 or Th0 clones in vitro may be independent of IL-2 and therefore resistant to the induction of tolerance.

Considerable controversy exists as to whether tolerogenic signals can silence the IL-4 pathway in vivo. Some studies have demonstrated that injection of mice with high concentrations of antigen, which results in suppression of IL-2 and IFN-γ production, primes for IL-4 production (24–26), whereas other studies have shown that similar treatment inhibits the development of IL-4–producing cells and decreases antibody production after administration of the peptide antigen in adjuvant (37, 38). Our data strongly suggest that induction or inhibition of IL-4 production critically depends on the dose of antigen as well as the amount of IL-4 supplied during T cell stimulation. One single injection of SEA (50 μg) together with IL-4 primed T cells for optimal IL-4 production, whereas two additional injection of SEA (50 μg) completely inhibited the development of IL-4–producing T cells. Similarly, $CD4^+$ T cells that had been primed for IL-4 production could be readily tolerized by reinjection of SEA, but this SEA-induced downregulation could be prevented by the simultaneous administration of IL-4. The susceptibility of primed Th2-like cells to tolerance induction in vivo has important implications for the design of therapeutic strategies for the immunomodulation of allergic diseases.

Our ability to reproducibly tolerize Th2-like cells afforded us the opportunity to reexamine the requirement for breaking T cell tolerance in vivo. We have previously shown that *N. brasiliensis* infection of animals tolerized with SEB resulted in a normal expansion of SEB-specific $CD4^+V\beta8^+$ T cells in vivo as well as an equivalent increase of SEB-reactive IL-4–producing T cells (29). We interpreted these studies as indicating that SEB injection rendered the IL-4–producing T cell population tolerant and that infection with *N. brasiliensis* resulted in breaking of the tolerant state by the induction of IL-4 in SEB-tolerant T cells. However, since we could not directly demonstrate that we had tolerized the IL-4 pathway in SEB-responsive cells, we could not exclude the possibility that infection with *N. brasiliensis* resulted in activation of a previously silent, but not tolerant, Th2-like population. The results of the present experiments directly demonstrate that infection with *N. brasiliensis* can break an established T cell tolerance. However, the signals required to induce IL-4 production in normal T cells, antigen and IL-4, were not capable of reversing SEA-specific tolerance in vivo. Furthermore, we could demonstrate that the major parameter that correlated with the capacity of infection with *N. brasiliensis* to break tolerance was the magnitude of the lymphoproliferation seen during the course of the infection. Thus, expansion of tolerant T (39–41) and B (42) cells appears to be one of the ways in which tolerance can be reversed in both T and B cells. Indeed, the polyclonal expansion of tolerant T cells seen during the course of infectious diseases or treatment with high doses of IL-2 may reactivate tolerized lymphokine pathways and has resulted in the induction of autoimmunity (43).

Although some studies suggest that the differentiation of $CD4^+$ T cells into Th1- or Th2-like subpopulations is established at the time of T cell priming and may become fixed in a short period of time (33), we have shown that Th1-like T cell clones can still be converted into Th2-like cells after 3 wk of culture (2). Similarly, during the course of an autoimmune disease, autoreactive T cells are continuously being generated and the administration of IL-4 might influence the lymphokine phenotype of these newly produced T cells. In this regard, we (Racke, M. K., A. Bonomo, D. E. Scott, B. Cannella, A. D. Levine, C. S. Raine, E. M. Shevach, and M. Röcken, manuscript submitted for publication) have recently shown that the induction of experimental allergic encephalomyelits by the transfer of Th1-like T lines can be markedly inhibited by the administration of IL-4 at the time of cell transfer. The beneficial effects of the administration of IL-4 are associated with the generation of a myelin basic protein–specific population of IL-4–producing cells in the recipients.

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