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Stat6 Regulation of In Vivo IL-4 Responses

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Although in vitro development of a Th2 response from naive CD4\(^+\) T cells is Stat6 dependent, mice immunized with a goat Ab to mouse IgD have been reported to produce a normal primary IL-4 response in Stat6-deficient mice. Experiments have now been performed with mice immunized with more conventional Ags or inoculated with nematode parasites to account for this apparent discrepancy. The ability of an immunogen to induce a primary in vivo IL-4 response in Stat6-deficient mice was found to vary directly with its ability to induce a strong type 2 cytokine-biased response in normal mice. Even immunogens, however, that induce strong primary IL-4 responses in Stat6-deficient mice induce poor memory IL-4 responses in these mice. Consistent with this, Stat6-deficient CD4\(^+\) T cells make relatively normal IL-4 responses when stimulated in vitro for 3 days with anti-CD3 and anti-CD28, but poor IL-4 responses if they are later restimulated with anti-CD3. Thus, Stat6 signaling enhances primary IL-4 responses that are made as part of a type 0 cytokine response (mixed type 1 and type 2) and is required for normal development or survival of Th2 memory cells. The Journal of Immunology, 2000, 164: 2303–2310.

Infectious agents can induce rodent and human T cells to secrete polarized sets of cytokines that influence the host’s ability to defend against the pathogen (1, 2). No single set of cytokines is universally protective for the host: protection against intracellular parasites is generally promoted by type 1 cytokines, cytokines is universally protective for the host: protection against gastrointestinal nematode parasites is promoted by the type 2 cytokines, IL-4, IL-9, and IL-13, but inhibited while protection against gastrointestinal nematode parasites is promoted by the type 2 cytokines, IL-4, IL-9, and IL-13, but inhibited by IFN-\(\gamma\) (2, 3).

Considerable progress has been made toward elucidating the mechanisms that regulate the differentiation of naive CD4\(^+\) T cells into Th1 or Th2 cells. Considerable progress has been made toward elucidating the mechanisms that induce type 1 cytokine production. Ligation of cellular receptors that recognize characteristics shared by multiple pathogens, called pattern recognition molecules (4 – 6), can stimulate APC to secrete IL-12, IL-18, IFN-\(\gamma\), and IFN-\(\beta\), which promote the development of a type 1 cytokine response and inhibit the development of a type 2 response (7–10).

Identification of the mechanisms that induce type 2 cytokine responses, in general, and IL-4 responses, in particular, has been more difficult. Several in vitro studies and some in vivo studies indicate that IL-4, itself, stimulates the development of a type 2 cytokine response and does so by binding to IL-4R\(\alpha\) and activating the IL-4R\(\alpha\)-associated signaling molecule, Stat6 (11–18). Observations that CD4\(^+\) T cells from Stat6-deficient mice fail to differentiate into Th2 cells when stimulated in vitro with Ag or anti-CD3 mAb \(\pm\) anti-CD28 mAb, even in the absence of IL-12 signaling (19); that a Stat6 binding site is present on the IL-4 promoter (16); and that occupancy of this site by Stat6 enhances IL-4 gene transcription (20, 21) are all consistent with the concept that Stat6 signaling is required to induce naive CD4\(^+\) T cells to differentiate into polarized Th2 cells.

The view that Stat6 activation by IL-4 is required to induce naive CD4\(^+\) T cells to differentiate into Th2 cells requires a source for the IL-4 that would prime the T cell response. Several sources for this initial IL-4 have been suggested, including mast cells, basophils, eosinophils, and NK T cells (22–26). Although each of these cell types can produce IL-4, none has been found to be necessary for the development of an in vivo IL-4 response (27–31). These observations are compatible with the possibility that the in vivo IL-4 response by conventional CD4\(^+\) T cells can be primed by multiple redundant sources of IL-4, so that deletion of any one source might have little effect on in vivo IL-4 production.

An in vivo observation, however, challenges the view that Stat6 signaling is required to induce IL-4 production: Stat6-deficient mice immunized with an affinity-purified goat Ab to mouse IgD (GaM\(\delta\))\(^4\) make an IL-4 response that has the same magnitude and kinetics as the response made by wild-type mice (31). It is unlikely that the large Stat6-independent IL-4 response in GaM\(\delta\)-immunized mice is made by cells other than CD4\(^+\) T cells (IL-4 production in GaM\(\delta\)-immunized wild-type mice is blocked by anti-CD4 mAb (32) and only CD4\(^+\) T cells from these mice express increased IL-4 mRNA (33)) or that it is coming from classic NK T cells (the response is \(\beta_2\)-microglobulin independent and class II MHC dependent (31)). Furthermore, studies of mice in which the absence of a functional IL-4R\(\alpha\) gene eliminated any possibility of IL-4 priming of T cell IL-4 production demonstrated that T cell IL-4 responses can be generated in the absence of IL-4R\(\alpha\) signaling (34).

\(^4\)Abbreviations used in this paper: GaM\(\delta\), affinity-purified goat anti-mouse IgD Ab; APF, Ascaris pseudocoelemic fluid; CCCA, Cincinnati cytokine capture assay; CFSE, carboxyfluorescein diacetate succinimidyl ester.

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The unexpected results of these studies led us to investigate whether Stat6-independent IL-4 production in GaMé-immunized mice remains IL-4Rα dependent and whether Stat6-independent IL-4 production can be induced in vivo by immunogens that are more conventional than GaMé. In addition, because host protection against gastrointestinal nematode parasites is Stat6 dependent (2, 3), we investigated the Stat6 dependence of IL-4 responses to these worms. Results of these studies demonstrate that the in vivo IL-4 response to GaMé is IL-4Rα independent as well as Stat6 independent and that all Ags tested can induce at least some IL-4 production in Stat6-deficient mice, although Ags that induce the strongest and most polarized type 2 responses in normal mice appear to depend least on Stat6 signaling to induce maximal IL-4 production. In contrast to primary IL-4 responses, secondary IL-4 responses are Stat6 dependent even when they are induced by stimuli that evoke large, Stat6-independent IL-4 primary responses. These observations suggest that Stat6 signaling is less important for the initial production of IL-4 by naive T cells than for the generation or survival of memory T cells that secrete IL-4.

Materials and Methods

Mice

Stat6-deficient mice on a mixed C57BL/6-129 background (14) were a gift of Dr. James Ihle (Memphis, TN); a separately produced line of Stat6-deficient mice (13), which had been bred onto a BALB/c background, was a gift of Dr. Michael Grusby (Cambridge, MA). IL-4Rα-deficient mice (55), which were produced on a BALB/c background, were bred at National Institutes of Health (Bethesda, MD). W/W, μM, and appropriate control mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Stat6-deficient mice and appropriate controls were bred in the laboratory animal facility at the Cincinnati Veterans Administration Medical Center (Cincinnati, OH). All mice were age and sex matched with controls in any given experiment.

Immunological reagents

GaMé and normal goat IgG were produced and purified as described (36). Hybridomas that secrete a cytotoxic rat IgG anti-mouse CD4 mAb (GK1.5 (37), a stimulatory anti-CD3ε mAb (145-2C11) (38), or a neutralizing anti-IFN-γ mAb (R46-A2) (39) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Hybridomas that produce neutralizing or nonneutralizing rat IgG anti-IL-4 mAbs (BV4D-ID1 and BV6D-24G2, respectively) (40) were obtained from the ATCC with the permission of the DNAX Research Institute (Palo Alto, CA) and the assistance of Dr. Robert Coffman (DNAX). Hybridomas that produce the control rat IgG2b mAb, J1.2, or a nonneutralizing anti-IFN-γ mAb (AN18) (41) were gifts of Dr. John Abrams and Anne O’Garra, respectively (DNAX). All of these hybridomas were grown as ascites in Pristane-primed athymic nude mice. All mAbs were purified from ascites by salt precipitation and ion-exchange chromatography, as described (42), using isotype-specific antisera (Miles Labs, Naperville, IL) and gel-double diffusion analysis to identify Ab-rich fractions. Human IL-2 and mAbs specific for mouse IL-4, IFN-γ, IL-2, and IL-3 were used in some experiments. Rabbit anti-IL-13Rα1 was a gift of Genzyme (Cambridge, MA). Some mAbs were biotin conjugated, using biotin N-hydroxy-succinimide (Calbiochem-Behring, La Jolla, CA), as described (32), at a 1:10 (w/w) biotin:protein ratio.

The pseudocelomonic fluid (APF), a strong allergen, was prepared as follows: Adult male and female worms were freshly recovered from the intestines of pigs at a local abattoir. The worms were kept at room temperature in a PBS solution in transit to the laboratory, where they were washed in PBS several times. The pseudocelom was cut with a scissors and the fluid drained into a collection flask. The fluid was clarified by centrifugation at 10,000 rpm for 20 min at 4°C and the supernatant stored frozen at −20°C until used.

Nematode parasites

Nippostrongylus brasiliensis larvae (L3), Heligmosomoides polygyrus larve (L3), and Trichinella spiralis muscle stage larvae were prepared as described (45–47). Mice were inoculated by s.c. injection with 500 N. brasiliensis L3 or by oral gavage with 200 H. polygyrus L3 or 50 T. spiralis muscle stage larvae. In experiments that examined responses to a second infection, primary infections were cured by treating mice by oral gavage with 1 mg of pyrantal pamoate (Strongid T; Pfizer, New York, NY) (47).

Cincinnati cytokine capture assay

The Cincinnati cytokine capture assay (CCCA) was used to monitor in vivo production of IL-4 and IFN-γ. This assay allows cytokines to accumulate in serum by capturing them in vivo with neutralizing IgG mAbs that inhibit their excretion, utilization, and catabolism (48). This increases the ability to detect IL-4 in serum by ~1000-fold and is specific (no IL-4 response is detected in IL-4-deficient mice). To capture secreted IL-4, mice were injected i.v. with 10 μg of biotin-BV4D-ID11. Mice were bled 2–4 h later, and serum levels of IL-4-biotin-anti-IL-4 complexes were determined by ELISA, using microtiter plates coated with BV6D-24G2.3. This mAb recognizes an IL-4 epitope that is distinct from that recognized by the neutralizing anti-IL-4 mAb BV4D-ID11, that was injected into mice. A CCCA for IFN-γ was performed similarly by injecting mice with 50 μg of biotin-R46-A2 and coating microtiter plates with AN18.

Serum IL-13 assay

Mouse IL-13 ELISA kits were purchased from R&D Systems (Minneapolis, MN) and were performed according to the manufacturer’s instructions, with the exception that rabbit IgG anti-IL-13Rα2 Ab (100 μg/ml) was added during the primary incubation period to 1/10 or 1/100 dilution of serum samples and incubated overnight at 4°C, to dissociate IL-13 from serum sIL-13Rα2. Duplicate serum samples were examined from each animal. OD readings were converted to pg/ml using a standard curve and the appropriate dilution factor. Rabbit IgG anti-mouse IL-13Rα2 had no effect on the assay curve for purified mouse IL-13. Addition of mouse serum to an IL-13 standard decreased assay sensitivity.

To determine whether production of rabbit anti-mouse IL-13Rα2 Ab would affect assay sensitivity, we compared the effect of adding rabbit IgG anti-mouse IL-13Rα2 (100 μg/ml) or an irrelevant rabbit IgG Ab during the primary incubation period to the IL-13 standard diluted in 50% serum. ELISAs were performed as described and demonstrated 100% recovery of IL-13 standard curve sensitivity, up to an IL-13 dose of 250 ng/ml.

In vitro stimulation of cytokine production and identification of IL-4- and IFN-γ-producing cells

Peripheral lymph node and spleen cells were depleted of CD8+ T cells, labeled with CFSE, and cultured with 2 μg/ml each of soluble anti-CD3 and CD28 mAbs plus 2.5 U of human IL-2, as described (43). After 3 days, cells were stimulated for 4 h with PMA and ionomycin, stained with tricolor-labeled anti-CD4 mAb, permeabilized, stained with PE-labeled anti-IL-4, and analyzed by flow cytometry for CFSE and PE staining of tricolor positive cells (43). A second aliquot of CD8+ T cell-depleted cells was cultured for 6 days with soluble anti-CD28 and anti-CD3 mAbs plus IL-2, washed, recultured for 24 h with plate-bound anti-CD3 mAb, stimulated for 4 h with PMA and ionomycin, permeabilized, and stained with FITC anti-IFN-γ and PE IL-4, then analyzed by flow cytometry for FITC and PE staining (43).

Results

IL-4Rα-deficient mice make a strong IL-4 response to GaMé

Our observation that Stat6-deficient mice make a normal IL-4 response to GaMé left open the possibility that IL-4 can prime CD4+ T cells to secrete IL-4 through a Stat6-independent mechanism. To
test this possibility, IL-4 secretion was measured following GaMδ injection in BALB/c mice that were heterozygous or fully deficient for a functional IL-4Ra allele (Fig. 1). IL-4 levels, as detected by the CCCA, were considerably higher in IL-4Ra-deficient mice than in IL-4Ra heterozygotes. This may reflect absent IL-4 utilization in IL-4Ra-deficient mice, rather than increased IL-4 production (GaMδ induces similar IL-4 mRNA responses in wild-type and IL-4Ra-deficient mice (N. Noben-Trauth, unpublished data)), but clearly demonstrates that no IL-4 signal is required for GaMδ induction of a strong IL-4 response.

The in vivo IL-4 response to a standard protein Ag is more Stat6 dependent than the response to a potent allergen

To determine whether the in vivo IL-4 response to a conventional Ag is as Stat6 independent as the GaMδ-induced IL-4 response, we used the CCCA to measure in vivo IL-4 production in wild-type and Stat6-deficient mice immunized with chicken OVA, an Ag that induces a type 2 cytokine response in mice (49). To avoid the use of adjuvant, which might modify cytokine responses, mice were injected i.p. once per day with 1 mg of OVA, a dose that we have previously found to induce a large IgG1 anti-OVA Ab response (our unpublished data). IL-4 production was detectable in OVA-immunized wild-type mice by day 3, peaked on day 6, and was still considerable on day 10 (Fig. 2, upper panel). In contrast, Stat6-deficient mice immunized and tested at the same time by the same protocol developed an IL-4 response that was only detectable at day 6 and was only 37% as large as the wild-type response at that time point. When immunized daily i.p. with 50 μl of APF, a potent allergen (50, 51), Stat6-deficient mice again made a smaller IL-4 response than wild-type mice; however, IL-4 responses were detectable in both strains at all time points, and peak values in the Stat6-deficient mice were closer (69%) to those in the wild-type mice (Fig. 2, lower panel).

Stat6-deficient mice make normal IL-4 responses to an initial N. brasilensis infection, but a reduced IL-4 response to an initial T. spiralis infection

Results of the previous experiment suggested that Stat6 signaling might be more important for the induction of an IL-4 response by a conventional Ag (OVA) than by Ags that induce a strongly biased type 2 cytokine response (GaMδ and APF) (33, 50, 51). To determine whether an analogous result would be observed if mice were inoculated with a gastrointestinal nematode parasite that induces a relatively pure type 2 response (N. brasilensis) (3, 52) or with a gastrointestinal nematode parasite that induces a more mixed cytokine response (T. spiralis) (53), primary IL-4 responses were assayed by CCCA at several time points in wild-type and Stat6-deficient mice following inoculation with either of these parasites. Identical IL-4 responses developed in wild-type and Stat6-deficient mice following inoculation with N. brasilensis; however, IL-4 responses in T. spiralis-infected mice were only ~10% as large as those in wild-type mice (Fig. 3). No differences were observed in the survival of either N. brasilensis or T. spiralis during the initial 6 days of infection, by which time IL-4 responses had reached peak or near-peak levels in wild-type mice, although wild-type mice cleared infections during the subsequent 4 to 8 days, while Stat6-deficient mice developed chronic infections (3 and data not shown).
type mice (data not shown). Thus, even though Stat6 signaling does not contribute to primary IL-4 or IL-13 responses to *H. polygyrus*, it appears to be required for the generation or survival of cells that make a normal memory type 2 cytokine response when mice are reinjected with this parasite. Studies of IL-4 production in wild-type and Stat6-deficient mice infected with *N. brasiliensis* and studies of IL-4 gene expression and secretion in BALB/c wild-type and Stat6-deficient mice infected with *Leishmania major* also demonstrated normal IL-4 expression in Stat6-deficient mice during a primary infection, but delayed or decreased IL-4 expression in these mice during a recall response (data not shown).

The memory IL-4 response produced by *H. polygyrus*-infected wild-type mice is CD4<sup>+</sup> T cell dependent and mast cell and Ab independent

These observations suggested that Stat6 signaling is required for the changes in cell physiology that allow memory T cells to rapidly produce IL-4 upon restimulation. An alternative possibility, however, is that the rapid IL-4 response that accompanies a second *H. polygyrus* infection results from the release of preformed IL-4 from non-T cells. Mast cells or basophils, for example, which can produce and store IL-4 (24), might be stimulated to release this cytokine by cross-linking *H. polygyrus*-specific IgE bound to their high affinity IgE receptors. This response might be defective in Stat6-deficient mice, which produce little or no IgE (13–15).

For this reason, experiments were performed to differentiate T cell from mast cell or basophil-mediated IL-4 production during the first day of a second *H. polygyrus* infection. First, the kinetics of IL-4 production following a second *H. polygyrus* infection was determined. Relatively little IL-4 was secreted 4–6 h after the challenge inoculation, while large quantities were released 8–10 h after inoculation and still larger quantities 12–14 h after inoculation (Fig. 5A). This result is more suggestive of new synthesis and secretion of IL-4 in response to worm inoculation, as opposed to rapid release of preformed IL-4 stores. Second, IL-4 responses were compared during first and second *H. polygyrus* infections in wild-type and W/W<sup>v</sup> mice, which have <1% of the normal number of mast cells (55). W/W<sup>v</sup> mice made normal IL-4 responses to primary and secondary infections with *H. polygyrus* (Fig. 5B). Because some basophils are present in W/W<sup>v</sup> mice (56) and these cells can secrete IL-4 in response to cell membrane FcεRI or FcγRIII (24), we also examined IL-4 responses to primary and second *H. polygyrus* infections in µMT mice that cannot secrete IL-4 through this mechanism because they lack B cells and Ig (57). µMT mice made normal IL-4 responses to both primary and secondary infections with *H. polygyrus* (Fig. 5C). To confirm that the rapid IL-4 response to a second *H. polygyrus* inoculation is CD4<sup>+</sup> T cell derived, we tested the ability of a cytotoxic anti-CD4 mAb (GK1.5), administered 1 day before a second *H. polygyrus* inoculation, to inhibit the rapid IL-4 response. In contrast to mast cell or Ig deficiencies, this treatment reduced the early IL-4 response to a second *H. polygyrus* infection to an undetectable level (Fig. 5D). Thus, the rapid, Stat6-dependent, memory IL-4 response to *H. polygyrus* appears to be derived from CD4<sup>+</sup> T cells, rather than mast cells or basophils.

**Stat6 promotes Th2 cell priming, but not the initial T cell IL-4 response, in vitro**

To determine whether the normal primary IL-4 response and defective memory IL-4 response that we observed in vivo in Stat6-deficient mice could also be demonstrated in vitro, we evaluated the Stat6 dependence of IL-4 production by CD8<sup>+</sup> T cell-depleted lymph node and spleen cells following primary stimulation with IL-2 plus soluble anti-CD3 and anti-CD28 mAbs, and secondary
The rapid IL-4 response made by mice given a second infection with *H. polygyrus* is CD4\(^+\) T cell derived. A, BALB/c mice (5/group) were inoculated orally with 200 infective, third stage *H. polygyrus* larvae. After 14 days, mice were treated with pyrantal pamoate to terminate infection. Mice were reinoculated orally with 200 infective, third stage *H. polygyrus* larvae 13 days later. IL-4 production was monitored by CCCA 2 days before and 4, 8, and 12 h after the second worm inoculation. B, Mast cell-deficient (W/W\(^{v}\)) mice or mice with a normal phenotype on the same background (10/group) were inoculated orally with 200 infective, third stage *H. polygyrus* larvae. After 14 days, mice were treated with pyrantal pamoate to terminate infection. Mice were reinoculated orally with 200 infective, third stage *H. polygyrus* larvae 14 days later. IL-4 production was monitored in five mice of each type by CCCA before and 1 day after the initial inoculation with *H. polygyrus* and, in additional groups of five mice of each type, immediately before and 1 day after the second inoculation with *H. polygyrus*. C, B cell-deficient (μMT) mice or normal mice on the same background (C57BL/6) (10/group) were treated and tested as in B. D, Stat6-deficient and wild-type mice on a BALB/c background (10/group) were inoculated orally with 200 infective, third stage *H. polygyrus* larvae. After 14 days, mice were treated with pyrantal pamoate to terminate infection. Fourteen days after that, mice were injected i.v. with 1 mg of either anti-CD4 mAb (GK1.5) or an isotype-matched control mAb (J1.2). One day later, all mice were reinoculated orally with 200 infective, third stage *H. polygyrus* larvae. IL-4 production was monitored in five mice of each group by CCCA 1 day after the second inoculation with *H. polygyrus*.

The rapid in vivo IL-4 response to anti-CD3 mAb is Stat6 independent

Our observations that Stat6-deficient mice are not primed by infection with a gastrointestinal nematode parasite to rapidly produce IL-4 upon reexposure to that parasite and that CD4\(^+\) lymph node cells from Stat6-deficient mice are not induced by culture with anti-CD3 and anti-CD28 mAbs to generate a rapid IL-4 response upon in vitro restimulation with anti-CD3 mAb raised the possibility that Stat6 signaling is universally required to promote the differentiation, survival, or growth of T cells that can rapidly be induced to produce IL-4. To investigate this possibility, we compared 4-h in vivo IL-4 responses in wild-type and Stat6-deficient mice to injection of anti-CD3 mAb. Most of the in vivo IL-4 response to this mAb is derived from T cells that express NK cell markers and are restricted by MHC class I-like CD1 molecules, rather than by conventional MHC class I- or MHC class II-restricted T cells (31, reviewed in Ref. 58). Stat6-deficient mice were found to make large, rapid IL-4 and IFN-γ responses to anti-CD3 mAb that are indistinguishable in magnitude from those made by wild-type mice of the same background strain (Fig. 7). Thus, Stat6-independent pathways exist for the generation, expansion, and survival of T cells that can rapidly produce IL-4, and NK T cells are

FIGURE 5. IL-4 expression, but not Th2 development, occurs in vitro in the absence of Stat6-mediated signaling. Lymph node and spleen cells from unimmunized wild-type and Stat6-deficient mice were depleted of CD8\(^+\) cells, labeled with CFSE, and stimulated with soluble anti-CD3 and anti-CD28 mAbs in the presence of IL-2. Cells were stained for surface CD4 and cytoplasmic IL-4 and analyzed by flow cytometry on day 3 for CFSE and intracellular IL-4 expression by CD4\(^+\) cells. CD8-depleted lymph node cells that were not labeled with CFSE were cultured as above, then washed and restimulated on day 6 with plate-bound anti-CD3 mAb, and analyzed the following day by flow cytometry for cytoplasmic IL-4 and IFN-γ expression by CD4\(^+\) cells.

FIGURE 6. IL-4 expression. 

FIGURE 7. CD3 stimulation induces similar in vivo IL-4 and IFN-γ responses in wild-type and Stat6-deficient mice. Wild-type and Stat6-deficient mice (5/group), on a mixed C57BL/6, 129 background (5/group), were injected i.v. with 10 μg of biotin-BVD4-1D11 (anti-IL-4) and 50 μg of biotin-R46-A2 (anti-IFN-γ) or with these mAbs plus 10 μg of 145-2C11 (anti-CD3e mAb). Mice were bled 4 h later, and serum levels of captured IL-4 and IFN-γ were determined by ELISA.
not a likely source of the Stat6-dependent, rapid, secondary IL-4 responses made in vivo by worm-inoculated mice and in vitro by CD4+ anti-CD3/anti-CD28 mAb-primed lymph node cells.

Discussion

Observations reported in this study demonstrate that in vivo antigenic stimulation can induce a primary Stat6-independent, IL-4Rα-independent IL-4 response that is normal in its magnitude and kinetics, and demonstrate that the Stat6 signaling requirement for in vivo generation of a primary IL-4 response is variable, but never, in our experience, absolute. We cannot totally rule out the possibility that the primary IL-4 responses that develop in immunized Stat6-deficient mice are made by cells other than CD4+ T cells. This possibility seems unlikely, however, because previous studies with mice immunized with GaM5 or inoculated with *N. brasiliensis* or *H. polygyrus* indicated that CD4+ T cells account for nearly all of the splenic and mesenteric lymph node IL-4 mRNA response during a primary immunization (Ref. 54 and K. B. Madden, unpublished data). It is also possible that special T cell populations, such as NK T cells, are responsible for the Stat6-independent IL-4 production that we have observed. It is unlikely that classical NK T cells, which are CD1 restricted (58), account for this IL-4 production, because previous studies have demonstrated normal IgE and IL-4 production in β2-microglobulin-deficient mice immunized with OVA and other soluble proteins (59–61), GaM5 (31), or *N. brasiliensis* (62), even though β2-microglobulin-deficient mice do not express CD1 (58). This does not eliminate the possibility that primary IL-4 responses in Stat6-deficient mice are made by nonclassical NK T cells, which are not CD1 restricted (63, 64). However, the slow development of in vivo primary IL-4 responses to soluble Ags or worm infection, as opposed to the rapid in vivo IL-4 responses to anti-CD3 mAb treatment (which stimulates IL-4 production in both classical and nonclassical NK T cells (31)) makes it less likely that even nonclassical NK T cells are responsible for primary IL-4 responses in Stat6-deficient mice.

In mice immunized with protein Ags or inoculated with gastrointestinal nematode parasites, the Stat6 dependence of the primary IL-4 response was greatest for immunogens that induce a mixed, type 0 cytokine response and least for immunogens that induce a strong, heavily biased type 2 cytokine response. This observation suggests that Stat6 signaling may promote IL-4 production during a primary response indirectly, by inhibiting the IL-4-suppressive effects of cytokines such as IL-12 and IL-18 (10), or that Stat6 enhances other stimuli that promote IL-4 production, such as CD4 or CD28 signaling (12, 65–72), when those signals are relatively weak, but not when those signals are strong. The simplest possibility is that strong T cell costimulation, in the absence of molecules such as IL-12, IL-18, and IFN-α/β/γ, which inhibit type 2 cytokine production, is all that is needed to induce an optimal type 2 cytokine response. This view is consistent with previous in vitro and in vivo observations that induction of primary mouse and human T cell IL-4 responses by Ag or anti-CD3 mAb requires CD4 and CD28 costimulation (12, 27, 65, 67, 70, 72–74) and in vivo observations that primary in vitro IL-4 responses are not always inhibited by anti-IL-4 or anti-IL-4R mAbs (12, 71, 72). This view is also consistent with the remarkable ability of GaM5 to induce Stat6-independent IL-4 production in vivo; in addition to inducing most B cells to process and present goat IgG, this Ab most likely optimizes CD4 and CD28 costimulation by directly increasing B cell MHC class II and CD86 expression (75, 76). Because little is known about the cells and costimuli that are involved in the presentation of Ags derived by gastrointestinal nematode parasites, it is not apparent why some of these parasites are such strong inducers of Stat6-independent type 2 cytokine responses. The ability of parasite-derived allergens, such as APF, to induce a strong IL-4 response in the absence of Stat6 signaling, even if administered i.p. rather than orally, makes it unlikely that mucosal Ag processing and presentation is critical for Stat6-independent elicitation of IL-4 production. Instead, worm-derived allergens, and probably the living parasites, may directly provide signals to T cells that substitute for Stat6 signaling or induce APCs to express molecules that have the same effect on T cells.

The ability of Stat6-deficient mice to produce in vivo IL-4 responses is consistent with suggestions that initial production of IL-4 by T cells may be a stochastic event that is promoted by rapid T cell proliferation (43, 77), rather than an event programmed by Stat6 signaling. Our observations clearly exclude Stat6 signaling as a requirement for the initial T cell IL-4 response, but leave open the possibility that another, as yet unidentified, factor is required to induce naive T cells to secrete IL-4.

The inability of Stat6-deficient mice to generate normal memory IL-4 responses following a secondary infection with *H. polygyrus* or *N. brasiliensis*, even though these parasites induce Stat6-deficient mice to make normal primary IL-4 responses, is consistent with a previous observation that T cells from *N. brasiliensis*-infected IL-4Rα-deficient mice make a markedly diminished IL-4 response when restimulated in vitro with anti-CD3 mAb (34). Taken together, these observations suggest that IL-4Rα-associated Stat6 signaling may have a nonredundant role in the generation of a normal memory Th2 cell population. This difference in the Stat6 requirements for the in vivo generation of primary type 2 cytokine responses vs a secondary Th2 response is also consistent with in vitro observations that anti-IL-4 or anti-IL-4Rα mAb does not inhibit IL-4 production by naive CD4+ T cells stimulated for the first time with Ag plus CD28 ligands or anti-TCR β and anti-CD28 mAbs, but prevents these cells from rapidly producing IL-4 and IL-5 upon restimulation (12, 71, 72) and that restimulation of T cells from Stat6-deficient mice with anti-CD3 or anti-CD4 and anti-CD28 mAbs fails to elicit IL-4 production (13–15).

It is possible, but unlikely, that the Stat6 requirement for development of normal secondary IL-4 and IL-13 responses results from absence of a positive feedback loop in which IL-4 stimulation of Stat6-deficient T cells fails to elicit IL-4 production, because previous studies have demonstrated that memory Th2 cell precursors, rather than orally, makes it unlikely that mucosal Ag processing and presentation is critical for Stat6-independent elicitation of IL-4 production. Instead, worm-derived allergens, and probably the living parasites, may directly provide signals to T cells that substitute for Stat6 signaling or induce APCs to express molecules that have the same effect on T cells.

The inability of Stat6-deficient mice to produce in vivo IL-4 responses following a secondary infection with *H. polygyrus* or *N. brasiliensis*, even though these parasites induce Stat6-deficient mice to make normal primary IL-4 responses, is consistent with a previous observation that T cells from *N. brasiliensis*-infected IL-4Rα-deficient mice make a markedly diminished IL-4 response when restimulated in vitro with anti-CD3 mAb (34). Taken together, these observations suggest that IL-4Rα-associated Stat6 signaling may have a nonredundant role in the generation of a normal memory Th2 cell population. This difference in the Stat6 requirements for the in vivo generation of primary type 2 cytokine responses vs a secondary Th2 response is also consistent with in vitro observations that anti-IL-4 or anti-IL-4Rα mAb does not inhibit IL-4 production by naive CD4+ T cells stimulated for the first time with Ag plus CD28 ligands or anti-TCR β and anti-CD28 mAbs, but prevents these cells from rapidly producing IL-4 and IL-5 upon restimulation (12, 71, 72) and that restimulation of T cells from Stat6-deficient mice with anti-CD3 or anti-CD4 and anti-CD28 mAbs fails to elicit IL-4 production (13–15).

It is possible, but unlikely, that the Stat6 requirement for development of normal secondary IL-4 and IL-13 responses results from absence of a positive feedback loop in which IL-4 stimulation of Stat6-deficient mice stimulates further IL-4 production. As seen in Figs. 3 and 4, the primary IL-4 responses to *H. polygyrus* and *N. brasiliensis*, which last several days, persist as long in Stat6-deficient mice as in wild-type mice. These responses are sufficiently long that they should provide time for a positive feedback loop to influence response magnitude. During the time that IL-4 is being actively produced (primary infection), there is no difference in the magnitude of the IL-4 response between the wild-type and Stat6-deficient mice. It is only when *H. polygyrus* infections are terminated by drug treatment, with cessation of IL-4 production, and mice are later reinoculated with *H. polygyrus* that the IL-4 response is defective. Because the difference between wild-type and Stat6-deficient mice appears when there is no detectable IL-4 production (the period between primary and secondary immunizations) rather than when IL-4 is being actively produced (the course of the primary immunization), it seems likely that defective generation of T cells that rapidly produce IL-4 upon restimulation with Ag, a failure of these cells to survive in the absence of continuing antigenic or IL-4 stimulation, or absent Stat6-mediated clonal expansion that is selective for memory cell precursors, rather than absent Stat6-mediated clonal expansion of IL-4-secreting cells, accounts for the defective rapid IL-4 response to a second *H. polygyrus* infection in

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Stat6-deficient mice. The possibility that Stat6 signaling is required to prevent memory cell death is consistent with considerable evidence that IL-4 has antiapoptotic effects, but less consistent with evidence that these antiapoptotic effects are Stat6 independent (78, 79). Recent observations that permanent commitment to type 2 cytokine secretion is accompanied by increased DNase sensitivity and demethylation of the IL-4 locus (80) leave open the possibility that these changes are not required to induce a primary IL-4 response. If so, the IL-4 locus may temporarily open in response to an acute Stat6-independent stimulus, but close once that stimulus terminates, preventing a rapid IL-4 response to restimulation. In contrast, Stat6 signaling may permanently open the IL-4 locus, allowing the generation of memory cells that rapidly produce IL-4 upon restimulation.

In sum, our data, taken together with previous observations (12, 43, 65, 66, 69, 71, 72), suggest the following scheme for the regulation of IL-4 production: Initial IL-4 production during a primary response may require CD4 and CD28 costimulation, but does not depend on IL-4 itself, although, in some circumstances, it is promoted by IL-4 activation of Stat6. Thus, to initiate production of IL-4 by non-T cells or specialized T cells should be necessary to induce conventional CD4+ T cells to produce IL-4. IL-4-induced Stat6 signaling during a primary response may enhance IL-4 production later on in that response, particularly if costimulation is limiting. Stat6 signaling is not universally required for the generation of a rapid T cell IL-4 response, but makes an important contribution to the differentiation, survival, and/or clonal expansion of conventional memory T cells that rapidly produce IL-4 upon restimulation.

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