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Activation of STAT Proteins and Cytokine Genes in Human Th1 and Th2 Cells Generated in the Absence of IL-12 and IL-4

Richard Moreigl,* Colette Kristovic,†† Bernd Kinzel,† Sinisa Volarevic,† Bernd Groner,* and Volker Brinkmann†‡

We have previously shown that human CD4+45RO− T cells could be primed for a Th2 phenotype independent of IL-4 if they were activated by anti-CD28 mAb plus IL-2. If additional TCR signals were provided, the cells differentiated toward Th1 independent of IL-12. Here we show that anti-CD28/IL-2-primed Th2 cells expressed high levels of activated STAT6, but no cytokine mRNA. Moreover, both Th1 and Th2 cells expressed active STAT1 and -3, but not STAT2, -4, and -5. Restimulation of Th1 or Th2 cells via CD3 plus CD28 induced production of IFN-γ or IL-4, respectively, but did not alter the activation status/DNA binding activity of STATs. Addition of IL-4 (or anti-IL-4 mAb) to restimulated Th2 cells did not modulate STAT6 activation or IL-4 expression, confirming the full commitment. However, Th2 cells remained responsive to IL-12, which repressed STAT6 DNA binding but activated STAT4, and this coincided with a suppression of IL-4/IL-5 and an induction of IFN-γ. In Th1 cells, IL-12 activated both STAT6 and STAT4, and IL-4 activated STAT6, but in both cases the Th1 phenotype remained. Together the data show that CD28/IL-2-dependent Th2 priming activated STAT6 without inducing IL-4 expression. The primed Th cells resembled memory cells and produced IL-4 upon the first CD3/CD28 costimulus without detectable modulation of STATs. Th2 cells remained responsive to IL-12, which repressed STAT6 DNA binding and activated STAT4, and switched the cells to Th1. The effects of IL-12 may depend on the commitment of the cells, since IL-12 phosphorylated STAT6 in Th1 and dephosphorylated STAT6 in Th2 cells.


A n imbalance in the numbers of Th2 cells secreting IL-4 and IL-5 vs Th1 cells secreting IL-2 and IFN-γ has been implicated in many immunologic diseases, including allergy, inflammation, and autoimmunity (1–6). This realization has been the impetus for a large number of studies aimed at defining the stimuli that determine the differentiation patterns of naive CD4 T cells into functionally committed Th1 and Th2 effector cells (4–12). Recent data suggested that the initiation of IL-4 production in naive T cells may provide limited TCR signaling (13–15) in costimulation with surface CD28 (13, 16) and CD40 ligand (13). Once IL-4 production is initiated, IL-4 feeds back on the differentiating cells and accelerates Th2 commitment in an autocrine manner (6–9, 17). In contrast, Th1 differentiation is favored by strong TCR signals in costimulation with CD28 (13–16), and this process is further supported by IL-12 (18, 19) and IFN-γ (20) secreted by cells of the monocyte/macrophage lineage.

It is now understood that binding of IL-4 and IL-12 to their receptors induces activation of latent cytoplasmic transcription factors, designated STATs (21, 22). Seven different mammalian members of the STAT family are known (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6), and all are encoded by different genes. IL-4 induces tyrosine phosphorylation of STAT6 (23–26), which after homodimerization via phosphotyrosine and SH2 domains (27) translocates to the nucleus, binds to the IgE promoter in B cells (25) or the IL-4 promoter in T cells (28), and supports gene transcription. In contrast, IL-12 activates STAT1 (29), STAT3 (19, 30), and STAT4 (31, 32), which support transcription of the Th1 cytokine IFN-γ (32, 33). Likewise, STAT6-deficient mice are defective in Th2/IgE immune responses (34), whereas STAT4-deficient animals display impaired IL-12 responses and enhanced development of Th2 cells (31, 35). Recent data suggested that the full trans-activation potential of DNA-bound/tyrosine-phosphorylated STATs requires additional phosphorylation on serine, which can be induced via the Ras/mitogen-activated protein kinase pathway, also known to be coupled to the TCR (36, 37).

The above data suggest a critical role of cytokines in Th cell commitment. However, we have recently shown that naive human CD4+45RO− T cells can be primed for a Th1 or a Th2 phenotype independent of IL-12 and IL-4 (16). Here we analyzed whether this IL-4/IL-12-independent Th2/Th1 cell differentiation would result in the activation of selected STAT proteins, and whether such Th1 and Th2 cells remained responsive to IL-4 and IL-12.

Materials and Methods

Culture medium

Cells were cultured in RPMI (Life Technologies, Paisley, Scotland) supplemented with sodium pyruvate (1 mM; Life Technologies), MEM non-essential amino acids (Life Technologies), 2-ME (0.5 μM), L-glutamine (2 mM), kanamycin (100 μg/ml; Life Technologies), Bacto-asparagine (20 μg/ml; Difco, Detroit, MI), human insulin (5 μg/ml; Sigma Chemical Co., St. Louis, MO), human transferrin (40 μg/ml; Sigma), and selected FCS (10%, HyClone Laboratories, Logan, UT).

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Purification of uncommitted naive CD4+ 45RO T cells

CD4+ 45RO T cells were sorted by FACS from PBMC stained with biotin-labeled anti-CD4 mAb plus streptavidin-Red 670 and phycoerythrin-labeled anti-CD45RO (Becton Dickinson, Mountain View, CA). We have shown previously (16) that sorted T cells were small based on the FACS scatter and homogeneously expressed CD3, CD4, CD45RA, and CD28, but did not express FACS-detectable levels of the activation markers CD45RO, CD25 (IL-2R p55 chain), CD71 (transferrin receptor), or HLA-DR (all Abs from Becton Dickinson). Uncommitted CD4+ 45RO T cells did not proliferate in response to either anti-CD3 or anti-CD28 or IL-2 alone, but responded to stimulation by anti-CD3 plus anti-CD28 (16).

Generation of Th1 and Th2 populations from naive CD4+ 45RO T cells

Cultures were performed in 96-well flat-bottom tissue culture clusters (Costar, Cambridge, MA). Th1 and Th2 subsets were prepared as described by us previously (16). To generate Th1 cells, uncommitted CD4+ 45RO T cells were stimulated in limiting dilution (500 cells/0.2 ml of culture) by anti-CD3 (OKT3, American Type Culture Collection, Rockville, MD; 2 µg/0.2 ml coated to plates) plus anti-CD28 (anti-Leu-28, Becton Dickinson; 2 µg/0.2 ml coated to plates) plus IL-2 (Novartis, Basel, Switzerland; 100 U/ml). To generate Th2 cells, CD4+ 45RO T cells were primed by anti-CD28 plus IL-2 (in the complete absence of TCR signals). The cloning efficiency during Th1 priming was 1/10, and that during Th2 priming was 1/20; both primings induced vigorous proliferation (clone sizes >200) (16). Th1 and Th2 populations were harvested after reaching culture densities of 10⁶ cells/ml (after 6–11 days). At this time, >99% of Th1 and Th2 cells expressed the T cell markers CD3 and CD4; the activation markers CD25, CD71, and HLA-DR; and the memory marker CD45RO (16). Th1 and Th2 populations did not contain detectable numbers of unprimed CD45RO+ T cells.

Restimulation of committed Th1 and Th2 populations

Th1 and Th2 cell populations were arrested for 2 h and restimulated (40,000 cells/0.2 ml of culture) as indicated in the respective experiments by anti-CD3 (2 µg/0.2 ml coated to plates), anti-CD28 (2 µg/0.2 ml coated), IL-2 (Novartis; 100 U/ml), IL-4 (Novartis; 200 U/ml), IL-12 (Phar Mingen, San Diego, CA; 100 U/ml), or their combinations. Some cultures contained the neutralizing anti-IL-4 mAb SF12 (Novartis; 20 µg/ml) (9). Restimulated cells and culture supernatants were analyzed as described below.

STAT analysis: preparation of whole cell extracts and electrophoretic mobility shift assays (EMSA)

Th1 and Th2 cell populations were left unstimulated or were restimulated (40,000 cells/0.2 ml of culture) for 1, 4, or 24 h by anti-CD3, anti-CD28, IL-2, IL-4, IL-12, or their combinations. Whole cell extracts were prepared from unstimulated or restimulated Th1 or Th2 cells by suspension of cell pellets in a buffer containing 400 mM NaCl, 50 mM KCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 20% glycerol, 1 mM DTT, 0.2 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 100 µM sodium orthovandate, and 20 µM PAO (phenyl arsenic oxide). After three freeze-thawes, lysates were centrifuged for 15 min at 4°C and 20,000 g. The supernatants were recovered for bandshift experiments. The protocol for the bandshift assays has been described (38). In the bandshift experiments the following high affinity STAT binding sites were used: for STAT1, STAT3, and STAT4, 5'-GATCCGGGAAAAGGGAAAACCGAAAACTGAAGCC-3' derived from the x-1-inducible element in the in the e-c-fos promoter (SIE-M67) (21, 29); for STAT1, STAT2, and p48, 5'-GATCCGGGAAAAGGGAAAACCGAAAACTGAAGCC-3' of the ISG-15 promoter (ISRE) (21); for STAT5a, STAT5b, and STAT6, 5'-AGATTTCCTAGAAATCTCAATC-3' from the bovine β-casein promoter (β-casein) (38); and for STAT6, 5'-GTCAACCTTCAGAAGCAAGA-3' from the human Ig heavy chain germ-line C region (39, 40). The STAT probes were end labeled with polynucleotide kinase to a sp. act. of 860,000 cpm/fmol.

Equal amounts of protein from whole cell extracts were introduced into bandshift assays. Proteins were measured at a wavelength of 595 nm with the Bradford protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions (41). The bandshift signals were quantitated with a PhosphoImager (Storm 860, Molecular Dynamics, Sunnyvale, CA). The ratio between the specific STAT DNA binding complex and the unspecific DNA binding complex, always shown in the lower part of the EMSAs, was calculated.

Abs for supershift analysis

The following Abs were used: murine mAb against the carboxyl terminal domain of STAT1 (Transduction Laboratories, Lexington, KY; the peptide used for immunization was comprised of amino acid positions 592–731 of human STAT1), polyclonal rabbit antiserum against the carboxyl-terminal domain of STAT3 (42) (the peptide used for immunization was comprised of amino acid positions 688–727 of murine STAT3), polyclonal rabbit antiserum against the carboxyl-terminal domain of STAT4 (20; Santa Cruz Biotechnology, Santa Cruz, CA; the peptide used for immunization was comprised of amino acid positions 730–749 of murine STAT4), polyclonal chicken antiserum against the carboxyl-terminal domain of STAT6 (43) (the peptide used for immunization was comprised of amino acid positions 633–837 of mouse STAT6), and polyclonal rabbit antiserum against the carboxyl-terminal domain of STAT16 (20, Santa Cruz Biotechnology; the peptide used for immunization was comprised of amino acid positions 828–874 of human STAT6). All antisera used for supershift experiments recognize the individual STAT proteins only and are not cross-reactive with any other STAT family member as examined with recombinant STAT expression in COS-7 cells followed by EMSA and supershift assays (data not shown). Supershifting of STAT-containing complexes was achieved by adding to the whole cell extracts 5 to 10 µg of antiserum against the individual STAT family members 10 min before the start of the binding reaction.

Northern blot analysis for cytokine mRNA

Th1 and Th2 cells were restimulated (40,000 cells/0.2 ml of culture) for 4 h by anti-CD3 plus IL-2, which resulted in optimal mRNA expression. Total cellular RNA was prepared from 2 × 10⁷ Th1 and Th2 cells using the RNeasy total RNA system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA samples (10 µg/lane) were separated by electrophoresis on a 0.8% agarose-formaldehyde gel, transferred to a zeta-blot membrane and probed with a polynucleotide kinase labeled (BPR216, R&D Systems, Abington, U.K.) probes that were 32P labeled using the Rediprime random primer labeling system (Amersham, Aylesbury, U.K.) or T4 polynucleotide kinase (New England Biolabs, Beverly, MA), respectively. Prehybridization and hybridization of RNA blots were performed as previously described (44). The hybridized membranes were imaged by placing them in contact with a Biomax MR x-ray film (Eastman Kodak, Rochester, NY) followed by three washes in 0.1× SSC/0.1% SDS. To verify the amounts of RNA applied, the same Northern blot was rehybridized with a control probe specific for glyceraldehyde-3-phosphate dehydrogenase as previously described (44).

Determination of secreted IL-4, IL-5, and IFN-γ by ELISA

Th1 and Th2 cells were restimulated (40,000 cells/0.2 ml of culture) by anti-CD3, anti-CD28, IL-2, IL-4, IL-12, or their combinations. Culture supernatants were harvested after 72 h and analyzed in ELISAs (46, 47). Ninety-six-well flat-bottom microtiter plates (Nunc, Maryland, NY) were coated with murine mAbs to human IFN-γ (43.11; 1 µg/well), IL-4 (4F12; 1 µg/well) (47), or IL-5 (JES-51D10; 0.5 µg/well; AMS Biotechnology, Lugano, Switzerland). Plates were blocked with 2% BSA in PBS containing 0.05% NaN₃ (1 h, 37°C). Plates were washed, and standards or test samples (50 µl/well) were added in the appropriate dilutions. Plates were incubated for 1 h and washed, and biotinylated murine mAbs to human IFN-γ (45.15) (46), IL-4 (3H4) (47), or IL-5 (JESI-51A10; AMS Biotechnology) were added (0.2 µg/well, 2 h, 37°C). Plates were washed; and avidin-alkaline phosphatase was added (Sigma; 20 ng/well, 2 h, 37°C). After additional washing, phosphatase substrate was added (disodium p-nitrophenyl phosphate; 0.1 mg/well; Sigma). The detection limit for IL-4 and IFN-γ was 0.02 ng/ml, and that for IL-5 was 0.3 ng/ml.

Results

Cytokine profile of committed human Th1 and Th2 cells

We have shown previously that naive human CD4+ 45RO T cells could be primed for massive production of Th2 cytokines if they were activated by immobilized anti-CD28 mAb plus IL-2 (16). Th2 priming was IL-4 independent, since it was not sensitive to suppression by neutralizing anti-IL-4 mAb. If additional TCR signals were provided, the cells defaulted to the Th1 pathway independent of IL-12. In this study we analyzed whether IL-4/IL-12-independent Th2/Th1 differentiation would result in the activation of selected STAT proteins. To this end, Th1 and Th2 populations were assessed.
Committed Th1 and Th2 cells were generated as previously described (16) (for details, see Materials and Methods). After priming, both populations (>99.5% of the cells) homogeneously expressed the T cell markers CD3, CD4, and CD28; the activation markers CD25, CD71, and HLA-DR; and the memory marker CD45RO, and no longer contained detectable numbers of CD45RO<sup>−</sup> (unprimed) cells (16). To induce and detect cytokine production, Th1 and Th2 cells were restimulated for 72 h by anti-CD3 plus either anti-CD28 or IL-2. Culture supernatants were analyzed for IL-4, IL-5, and IFN-γ by ELISA (representative of three experiments performed with T cells from different individuals; mean of triplicates ± SD).

**FIGURE 1.** Cytokine pattern of committed human Th1 and Th2 populations. Committed Th1 and Th2 cells were restimulated (40,000/0.2 ml) by plate-bound anti-CD3 plus IL-2 for 72 h, and culture supernatants were analyzed for IL-4, IL-5, and IFN-γ by ELISA (representative of three experiments performed with T cells from different individuals; mean of triplicates ± SD).

Overlapping and distinct sets of STAT proteins are active in committed Th1 and Th2 cells

To assess the activation status of STAT proteins in committed Th cells by various stimulatory signals, Th1 and Th2 cells were either left unstimulated or were restimulated for 1, 4, or 24 h by anti-CD3, anti-CD28, IL-2, and their combinations. Whole cell extracts were prepared and introduced to EMSAs with STAT-specific 32P-labeled oligonucleotides. Briefly, the sis-inducible element from the e-fos promoter (SIE-M67 probe) served as the high affinity site for STAT1, STAT3, and STAT4 (29). The STAT6 element from the Ig κ heavy chain e promoter (Iκe probe) served as the high affinity site for STAT6; other STAT proteins are unable to bind this probe (43). Unspecific DNA binding complexes in the lower part of the EMSA are given to estimate the relative positions of the complexes on the native gels.

Figure 3 shows that overlapping and distinct sets of STAT proteins were active in committed Th1 and Th2 cells, and that the STAT activation profile did not change significantly with restimulation. Th1 cells contained two major STAT complexes and one minor complex binding the SIE-M67 probe (Fig. 3A, lanes 1–7, and Fig. 3C, lanes 1 and 5), but no complexes binding the Iκe probe (Fig. 3B, lanes 1–7). Th2 cells contained similar amounts of the SIE-M67 binding complexes (Fig. 3A, lanes 8–14), but in addition contained two complexes binding the Iκe probe (Fig. 3B, lanes 8–14). The STAT activation profile shown in Figure 3 was maintained for 24 h (the latest time point examined) if cells were restimulated by anti-CD3 alone or in combination with anti-CD28 or IL-2 or by anti-CD28 plus IL-2 (data not shown). If cells were left unstimulated or were stimulated by anti-CD28 or IL-2 alone, they rapidly died off and therefore were not examined at later time points.

To identify the molecular compositions of the detected complexes, supershift analysis was performed with STAT-specific Abs. The specific Abs used did not show cross-reactivity to other STAT family members (data not shown). Figure 3C shows that the two major complexes and the minor complex binding to the SIE-M67 probe were SIE-1-1 (STAT1 homodimer), SIE-1-3 (STAT1-STAT3 heterodimer), and SIE-3-3 (STAT3 homodimer), and that these complexes were present in both Th1 and Th2 cells (lanes 1 and 5). Anti-STAT1<sub>192–731</sub> completely supershifted the SIE-1-1 complex (lanes 2 and 6) and partly supershifted the SIE-1-3 complex. In the presence of anti-STAT<sub>3</sub><sub>688–727</sub> (which interferes with DNA binding of STAT3-containing complexes), the SIE-1-3 and SIE-3-3 complexes disappeared (lanes 3 and 7). Addition of anti-STAT1 and anti-STAT3 completely supershifted/inhibited the DNA-bound complexes in Th1 and Th2 cells (lanes 4 and 8). None of the complexes could be supershifted with Abs recognizing other STAT family members (not shown). The data show that both Th1 and Th2 cells contained a comparable composition of complexes composed of STAT1 and STAT3.

To further examine the molecular composition of the two complexes binding the Iκe probe, we performed supershift analysis with two different anti-STAT6 Abs recognizing the extreme carboxyl terminus of STAT6 (Fig. 3D). Anti-STAT6<sub>633–837</sub> completely supershifted both STAT6 complexes on the Iκe probe (lane 2). In contrast, anti-STAT6<sub>628–847</sub> supershifted only the upper complex, whereas the lower complex remained unaffected (lane 3). The complexes were not shifted by Abs recognizing other STAT family members (not shown). The data show that in contrast to Th1 cells, Th2 cells express two complexes containing activated STAT6. The faster migrating complex may represent an insufficiently/alternatively spliced form of STAT6, since the last exon is...
often substituted for or alternatively spliced from various STAT family members (44, 48, 49).

IL-4 and IL-12 differentially modulate STAT activation in Th1 and Th2 cells

In the next set of experiments we determined whether IL-4 and IL-12 would differentially modulate STAT activation in committed Th1 and Th2 cells. To this end, Th cell subsets were restimulated for 1 h by anti-CD3 plus IL-2 in the presence or absence of IL-4, IL-12, or IL-4 plus IL-12 as indicated. Whole cell extracts were prepared and introduced to STAT-specific probes in EMSA and supershift analysis.

Addition of IL-4 to restimulated Th1 cells induced activation of STAT6 (Fig. 4A, lanes 1 and 3), whereas the (already optimal) activation status of STAT6 in Th2 cells remained unchanged (lanes 9 and 11). Like IL-4, IL-12 induced significant activation of STAT6 in Th1 cells (quantitation by Phospholmager revealed

**FIGURE 3.** Committed Th cells express steady state levels of activated STAT proteins. Committed Th1 and Th2 cells were left unstimulated or were restimulated (40,000/0.2 ml) for 1 h by anti-CD3, anti-CD28, IL-2, or their combinations as indicated. Whole cell extracts were prepared and introduced in EMSAs with the 32P-labeled SIE-M67 probe binding STAT1, STAT3, and STAT4 (A and C) or with the 32P-labeled Ie probe binding STAT6 (B and D). In C, supershifts were performed with anti-STAT1 (lanes 2 and 6), anti-STAT3 (lanes 3 and 7), or anti-STAT1 plus anti-STAT3 (lanes 4 and 8). In D, supershifts were performed with anti-STAT6 (lanes 2) or anti-STAT6 (lanes 3).

**FIGURE 4.** Differential regulation of STAT activation in Th1 and Th2 cells by IL-4 and IL-12. Committed Th1 and Th2 cells were restimulated (40,000/0.2 ml) by anti-CD3 plus IL-2 for 1 h in the presence or the absence of IL-4, IL-12, or IL-4 plus IL-12 as indicated. Whole cell extracts were prepared and introduced to EMSAs with the 32P-labeled Ie probe (A) or the 32P-labeled SIE-M67 probe (B). To confirm the specificity of the STAT-containing complexes, supershift analysis was performed with STAT-specific Abs (representative of three experiments).
FIGURE 6. Regulation of cytokine secretion in Th1 and Th2 cells by IL-4 and IL-12. Committed Th1 and Th2 cells were restimulated (40,000 cells/0.2 ml of culture) for 4 h by anti-CD3 plus IL-2 in the presence or the absence of IL-4, IL-12, or IL-4 plus IL-12. Total cellular RNA was prepared from 2 × 10^6 Th1 and Th2 cells, and Northern blot analysis performed as described in Materials and Methods.

FIGURE 5. Regulation of cytokine gene transcription in Th1 and Th2 cells by IL-4 and IL-12. Committed Th1 and Th2 cells were restimulated (40,000 cells/0.2 ml of culture) for 4 h by anti-CD3 plus IL-2 in the presence or the absence of IL-4, IL-12, or IL-4 plus IL-12. Total cellular RNA was prepared from 2 × 10^6 Th1 and Th2 cells, and Northern blot analysis performed as described in Materials and Methods.

that unstimulated Th1 and Th2 cells did not express detectable levels of cytokine mRNA (lanes 1 and 6). Restimulation of Th1 cells by anti-CD3/IL-2 rapidly induced transcription of IFN-γ, but not IL-4 (lane 2), whereas restimulation of Th2 cells induced transcription of IL-4, but not IFN-γ (lane 7), confirming the pure phenotypes of the responding cells. Addition of IL-4 during the restimulation did not induce or increase IL-4 transcription in Th1 or Th2 cells, respectively (lanes 3 and 8), and did not suppress transcription of IFN-γ in Th1 cells (lane 3). In contrast, IL-12 rapidly increased IFN-γ transcription in Th1 cells (lane 4) and induced IFN-γ mRNA in Th2 cells (lane 9). Moreover, IL-4 did not antagonize the activity of IL-12 on Th1 or Th2 cells (lanes 5 and 10). Together the data show that IL-12 rapidly increased/induced IFN-γ mRNA in Th1/Th2 cells.

Regulation of cytokine production in Th1 and Th2 cells by IL-4 and IL-12

We have shown above that both IL-4 and IL-12 readily modulated STAT phosphorylation in Th1 and Th2 cells within 1 h of restimulation. However, this may not have immediate consequences on the levels of detectable cytokine mRNA, which also depends on the mRNA turnover. Therefore, we analyzed whether prolonged exposure of Th1 and Th2 populations to IL-4 or IL-12 could modulate the secreted cytokine pattern according to the observed modulation of STAT proteins.

Committed Th1 and Th2 cells were restimulated for 72 h by anti-CD3 plus IL-2 in the presence of IL-4, IL-12, or IL-4 plus IL-12, and culture supernatants were analyzed for cytokines by ELISA. Figure 6 shows that in Th2 cells, IL-12 suppressed the production of IL-5 by >80% and that of IL-4 by 25%, and induced the production of large amounts of IFN-γ. The suppressive effects of IL-12 could not be reversed by addition of exogenous IL-4. Moreover, IL-12 increased the production of IFN-γ in Th1 cells, and this could not be suppressed by IL-4. In Th2 cells, addition of IL-4 did not further increase IL-5 production.

The effects of cytokines were even more pronounced if primed Th cells were restimulated for 72 h in the presence of IL-4 or IL-12
and restimulated in the absence of cytokines. Figure 7 shows that Th2 cells restimulated in IL-4 did not secrete larger amounts of IL-4 upon a second restimulation. Furthermore, restimulation of Th2 cells in the presence of a neutralizing anti-IL-4 mAb did not reduce, but rather, increased, the amounts of IL-4 secreted upon a second restimulation. These data show that production of IL-4 was independent of endogenous IL-4 signaling, which supports the full commitment of Th2 cells. However, IL-12 suppressed production of IL-4 by 80%, and the effects of IL-12 could not be reversed by addition of exogenous IL-4.

Together the data show that in Th2 cells, IL-12 induced a rapid loss of STAT6 DNA binding activity (Fig. 4a), but induced DNA binding activity of STAT4 (Fig. 4b). This coincided with rapid induction of IFN-γ and suppression of IL-5, and delayed suppression of IL-4 (Figs. 6 and 7). In Th1 cells, the IL-12-induced activation of STAT6 and STAT4 (Fig. 4a) coincided with increased IFN-γ production, but did not result in detectable Th2 cytokine expression. Similarly, the activation of STAT6 in Th1 cells by IL-4 did not lead to Th2 cytokine expression, whereas in Th2 cells, expression of IL-4 and IL-5 always coincided with activation of STAT6.

### Discussion

The cytokines IL-4 and IL-12 play a crucial role in the differentiation of Th2 and Th1 effector cell populations, respectively (1–6). However, we have recently shown that naive human CD4+ CD25− T cells can be primed for massive production of Th2 cytokines independent of IL-4 if they are activated in limiting dilution by immobilized anti-CD28 mAb plus IL-2 (16, 50). Th2 priming induced vigorous proliferation (clone size $>200$) and was completely resistant to suppression by a highly neutralizing anti-IL-4 mAb (16). If additional TCR signals were provided, the cells defaulted to the Th1 pathway, and this process was independent of IL-12. We now analyzed whether the IL-4/IL-12-independent Th2/Th1 cell differentiation would result in the activation of selected STAT proteins, and whether such Th1 and Th2 cells remained responsive to IL-4 and IL-12.

Both Th1 and Th2 populations (>99.5% of the cells) homogeneously expressed the T cell markers CD3 and CD4; the activation markers CD25, CD71, and HLA-DR; and the memory marker CD45RO (16), but no longer contained unprimed CD45RO− cells. Both populations required restimulation via the TCR (by anti-CD3) in combination with either anti-CD28 or IL-2 to express cytokine. Th1 cells expressed IFN-γ but no IL-4 and IL-5, whereas Th2 cells expressed IL-4 and IL-5 but little IFN-γ. In restimulated Th2 cells, the high levels of IL-4 mRNA combined with the barely detectable amounts of IFN-γ mRNA confirm the high purity of the Th2 population. Moreover, the massive amounts of secreted IL-4 (150 ng/10^6 Th2 cells) underline the high efficiency of the priming procedure.

Although committed Th2 cells did not express IL-4 mRNA or protein without TCR restimulation (Fig. 5), such Th2 (but not Th1) cells expressed high levels of the active form of STAT6, a transcription factor known to be involved in IL-4 gene activation (23–26, 34). We have shown previously that CD28/IL-2-dependent Th2 priming was insensitive to suppression by a highly neutralizing anti-IL-4 mAb, but that the proliferation associated with the stimulation could be blocked completely by adding small amounts of exogenous IL-4 to the cultures (16, 50). These data demonstrate that STAT6 could be activated in response to signals delivered via CD28 and the IL-2R independent of IL-4. The fact that activation of STAT6 occurred in the absence of TCR/CD3 signals suggested that active STAT6 may help to maintain Th2 commitment and may accelerate the response to Ag challenge. A constitutive tyrosine phosphorylation of STAT proteins has been reported previously in lymphoid leukemia cells and lymphoma cell lines (51).

We found that restimulation of Th2 cells by anti-CD3 plus either anti-CD28 or IL-2 rapidly induced large amounts of IL-4 mRNA and protein, but did not modulate the tyrosine phosphorylation of STAT6. The fication of CD3/CD28 may be required to fully trans-activate DNA-bound STATs through phosphorylation on serine, which can be induced via the Ras/mitogen-activated protein kinase pathway that is also known to be coupled to the TCR (36, 37). Alternatively, the TCR signal may induce/activate STAT-unrelated transcription factors. In Th2 cells, a possible candidate is the proto-oncogene c-maf, which is induced in murine Th2 (but not Th1) clones via CD3 and acts as a specific IL-4 transcription factor in synergy with nuclear factor-ATp (52) and NIP45 (53). Taken together the constitutive activation of STAT6 may explain why IL-4 production by committed human Th2 cells was uncoupled from the autocrine IL-4 signaling pathway and was insensitive to suppression by neutralizing anti-IL-4 mAb. Likewise, chronic Th2 immunity in mice is relatively resistant to suppression by anti-IL-4 mAb (54), and in vitro IL-4 production by differentiated murine CD4 T cells is cytokine autonomous (55).

Both Th1 and Th2 populations expressed activated STAT1 and STAT3 (without expressing mRNA for IFN-γ or IL-4), whereas active STAT2, STAT4, and STAT5 were not detectable. STAT1 and STAT3 are known to be activated by a large number of ligands, including those with receptors that have intrinsic tyrosine kinases as well as members of the cytokine receptor family that recruit tyrosine kinases of the JAK family (21, 22), and this may explain their activation in both Th1 and Th2 cells. However, STAT1 and STAT3 can be activated by IL-12 and IFN-α (21), two major players in the induction of Th1 immunity (18, 20), but are not activated by IL-4 (21), the cytokine driving Th2 cell differentiation. Accordingly, STAT1-deficient mice are highly sensitive to infection by microbial pathogens and viruses (56, 57). However, the precise roles of STAT1 and STAT3 in Th1 vs Th2 immunity remain elusive.
In primary TCR-stimulated T cells, addition of IL-12 leads to activation of STAT4 (but not STAT6), and this coincides with strong induction of IFN-γ production (21, 22). We observed that efficient production of IFN-γ occurred in anti-CD28/anti-CD3/IL-2–primed Th1 cells in the absence of IL-12 and without detectable tyrosine phosphorylation of STAT4. This correlates to the finding that IL-12-deficient mice are impaired, but not lacking in the ability to produce IFN-γ or to mount a Th1 response in vivo (58). In human Th1 cells, IL-12 activated both STAT6 and STAT4, which coincided with a rapid and massive increase in IFN-γ production, but did not lead to expression of IL-4. The human data correlate to murine systems in which optimal production of IFN-γ by fully differentiated murine CD4 T cells was still cytokine dependent (55). In human Th1 cells, the activation level of STAT6 induced by IL-12 was about one-third of that induced by IL-4, and IL-4 and IL-12 strongly synergized in the activation of STAT6. However, the lack of Th2 cytokine induction in such Th1 cells was not related to a dominant negative STAT6 variant (48, 59), which would be visible in bandshift assays, since the Abs used for supershift analysis recognized the individual trans-activation domain of STAT6, which resides at the extreme carboxyl terminus (43). Therefore, CD3/IL-2/IL-4–restimulated Th1 cells may require additional signals to express Th2 cytokine genes. Such signals may be provided by accessory cells, since murine Th1 cells could be converted to Th2 in the presence of APC and IL-4 (11).

In striking contrast to the situation in Th1 cells, IL-12 readily and completely inactivated STAT6 DNA binding activity and activated STAT4 in the Th2 population. The inactivation of STAT6 may have been related to an activation of nuclear tyrosine phosphatases or proteolysis mechanisms (59–62). The modulation of STAT activation coincided with a rapid induction of IFN-γ and a suppression of IL-5. The delayed suppression of IL-4 may have been related to a slow turnover of the IL-4 mRNA. However, the data demonstrate that CD28/IL-2–primed Th2 cells retained their potential to down-regulate Th2 cytokines in response to IL-12. The remaining IL-12 responsiveness may relate to the fact that CD28/IL-2–dependent Th2 priming did not lead to detectable production of IL-4 despite the activation of STAT6, but that IL-4 would be required to down-regulate IL-12R (63). Recent reports suggested that even conventional (TCR/IL-4–primed) Th2 cells may up-regulate IL-12R if persistently exposed to IFN-γ (45, 55, 63–65).

In vivo, CD28/IL-2–primed Th cells may behave like memory T cells (rather than like primary T cells) when they are confronted with the Ag for the very first time. Such primed T cells could readily function as Th2 effector cells, leading to increased humoral responses, or support cellular Th1 immunity, depending on whether the specific Ag is presented on B cells (not producing IL-12) or on phagocytes/dendritic cells (producing IL-12). It is tempting to speculate that Ag-independent Th2 priming may occur in inflammatory tissues, where T cells are in intimate contact with large numbers of accessory cells bearing the natural CD28 ligands B7.1 and B7.2 and where these T cells are simultaneously exposed to high concentrations of IL-2 (16, 50). A shift from Th1 to Th2 immunity at the end of an infection (with decreasing Ag concentrations) may, in general, help to terminate Th1–driven inflammatory reactions.

In summary, our data show that TCR/IL-4–independent Th2 priming of naive human CD4+45RO T cells resulted in activation of STAT6 DNA binding activity without inducing detectable cytokine (IL-4) expression. The primed Th2 cells resembled memory cells and responded with massive production of Th2 cytokines upon the first costimulation via CD3 and CD28. However, they maintained their potential to down-regulate Th2 cytokines in response to IL-12, which coincided with a rapid repression of STAT6 and an activation of STAT4. The CD3/CD28 signals required to initiate cytokine gene expression did not modulate the activation status of STATs, but may modulate their trans-activation potential or may provide STAT-unrelated signals. The effects of cytokines on STAT activation may vary depending on the commitment of the responding cells, since IL-12 was able to activate STAT6 in Th1 and to repress STAT6 in Th2 cells.

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