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.
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 primes 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 [NIH, NIAID], NIH. All mice were used between 6 and 15 wk of age and were cared for according to NIH guidelines.

Antibodies, Cytokines, and Supemntigens. 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, NJ) was a generous gift from Dr. W. E. Paul (NIH, NIAID, NIH). Rat anti–mouse FeR 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 × 10^3 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, from Cetus Corp. (Emeryville, CA). SEA was purchased from Toxin Technologies (Sarasota, FL) and SEB from Sigma Chemical Co. (St. Louis, MO). 1 μg of Ib4 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 × 10^3 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).

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 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).

1Abbreviation 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 \(\mu\)g 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 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 (V\(\beta\)10) 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\(^+\)V\(\beta\)10\(^+\) 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).

![Figure 1](image1.png)  
**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 \(\mu\)g), 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 \(\mu\)g every 8 h, i.p., [A] or 0.1-2 \(\mu\)g [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 \(\mu\)g/ml) or SEB (10 \(\mu\)g/ml) in the presence of rIL-2 (5 U/ml). (A) 1 of 10 similar experiments.

![Figure 2](image2.png)  
**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 \(\mu\)g 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.

**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
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).

**Tolarization 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 tolerate 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 re-stimulated in vitro, they produced no detectable IL-4 in response to SEA (Fig. 5 B). This downregulation of the IL-4 pathway 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.

### 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 3 | Day 6 | Lymph nodes | Spleen |
|-------------------|------------------|------|------|-------------|--------|
| None              | None             | None | None | 7.9         | 6.4    |
| SEA + IL-4        | None             | 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.
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
was only moderately increased, whereas the number of lymphocytes from control animals, produced large amounts of IL-4 when stimulated in vitro with SEA. Thus, sufficient IL-2 may have been generated at injection of SEA and IL-4 to facilitate induction of IL-4 in 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

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 Vβ 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.

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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 tolerate 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+ Vβ8+ 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 encephalomyelitis 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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