Interleukin 2 plays a central role in Th2 differentiation

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Differentiation of naïve CD4 T cells into T helper (Th) 2 cells requires signaling through the T cell receptor and an appropriate cytokine environment. IL-4 is critical for such Th2 differentiation. We show that IL-2 plays a central role in this process. The effect of IL-2 on Th2 generation does not depend on its cell growth or survival effects. Stat5a−/− cells show diminished differentiation to IL-4 production, and forced expression of a constitutively active form of Stat5a replaces the need for IL-2. In vivo IL-2 neutralization inhibits IL-4 production in two models. Studies of restriction enzyme accessibility and binding of Stat5 to chromatin indicate that IL-2 mediates its effect by stabilizing the accessibility of the Il4 gene. Thus, IL-2 plays a critical role in the polarization of naïve CD4 T cells to the Th2 phenotype.

Differentiation of naïve CD4 T cells to the T helper (Th) 2 phenotype requires activation in the presence of costimulatory signals. The cytokine environment during priming is critical; IL-4 markedly enhances Th2 priming through a Stat6-dependent pathway involving the up-regulation of GATA-3 (1, 2) and increased accessibility of the Il4 gene (3–8).

The role of other cytokines in Th2 priming has not been clear, and IL-2 has been reported to be needed for such priming (9, 10). Much of the effect of anti-CD28 in in vitro Th2 priming is through its stimulation of IL-2-production (11). The concern has been that the role of IL-2 might have been to promote the selective outgrowth of cells differentiating to the Th2 phenotype, but because IL-2 also mediates a program of gene activation (12), it may be directly involved in the Th2 differentiation process.

Expressing a constitutively active form of Stat5 in recently activated CD4 T cells allows the acquisition of competence to produce IL-4 (13). Here we show that IL-2 plays an essential role in Th2 priming, and its effects cannot be explained simply by its proliferation-inducing activity. IL-2 stabilizes the accessibility of the Il4 gene, and STAT5, a key transducer of IL-2 function, binds to sites in the second intron of the Il4 gene. We also demonstrate that anti-IL-2 inhibits priming for IL-4 production in two in vivo models.

Materials and Methods

Mice, Reagents, and Antibodies. 5C.C7/RAG-2−/− B10.A mice, expressing a T cell antigen receptor specific for a pigeon cytochrome c (PCC) peptide (88–104), DO11.10 BALB/c mice expressing a T cell antigen receptor specific for an ovalbumin peptide, C57BL/6 mice, and IL-2-deficient mice (IL-2−/−) backcrossed to 5C.C7 19 times (14) were obtained from Taconic Farms. Stat5a−/− C57BL/6 mice were the gift of W. J. Leonard (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda). Mice were maintained in pathogen-free conditions and were used at 6–12 weeks of age.

CD4+ Cells and Antigen-Presenting Cells (APCs). Naïve lymph node CD4+ T cells were purified by negative selection, and purity was >98%. APCs were T-depleted, irradiated splenocytes (see Supporting Text, which is published as supporting information on the PNAS web site).

Cell Culture and Priming. CD4+ T cells (2.5–3 × 105 per ml) were cultured in tissue culture dishes coated with anti-CD3 (0.2 μg/ml) and anti-CD28 (3 μg/ml) or with irradiated APCs in the presence of soluble anti-CD3 (1 μg/ml) plus anti-CD28 (3 μg/ml). Naïve 5C.C7 CD4+ T cells (3 × 106 per ml) were cultured with PCC peptide plus 1.5 × 106 APC with or without anti-CD28 (3 μg/ml). For Th1 priming, cells were cultured in the presence of IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml). For Th2 priming, cells were primed in the presence of IL-4 (1,500 units/ml), anti-IFNγ (10 μg/ml), and anti-IL-12 (10 μg/ml). For priming under Th neutral conditions, no cytokines were added. Unless specified, rhIL-2 was added at 100 units/ml. After 3–4 days of priming, cells were washed and analyzed by intracellular staining.

Western Blot Analysis. Cell lysates (5 × 106 per sample) were separated on an SDS–8% polyacrylamide gel, transferred to Immobilon-P (Millipore) membranes and probed with anti-phospho-Stat5 (Tyr-694) rabbit antibody followed by horseradish peroxidase-labeled secondary antibody (see Supporting Text).

5,6-Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Labeling, Fluorescence-Activated Cell Sorter Analysis, and Cytokine Intracellular Staining. Cells were labeled with CFSE (15). For intracellular cytokine detection, cells were stimulated with immobilized anti-CD3 (3 μg/ml) plus anti-CD28 (3 μg/ml) for 6 h; monensin (2 μM) was added for the last 2 h. Harvested cells were fixed, permeabilized, and stained (see Supporting Text).

Purification of IL-4-Producing Cells. IL-4+ cells were purified by cell sorting after detecting IL-4-secreting CD4+ T cells with the mouse IL-4 secretion assay detection kit (Miltenyi Biotec, Auburn, CA) (see Supporting Text).

RNA Preparation and Real-Time PCR Analysis. RNA was purified by using TRIzol Reagent (Invitrogen) followed by an Rneasy total RNA purification kit (Qiagen, Valencia, CA). cDNAs were made from total RNA, and real-time PCR was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) (see Supporting Text for details).

Restriction Endonuclease Accessibility Assay (REA). REA was carried out as described (15).

Abbreviations: Th, T helper; APC, antigen-presenting cell; PCC, pigeon cytochrome c; REA, restriction endonuclease accessibility assay; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; HSII and HSIII, hypersensitivity regions II and III.

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Chromatin Immunoprecipitation Analysis. Chromatin immunoprecipitation assays were carried out as described (13).

Confocal Fluorescence Microscopy. Fixed and permeabilized cells were stained with monoclonal anti-GATA-3. The primary antibody was revealed by using Cy5-donkey F(ab')2 anti-mouse IgG. Stained cells were visualized with a Leica TCS-NT/SP confocal microscope (Leica, Deerfield, IL) with a ×63 oil immersion objective. Twenty to thirty z sections separated by 0.2 μm were acquired. Reconstructions of images were performed by using the IMARIS software systems from BitPlane (Zürich, Switzerland) and PHOTOSHOP software (Adobe Systems, Mountain View, CA).

Retrovirus Infection. Infection of T cells with the retroviruses GFP-RV, GFP-RV-GATA-3, NGFR-RV, or NGFR-RV-STAT5A1*r was performed as described (16) (see Supporting Text). Three days after infection, the frequency of retrovirus-positive cells and IL-4-secreting cells was analyzed after 6 h of restimulation with immobilized anti-CD3 plus anti-CD28.

In Vivo Priming of CD4 T Cells for IL-4 Production. BALB/c mice (four per group) were implanted with miniosmotic pumps (Alzet 2001; 1 μl/h, Durect, Cupertino, CA) containing 1 mg of ovalbumin (Sigma) in PBS (day −1). At day 0, mice received 5 × 10⁶ purified CFSE-labeled DO11.10 CD4 T cells i.p. Mice received 500 μg of either anti-IL-2 (S4B6) or isotype control or PBS on days −1, 0, 2, and 3.5 and were killed 4.5 days after cell transfer. Draining lymph node cells were restimulated with 3 μM OVA323–339 peptide (American Peptide, Sunnyvale, CA) and soluble anti-CD28 (3 μg/ml) for 8 h; monensin was added for the last 4 h. IL-4 content and CFSE dilution of KJ1−26¢ CD4+ cells were determined by flow cytometry as described above. The statistical differences among the groups were analyzed by using Student’s t test.

Results

Neutralizing IL-2 Inhibits Th2 Differentiation. 5C.C7 CD4 lymph node T cells labeled with CFSE were cultured with APC for 86 h under Th2 conditions with PCC peptide and anti-CD28. Anti-IL-2 reduced IL-4-producing cells from 46.5% to 1.2% without diminishing the cell yield or altering the division history of the cells (Fig. 1a). If anti-CD28 is omitted, cell yield in the presence of anti-IL-2 is diminished 2- to 3-fold, but IL-4 priming is still inhibited. Anti-IL-2 also inhibited priming for IL-4 production when soluble or immobilized anti-CD3 and anti-CD28 were used (Fig. 8, which is published as supporting information on the PNAS web site). If endogenous IL-2 is neutralized with anti-IL-2, maximal restoration of priming for IL-4 production required addition of 100–1,000 units/ml human IL-2, substantially more than required for optimal IL-2-mediated cell yield (Fig. 9, which is published as supporting information on the PNAS web site).

Neutralizing IL-2 diminished priming for IL-5, IL-6, IL-10, IL-13, and granulocyte/macrophage colony-stimulating factor production (Fig. 1b) as judged by mRNA levels at 84 h of culture. Priming for IL-9 production was strongly inhibited when anti-IL-2 was used in the cultures (data not shown). Cells primed in the absence of IL-2 showed no diminution in their level of IL-2 mRNA or in IL-2 production on subsequent challenge (data not shown).

When CD4 T cells from IL-2−/− 5C.C7 Rag2−/− mice were cultured under Th2 conditions without added IL-2, ~3% of the cells produced IL-4 in comparison with 21% of conventional cells cultured under the same conditions. Addition of IL-2 (100 units/ml, which fully restored priming for IL-4 production) WT and knockout cells showed similar yields and similar proliferative rates with or without added IL-2 (Fig. 2).

IL-2 Cannot Be Replaced by Another Cytokine. 5C.C7 CD4 T cells were cultured with APC, 0.2 μM PCC peptide, IL-4, anti-IL-12, and anti-IFNγ and anti-IL-2. Adding IL-3, IL-6, IL-9, IL-10, IL-11, IL-13, IL-18, IL-21, tumor necrosis factor α, tumor necrosis factor β, transforming growth factor β1, or transforming growth...
factor β2 failed to restore priming for IL-4 production (Fig. 3a). IL-15 caused a modest restoration of priming; its limited effect may be explained by a relatively poor capacity of purified CD4 T cells to respond to IL-15. IL-2 (100 units/ml) caused substantially more phosphorylation of Stat5 than did IL-15 (100 ng/ml; Fig. 3b). IL-7 also caused modest Stat5 phosphorylation and modest restoration of Th2 priming.

**IL-2 Mediates Its Function Late in the Period of Th2 Priming.** In cultures treated with anti-IL-2, adding human IL-2 as late as 46 h of culture almost fully restored priming for IL-4 production. Delaying addition of anti-IL-2 until 46 h of culture almost completely inhibited priming for IL-4 production (Fig. 10, which is published as supporting information on the PNAS web site). Thus, IL-2 is needed in the second half of the priming culture and is unnecessary during the first 46 h.

IL-2 was also needed in later rounds of Th2 culture. 5C.C7 CD4 T cells were primed in the presence of IL-2 and IL-4. A striking increase in IL-4-producing capacity was observed in cells reactivated with peptide and IL-2 and IL-4 or with IL-2 and anti-IL-4; cells cultured with IL-4 and anti-IL-2 showed no increase in IL-4-producing capacity (Fig. 10).

**IL-2 Is Not Required for GATA-3 Induction.** GATA-3 plays a major role in Th2 priming (1, 2). GATA-3 mRNA induction was not blocked by anti-IL-2 in DO11.10 CD4 T cells cultured with immobilized anti-CD3 and anti-CD28 under Th2 conditions.

Cells cultured under similar conditions were fixed, permeabilized, and stained for GATA-3 at 72 h. No differences were observed in cells cultured with IL-2 or with anti-IL-2 (Fig. 4).

**IL-2 Maintains Il4 Gene Accessibility.** 5C.C7 CD4 T cells were stimulated with APC and 0.2 μM PCC peptide under Th2 conditions. At 86 h, an RsaI site in DNase I hypersensitivity region II (HSII), in the second intron of the Il4 gene, was accessible, by REA, in DNA from cells cultured in the presence of IL-2 but not in cells that had been cultured with anti-IL-2. This site was not accessible in Th1 cells (Fig. 5a).

We measured REA by using real-time PCR in HSII and hypersensitivity region III (HSIII) at the outset and at 48 and 96 h of Th2 priming. DNA from cells cultured with IL-2 or anti-IL-2 had similar degrees of accessibility at 48 h, but REA values fell dramatically if IL-2 was not present during the last 48 h (Fig. 5b).

REA at HSII was the same in IL-2-treated DO11.10 CD4 T cells to which cyclosporin A was or was not added for the last 48 h of culture (Fig. 5c), suggesting that the nuclear factor of activated T cells was not required to maintain accessibility of the Il4 gene.
To rule out the possibility that IL-2 mediates its effects by causing preferential survival of IL-4-producing cells, we primed cells for one round under Th2 conditions. IL-4-producing cells were purified by cytokine capture. Of purified IL-4 producers recultured in the presence of IL-2, 46% produced IL-4 on challenge; only 11% of those recultured in the absence of IL-2 produced IL-4 (Fig. 5d). REA at HSII and HSIII showed a similar diminution; at HSII, REA fell from 7.1 for cells cultured in IL-2 to 3.2 in cells cultured in the absence of IL-2; at HS III, REA fell from 0.84 to 0.22. Thus, IL-2 played a role in maintaining both IL-4-producing capacity and the open conformation of the Il4 gene, even among cells that had all been IL-4 producers.

Involvement of Stat5 in Priming for IL-4 Production. Inhibitors of phosphatidylinositol 3-kinase, mitogen-activated protein kinase kinase, p38, or c-Jun N-terminal kinase added with IL-2 at 48 h of Th2 culture failed to block priming for IL-4 production (Fig. 11, which is published as supporting information on the PNAS web site). By contrast, only 1% of CD4 T cells from STAT5a−/− mice primed with soluble anti-CD3 and anti-CD28 with added IL-2 produced IL-4 on subsequent stimulation, whereas 10.5% of control C57BL/6 CD4 T cells produced IL-4 (Fig. 6a).

5C.C7 cells infected with a retrovirus expressing a constitutively active form of Stat5A (STAT5A1*6) were primed for IL-4 production in the presence of IL-2-neutralizing antibodies (Fig.
fig. 6. Stat5a and IL-4 production. (a) C57BL/6 Stat5a−/− and WT CD4 T cells primed for 86 h with soluble anti-CD3 (0.5 μg/ml) and anti-CD28 (3 μg/ml) under Th-neutral conditions with either anti-IL-2 and anti-IL-2R or with added IL-2. KO, knockout. (b) and (c) SC.C7 CD4 T cells were activated for 40 h before retrovirus infection. Cells were infected with a GATA3- (b) or a Stat5a1*6- (c) encoding retrovirus and cultured for an additional 3 days under Th2 conditions with IL-2 or anti-IL-2, anti-IL-2Rα, and anti-IL-2Rββ. Intra-cellular IL-4 expression was analyzed after stimulation in CD4 T cells (a) or STAT5A1*6-, GATA-3-, or control retrovirus-infected cells (b and c). NGFR, nerve growth factor receptor. (d) SC.C7 CD4 T cells were stimulated under Th1 or Th2 conditions with APC and 1 μM PCC peptide for three rounds. Each round consisted of 4 days priming and 3 days resting in IL-2. Chromatin immunoprecipitation (ChIP) assays were carried out by using anti-Stat5a and control serum. Amounts of immunoprecipitated DNA sequences from HSII, HSIII, and SOCS-3 promoter Stat-binding motif (S3PS) were assessed by real-time PCR. Relative enrichment of the sequences were normalized by using a 3′-UTR t-bet sequence, which does not have a Stat-binding site.

IL-2 Enhances in Vivo Priming for IL-4 Production. CFSE-labeled CD4+ DO11.10 cells (5 × 10^6) were injected i.p. into BALB/c mice 1 day after implantation of a miniosmotic pump containing 1 mg of ovalbumin. Anti-IL-2, control Ig, or saline were injected 1 day before cell transfer, on the day of transfer, and 2 and 3.5 days later. Mice were killed 4.5 days after transfer; draining lymph node cells were restimulated with ovalbumin peptide and soluble anti-CD28. Yields of DO11.10 cells were similar in the three groups; and, in general, division rates were similar, but the frequency of IL-4+ cells was 5-fold lower in the anti-IL-2-treated group than in the control Ig group (Fig. 7).

Bone marrow derived bm12 dendritic cells (0.5 × 10^6) were injected into each hind foot pad of C57BL/6 mice; anti-IL-2 or control IgG were injected on days 0, 2, and 4. Mice were killed on day 6, and draining lymph node cells were stimulated with anti-CD3/anti-CD28. The frequency of IL-4+ cells among CD4+ CD44+ cells was 3.6% in the control Ig group and 1.4% in the anti-IL-2 group (Fig. 12, which was published as supporting information on the PNAS web site).

Discussion

IL-2 is important in Th2 priming (9, 10). Because it is a T cell growth factor and efficient priming for IL-4 production is associated with proliferation (4), the role of IL-2 might be limited to growth and/or survival. We showed that neutralizing IL-2 inhibits in vitro and in vivo priming for IL-4 production and in vitro priming for production of essentially all Th2 cytokines, even when no reduction in T cell proliferation or total cell expansion occur. Other arguments against IL-2 acting as a growth factor for IL-4 production include the ability of CD4 T cells to differentiate into IL-4 producers in the presence of G2/M cell cycle inhibitors (18). Furthermore, removal of IL-2 from rested, antigen-stimulated Th2-primed cells for 24 h, during which cell division occurs, still results in striking inhibition in IL-4-producing capacity (J.C.-S., unpublished observations). Finally, anti-IL-2 inhibited in vivo priming of transferred DO11.10 cells to become IL-4 producers without reducing their division rate or total expansion.

Our analysis of the mechanism through which IL-2 mediates its Th2-priming function implicates the Stat5 pathway. Infection with a retrovirus encoding a constitutively active form of Stat5a renders cells capable of developing into IL-4 producers in the absence of IL-2. In agreement with a recent study (19), we showed that in vitro priming for IL-4 production is impaired in Stat5a−/− T cells under “neutral” conditions.

IL-4 may cause Stat5 phosphorylation (20), but its magnitude is modest (see Fig. 3b), consistent with the inability of IL-4 to replace IL-2 function. Receptors for several other growth factors that activate Stat5 (21–23) are not expressed in naïve and recently activated CD4 T cells. However, receptors for prolactin (24) and growth hormone (24), which also activate Stat5, are present on lymphoid cells (25–27). Prolactin increases proliferation of lymphocytes (28, 29) and enhances IL-4 production (30).

Accessibility in the second intron of Il4 in regions overlapping the HSII and HSIII DNase I-hypersensitive sites occurred independent of IL-2 during the initial 48 h of culture. However, cells maintained for the last 48 h without IL-2 showed REAs at HSII and HSIII similar to those of naïve CD4 T cells, consistent with IL-2 being needed to maintain accessibility in this portion of the Il4 gene.

The rate at which cells differentiating along the Th2 pathway acquire accessibility at HSII, HSIII, and other sites in the Il4 gene is far faster than the rate at which CpGs in this region of
the gene become demethylated (8). Thus, in the period before stabilization of the accessible state of the chromatin by CpG demethylation, IL-2 may be essential for maintaining accessibility at the If4 locus. Indeed, cells primed under Th2 but not Th1 conditions show association of Stat5A with HSII and HSIII. We have also shown that cells infected with the Stat5A1*6 retrovirus acquire IL-4-producing capacity and that Stat5 is associated with DNA elements near HSII and HSIII (13). It had previously been shown that deletion of HSII led to reduction in transcription activity in a transient transfection system (31).

Although IL-2 was essential for priming for IL-4 production, it could not replace IL-4 (Fig. 10b). However, retroviral-mediated expression of Stat5A1*6 in cells primed in the absence of IL-4 allowed those cells to develop into IL-4-producing (13). This finding may be explained by the high levels of active Stat5A1*6 in cells primed in the absence of IL-4, such as in infection with Nipponstrongylus brasiliensis (32) or with Schistosoma mansoni (33).

Anti-IL-2 strikingly inhibits in vivo priming of transferred DO11.10 cells to become IL-4 producers in response to immunization or infection with BALB/c mice with a miniosmotic pump containing ovalbumin, without any diminution in proliferation rate or expansion. Hwang et al. (34) reported that anti-IL-2Rα/anti-IL-2Rβ antibody treatment of DO11.10 mice diminished in vivo priming for IL-4 production by ovalbumin emulsified in complete Freund’s adjuvant.

On the other hand, IL-2- (35, 36), IL-2Rα- (37), and IL-2Rβ-knockout (38) mice, when young, express elevated levels of IgG1 and IgE isotypes that generally depend on IL-4 for their expression (39). The interpretation of these experiments is clouded by the fact that such mice are deficient in regulatory T cells and develop inflammatory bowel disease and autoantibodies; a large proportion of their CD4 T cells are in an activated state, as judged by expression of CD44 (37, 40) and CD69 (38, 40). Under these circumstances, IL-2 function may be replaced by other factors that have not yet been identified.

Optimal induction of Th2-dominated responses will depend on availability of adequate amounts of either IL-2 or alternative factors that can replace its function. Considerations regarding the need for an If4-stabilizing function, as mediated by IL-2, should be part of any vaccine strategy aimed at an IL-4-dominated response or of efforts to elicit Th2 responses to ameliorate tissue-damaging autoimmunity. Strategies intended to block IL-2 signaling could have clinical implications for the treatment of allergic diseases.

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