

Interleukin 12 Signaling in T Helper Type 1 (Th1) Cells Involves Tyrosine Phosphorylation of Signal Transducer and Activator of Transcription (Stat)3 and Stat4

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Summary

Interleukin 12 (IL-12) initiates the differentiation of naive CD4⁺ T cells to T helper type 1 (Th1) cells critical for resistance to intracellular pathogens such as *Leishmania major*. To explore the basis of IL-12 action, we analyzed induction of nuclear factors in Th1 cells. IL-12 selectively induced nuclear DNA-binding complexes that contained Stat3 and Stat4, recently cloned members of the family of signal transducers and activators of transcription (STATs). While Stat3 participates in signaling for several other cytokines, Stat4 was not previously known to participate in the signaling pathway for any natural ligand. The selective activation of Stat4 provides a basis for unique actions of IL-12 on Th1 development. Thus, this study presents the first identification of the early events in IL-12 signaling in T cells and of ligand activation of Stat4.

Cytokines regulate the development of Th1 and Th2 subsets during primary T cell activation (1–15). We and others recently showed that IL-12 induces development of Th1 cells, which secrete IL-2, IFN- γ , and lymphotoxin and promote cell-mediated immune responses (1, 2, 16–18). In contrast, IL-4 induces development of Th2 cells, which secrete IL-4, -5, -6, and -10 (1, 2, 19, 20). IL-12 is a covalent heterodimer of p35 and p40 subunits (21–23) that binds to cell surface receptors on lymphocytes (24). Besides inducing Th1 differentiation (1, 2), IL-12 can augment Th1 cytokine (e.g., IFN- γ) production and cellular proliferation during antigenic stimulation (25–27). While IL-12 and IL-4 are critical in directing the development of naive T cells, little is known about the intracellular signals delivered by these cytokines to promote differentiation.

Several cytokines, including IFN- α , IFN- γ , IL-6, prolactin, epidermal growth factor, and platelet-derived growth factor (PDGF),¹ deliver signals through the Jak-STAT family of signal transduction molecules (4–9, 28, 29). Receptor activation leads to rapid recruitment and tyrosine phosphorylation of specific members of the STAT family, inducing their dimerization, nuclear translocation, and binding to specific

DNA sequences in target genes (4, 30–32). Besides forming homodimers, STAT members can also form heterodimers, such as the epidermal growth factor-induced SIF-B complex, a heterodimer of Stat1 and Stat3 (9). While several of these cytokines activate Stat1, 2, 3, 5, and 6, none have been shown to activate Stat4 (5, 6).

In this report we examined the signaling pathway used by IL-12 in Th1 cells. We report that IL-12 induces the rapid tyrosine phosphorylation of Stat3 and Stat4 and formation of nuclear complexes capable of binding to DNA sequences related to IFN- γ activation site (GAS) elements. This report provides the first identification of a ligand to activate Stat4 and of a transcription complex induced by IL-12. These results open the way to examination of the molecular basis of T helper cell development and commitment.

Materials and Methods

Reagents and Cytokines. Genistein, PMA, and ionomycin were purchased from Sigma Chemical Co. (St. Louis, MO); poly-dIdC was from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ); and media were from Washington University Tissue Culture Support Center (St. Louis, MO). Human rIL-2 was supplied by Takeda (Osaka, Japan); murine IL-4 was produced as high titer culture supernatant of transfected P815 mastocytoma cells; murine rIL-12 was supplied by Genetics Institute (Cambridge, MA), human rIFN- α A/D was supplied by Hoffmann-La Roche (Geneva, Switzerland); murine rIFN- γ was supplied by Genentech, Inc. (South San Fran-

¹ Abbreviations used in this paper: EMSA, electrophoretic mobility shift assay; PDGF, platelet-derived growth factor; STAT, signal transducer and activator of transcription.

cisco, CA); and PDGF-BB was supplied by the National Institutes of Health (Bethesda, MD). IL-6 and antiphosphotyrosine RC20 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-STAT antisera were produced as described (5) using fusion proteins of glutathione S-transferase to the less homologous COOH-terminal regions of STAT family members.

Animals and Cell Lines. The Th1 clone 3F6 was derived by OVA stimulation from DO11.10 transgenic splenocytes in the presence of anti-IL-4 followed by limiting dilution cloning (33, 34), and it was maintained by 3-d stimulation of 10^7 T cells with 3×10^8 Balb/c splenocytes, 500 nM OVA, and 40 U/ml IL-2 in supplemented IMDM with 10% FCS, followed by a 7-d period of expansion in media with IL-2 alone. For cytokine assays (34), 3F6 cells were stimulated in 2-ml cultures with 2×10^5 T cells and 5×10^6 splenocytes. For nuclear extracts and immunoprecipitations, 3F6 cells were harvested at approximately day 10 after stimulation and were free of contaminating feeder cells. The murine fibroblast line 3T3, the murine thymoma line EL4, and the murine hepatocyte cell line HepG2 were grown in DMEM with 10% FCS. Female Balb/c mice were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN).

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA). 3F6 cells were stimulated for 30 min with 200 U/ml IL-4, 10 U/ml IL-12, 1,000 U/ml IFN- γ , or 300 U/ml IFN- α , and nuclear extracts were prepared as previously described (35). 3T3 or HepG2 cells were stimulated for 15 min with 100 ng/ml PDGF-BB or 30 ng/ml IL-6 before preparation of extracts as described (31). EMSAs were performed as described (35). Briefly, nuclear extracts (3 μ g) were incubated at room temperature with 32 P-labeled m67 or Fc γ RI probe (1 ng, 2×10^5 cpm) in binding buffer with 1 μ g poly-dIdC for 30 min before addition of gel loading buffer and electrophoresis at room temperature on polyacrylamide gels (4.5% with 2.5% glycerol in 0.4 \times TBE). Antibody supershifts were performed by adding 2 μ l of diluted antiserum (1:10 in binding buffer) to binding reactions for 30 min before electrophoresis.

Immunoprecipitation and SDS-PAGE. Total cellular lysates of $2-5 \times 10^7$ cells were immunoprecipitated as described (32) and separated by electrophoresis on 7% SDS-PAGE. After transfer to nitrocellulose, blots were probed with RC20 (1:2,500). Blots were then stripped and reprobed with anti-STAT antisera (1:3,000).

Oligonucleotide Probes and Competitors. The following double-stranded oligonucleotides were used in EMSA experiments: m67, GTCGACATTTCCCGTAAATCGTCGA; Fc γ RI, TCGACGCGATGTTTCAAGGATTTGAGATGTATTTCCCGAAAAGGCTCGA; Fc γ RI.2, TCGACGTATTTCCCGAAAAGGAACCTCGA; ISRE, GATCCTTCAGTTTCGGTTTCCCTTTCC; P γ 1, GATCCAAGCCCAAGAGTTTCCTTTTATTCGTCGA; P γ 2, TCGACTGCTTTCAGAGAATCCACACTCGA; P γ 3, GAAGTAAAA-GTGCTTTCAGAGAATCCACAGAATGGCAC; P γ 4, GGC-TGTCTCATCGTCAGAGAGCCCAAGGAGTCGA; P γ 5, GGC-CAAGGAGTCGAAAAGGAACTCTAACATGCC; P γ 6, TGT-AACATGCCACAAAACCATAGCTGTAATGCAAAAG; P γ 7, CCCCCACCTATCTGTCAACCATCTTAACC; and P γ 8, GGA-TTACGTATTTTCCACAAGTTTTTTTCC.

Luciferase Reporter Constructs and Transient Transfections. Various regions of the IFN- γ promoter were amplified by PCR from genomic templates (provided by Dr. H. Fox, Department of Immunology, Scripps Clinic, La Jolla, CA) and placed into the SalI/BglII sites of the luciferase reported plasmid pBS-Luc (35). These sequences have been previously reported (35a). Promoter constructs spanned the region from -3448 to +36, -178 to +36, or -56 to +36. All plasmid constructs were cesium chloride-banded twice and verified by sequence. Electroporation, stimulation, and luciferase assays were performed as described (35). Cells were stimu-

lated 12 h after transfection with 50 ng/ml PMA and 1 μ M ionomycin and assayed for luciferase activity 5 h later. For cytokine stimulation, IL-12 was added at 10 U/ml 2 h after transfection. Data are presented as the mean of duplicate determinations of relative light units (RLU) normalized for transfection efficiency by CMV-CAT reporter plasmid (35). Each experiment was repeated at least three times.

Results

Identification of a Cellular Model for IL-12 Signaling. Freshly isolated naive CD4 $^+$ T cells are an ideal model of IL-12 responsiveness (2), but isolation of sufficient cell numbers limits their use for Western blot and EMSA analysis. Immortalized cell lines are convenient for analysis of signaling pathways, but none of the transformed T cell lines we have tested responded to IL-12 by IFN- γ production or proliferative responses. However, we identified several antigen-dependent CD4 $^+$ T cell clones responsive to IL-12. For the Th1 clone 3F6 (34, 35), addition of IL-12 during antigen activation with OVA produced a threefold augmentation of IFN- γ production (Fig. 1). This degree of augmentation by IL-12 is typical of many Th1 cells, which, unlike naive T cells, produce IFN- γ even without the addition of IL-12 (2, 34, 36). Thus, 3F6 appeared to be a useful system for examining IL-12 signaling.

IL-12 Induces Tyrosine Phosphorylation of Stat3 and Stat4. Cytoplasmic extracts of IL-12-treated or untreated 3F6 cells were precipitated using one of four STAT-specific antisera, separated by SDS-PAGE, and examined for phosphotyrosine incorporation into proteins of the 90–100-kD size range (Fig. 2). IL-12 induced tyrosine phosphorylation of both Stat3 and Stat4, whereas IFN- α caused tyrosine phosphorylation of neither. We confirmed the presence of non-tyrosine-phosphorylated Stat3 and Stat4 in unstimulated cells by reprobing stripped blots with antisera to Stat3 and Stat4 (Fig. 2 A). 3F6 secretes IFN- γ after antigen activation, resulting in the constitutive phosphorylation of Stat1 in these cells (Fig. 2 B). 3F6 cells express low levels of Stat2, which is not activated by IL-12 (data not shown).

To determine whether tyrosine phosphorylation of Stat3 and Stat4 by IL-12 results in their homodimerization or heterodimerization, we precipitated complexes from IL-12-treated 3F6 cells using Stat3- and Stat4-specific antisera and probed Western blots with various anti-STAT antisera or the antiphosphotyrosine reagent RC20 (Fig. 2 B). As expected, Stat3 and Stat4 complexes immunoprecipitated from IL-12-treated cells contained phosphotyrosine. The overall amount of phosphorylated Stat4 appeared significantly greater than phosphorylated Stat3 or Stat1. Antiphosphotyrosine RC20 recognized bands of two distinct molecular weights in complexes immunoprecipitated by anti-Stat3 antisera. Complexes precipitated by anti-Stat3 also contained Stat4, clearly distinguishable by its higher molecular weight and reactivity with Stat4 antiserum (Fig. 2 B). This coprecipitation of Stat4 with Stat3 is specific to IL-12 treatment, suggesting that it results from cytokine activation rather than simply antibody cross-reactivity. In contrast, Stat3-immunoreactive material was not detect-

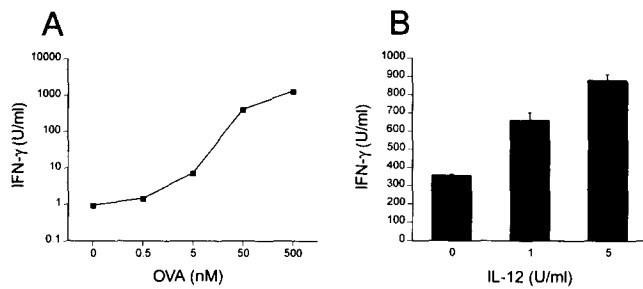


Figure 1. IL-12 augments production of IFN- γ by the OVA-reactive Th1 clone 3F6. (A) 3F6 cells were stimulated with splenocytes, IL-2, and OVA peptide. Supernatants were collected after 36 h and analyzed by ELISA for IFN- γ production. (B) 3F6 cells were stimulated with splenocytes, IL-2, 50 nM OVA, and IL-12. 3F6 cells used for all experiments were harvested in the quiescent phase at approximately day 10 after passage with OVA and IL-2 and were nonproliferative before stimulation. The sensitivity of the IFN- γ ELISA is ~ 1 U/ml.

able in complexes precipitated with anti-Stat4 antisera, suggesting a relative abundance of phosphorylated Stat4 production compared with phosphorylated Stat3. These results indicate that IL-12-induced phosphorylation of Stat3 and Stat4 generates a heterodimer of Stat3 and Stat4 and a probable homodimer of Stat4. Without additional experiments, however, we cannot rule out the association of either STAT with a currently unidentified component.

IL-12 Activates Stat3 and Stat4 for DNA Binding. To determine whether the IL-12-induced STAT factors could bind DNA, we used two oligonucleotide probes. The m67 probe, derived from the serum-inducible element of the *c-fos* promoter (37), and the Fc γ RI probe, from the IFN- γ response region of the high affinity Fc γ receptor promoter (38), have distinct binding specificity for various STAT family members. IL-12 induced the formation of nuclear complexes with the Fc γ RI probe within 10 min of stimulation (Fig. 3 A) at as little as 1 U/ml IL-12 (Fig. 3 B). The formation of these IL-12-induced complexes was dependent upon tyrosine phosphorylation, since the protein tyrosine kinase inhibitor genistein blocked their generation (Fig. 3 C).

In addition, we used treatment of cells with other cytokines to compare the migration of IL-12-induced STATs with various known STAT factors. Using the m67 probe, IFN- α induced the expected Stat1 homodimer (SIF-C) (Fig. 4), and

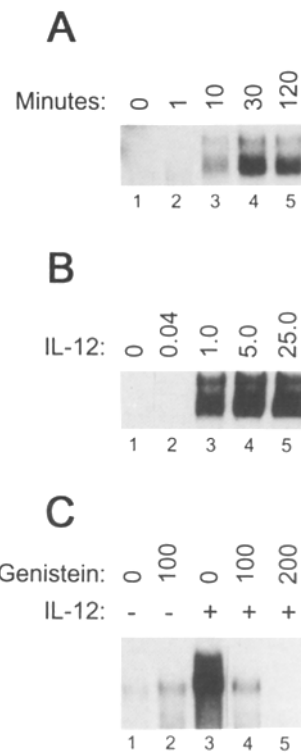


Figure 3. IL-12 induction of nuclear complexes binding the Fc γ RI probe requires tyrosine phosphorylation. 3F6 cells were treated (A) with 10 U/ml IL-12 for the indicated number of minutes, or (B) for 10 min with 0, 0.04, 1, 5, or 25 U/ml IL-12, and nuclear extracts were prepared. (C) 3F6 cells were pretreated for 3 h with the indicated concentrations (100 or 200 μ g/ml) of genistein before 10-min stimulation with IL-12 (10 U/ml). EMSA was carried out with the Fc γ RI probe.

IL-6 induced the expected Stat3 homodimer (SIF-A). Each of these was clearly distinct from the pattern of complexes induced by IL-12 (Fig. 4 A). PDGF induced the expected three complexes, Stat3 homodimers (SIF-A), Stat1 homodimers (SIF-C), and Stat1/Stat3 heterodimers (SIF-B, Fig. 4 A) (4). By comparison, IL-12 induced one lower band migrating with SIF-C, but with a closely spaced upper doublet with mobility differing from SIF-A and SIF-B (Fig. 4 A, lane 3). Thus, with the m67 probe, it was difficult to distinguish the IL-12-induced complexes from SIF-A and SIF-B induced by PDGF.

However, the Fc γ RI probe allowed clearer differentiation of the complexes induced by these cytokines. Using the Fc γ RI probe, IL-12 induced a broad complex of slow mobility (Fig. 4 B), whereas IL-6-, PDGF-, and IFN- α -induced complexes did not bind well to this probe. The IL-4-induced complex, which bound poorly to the m67 probe, produced a strong

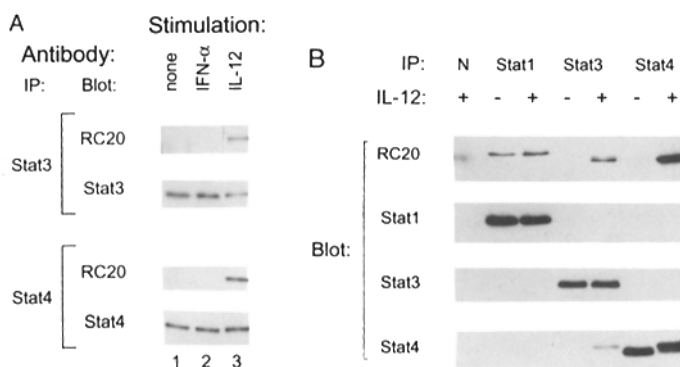


Figure 2. IL-12 treatment induces tyrosine phosphorylation of Stat3 and Stat4. Whole-cell lysates were prepared from 3F6 cells treated with IFN- γ or IL-12 and immunoprecipitated with anti-STAT antisera. After separation by SDS-PAGE (7% gel) and transfer to nitrocellulose, the blot was probed with antiphosphotyrosine RC20 and then stripped and reprobed with anti-STAT antisera. (A) IFN- α or IL-12-stimulated 3F6 extracts were precipitated with anti-Stat3 or anti-Stat4 and probed with RC20, followed by stripping and reprobing with respective STAT antisera. (B) Control or IL-12-stimulated extracts, precipitated with normal serum (N) or anti-Stat1, 3, or 4 antisera, were probed with RC20 or with each STAT antisera as indicated.

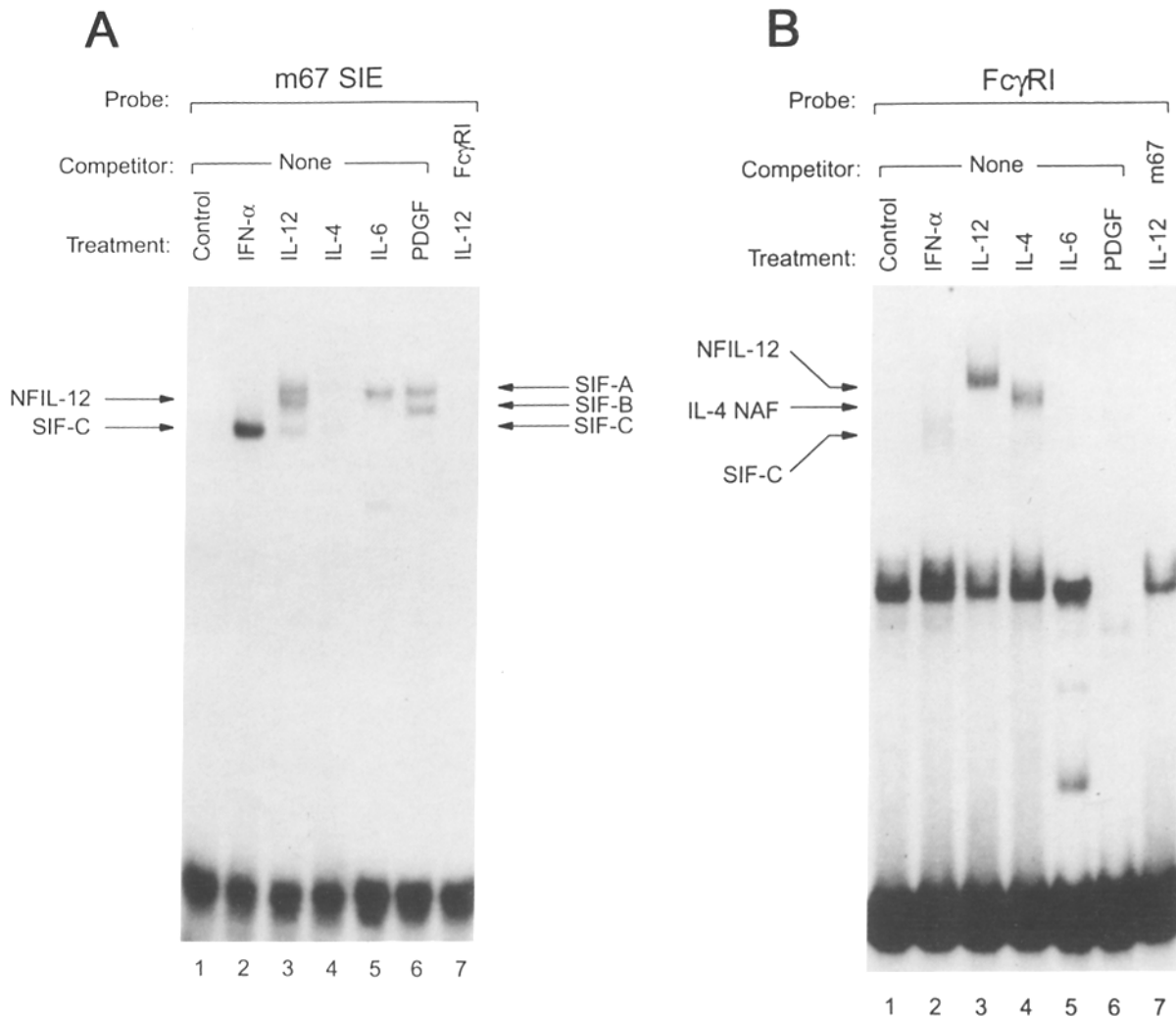


Figure 4. IL-12 induces a unique pattern of nuclear complexes binding to (A) the m67 and (B) FcγRI oligonucleotides. Extracts were prepared from 3F6 cells treated with media, IFN-α, IL-12, or IL-4; 3T3 cells treated with PDGF; and HepG2 cells treated with IL-6.

band with a faster mobility than the IL-12-induced complex with the FcγRI probe. In summary, IL-12 induced nuclear complexes with a distinct pattern of mobility and probe specificity from factors composed strictly of Stat1, Stat3, or IL-4 Stat (Stat6). (During the preparation of this manuscript, a new protein termed IL-4 Stat, distinct from Stat1 through Stat5, has been described [39]; presumably, the IL-4-induced protein complex we observed contains this new protein.)

To further characterize these complexes, we determined their reactivity to various anti-STAT antisera, using both m67 and FcγRI probes. With the FcγRI probe, Stat4 and Stat3 antisera removed the majority of IL-12-induced complexes (Fig. 5 A, lanes 6 and 7), whereas Stat1 and Stat2 antisera were ineffective. By contrast, the IL-4-induced complex was unaffected by any of these four antisera. With the m67 probe, the IL-12-induced complexes reacted specifically with Stat1, 3, and 4 antisera (Fig. 5 C). The lower band, comigrating with SIF-C, was affected only by Stat1 antiserum (Fig. 5 C,

lane 2). This Stat1-containing complex is not seen with the FcγRI probe because the relative affinity of that probe for Stat1 is much lower. The upper doublet shifted completely with Stat4 antiserum and nearly completely with Stat3 antiserum (Fig. 5 C, lanes 4 and 5). Stat2 antiserum had no effect on these bands. As controls, the supershifts of PDGF-treated extracts yielded the expected shift pattern with Stat1 and Stat3 antisera and showed no effect with Stat4 antiserum (Fig. 5 D).

No Evidence for Direct Interaction of Stat3/Stat4 with the IFN-γ Promoter. IL-12's augmentation of IFN-γ production in T cells and NK cells could be mediated either by direct action of STAT factors on the IFN-γ promoter or by an indirect pathway involving the modification and/or induction of secondary intermediate proteins. To determine whether the IL-12-induced STAT factors identified above could be interacting directly with *cis*-acting elements within the proximal IFN-γ promoter region, we have initiated studies on this promoter

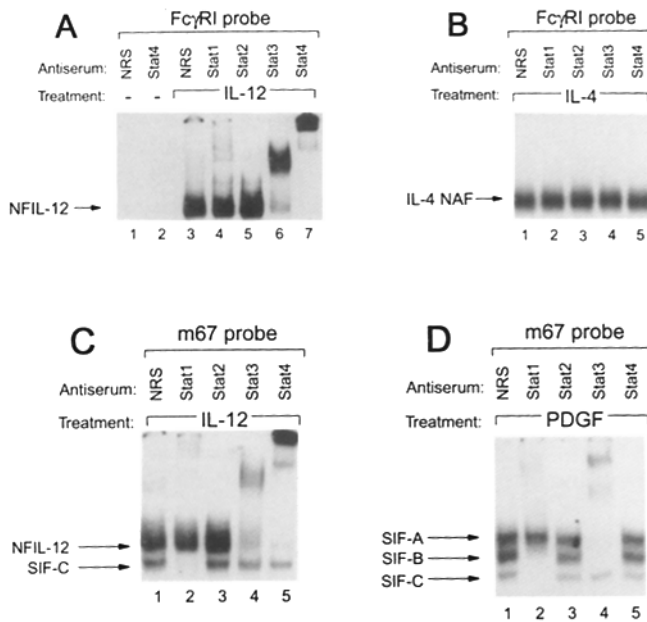


Figure 5. IL-12-induced nuclear complexes contain Stat3 and Stat4. Nuclear extracts of 3F6 cells were prepared after (A) IL-12 treatment, or (B) IL-4 treatment. EMSA was performed with the Fc γ RI probe and antisera to Stat1, 2, 3, and 4. (C) IL-12-treated 3F6 nuclear extracts, or (D) PDGF-treated 3T3 cell extracts were supershifted using the m67 probe.

region. Previously, others have found that an immediate 5' flanking region from -108 to -40 bp mediates inducible reporter activity in human T cells (40). We have produced a series of luciferase-based mouse reporter constructs to confirm these findings in the mouse system and to examine the responses of this region to IL-12 (see Fig. 7). As reported for the human promoter (40), we find that PMA and ionomycin responsiveness lie within the -178 to -56 bp region of the proximal promoter. However, this region of IFN- γ promoter does not appear to confer IL-12 responsiveness to the reporter when expressed either in the murine thymoma EL4 (Fig. 6), in the transfectable IL-12 responsive OI17, or in the human T cell line Kit225 (data not shown). Furthermore, we examined the IFN- γ promoter (Fig. 7 A) for direct interaction with IL-12-induced STAT factors in EMSA using the m67 probe and IL-12-stimulated 3F6 nuclear extracts (Fig. 7 B). For this analysis we examined oligonucleotides spanning several hundred base pairs of the proximal promoter important for inducible activity. None of the competitors from the IFN- γ promoter inhibited IL-12-induced STAT factor binding to the m67 probe (Fig. 7 B). As controls for inhibition, we showed that m67 and Fc γ RI competitors blocked complex formation (Fig. 7 B). These results suggest the possibility that the effect of IL-12 on the IFN- γ promoter may be indirect or mediated by sites outside the immediate 5' promoter.

Discussion

IL-12 induces Th1 development (1, 2) and is critical to pathogen resistance by promoting cell-mediated immunity

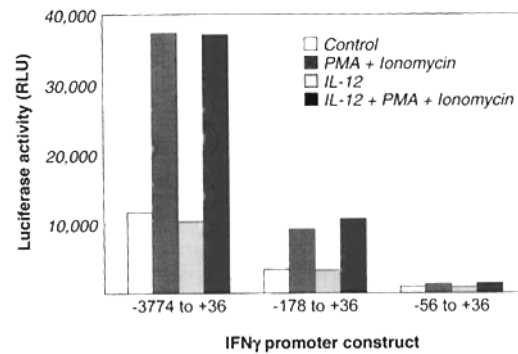


Figure 6. The 5' promoter of IFN- γ is responsive to PMA and ionomycin but not to IL-12 stimulation. EL4 cells were transiently transfected with murine reporter constructs spanning from -3447 , -178 , to -56 bp in the IFN- γ promoter and stimulated with PMA and ionomycin (5 h) and/or IL-12 (15 h). Luciferase assay measurements are reported in RLU.

via both T cells and NK cells (13–15). One subunit of IL-12R has been cloned and is homologous to gp130, with box 1 and box 2 motifs (41, 42). Additional receptor components are likely to be necessary for high affinity binding, however (42). Bacon et al. recently found that IL-12 activates Jak2 and Tyk2 tyrosine kinases in activated human T cells (43). IL-12 may also induce tyrosine phosphorylation of putative mitogen-activated protein kinases (44), but, in our Western blots of 3F6 lysates, IL-12-induced phosphotyrosine was present only in proteins of ~ 90 kD (data not shown). Taken together, current data support the hypothesis that IL-12 uses specific members of the Jak-STAT pathway for signal transduction.

Stat4 was recently cloned in two laboratories by its homology to Stat1 (5, 6) but was not found to be activated by any natural ligand in numerous cell types. While Stat1 is widely expressed, Stat4 expression was restricted to the testis and a few other tissues including the spleen. Stat4 is

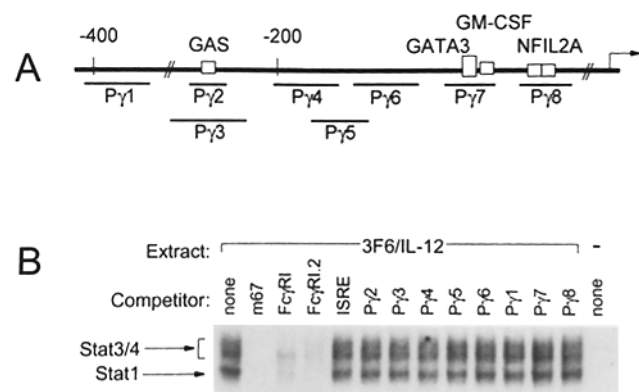


Figure 7. IL-12-induced nuclear factors do not bind to oligonucleotide competitors from the IFN- γ promoter. (A) Oligonucleotide probes P γ 1–P γ 8 span the proximal ~ 400 bp of IFN- γ promoter and include putative GATA-3, NFIL2A-like, and GMCSF-like sites described by Penix et al. (41). (B) Nuclear extracts from IL-12-induced 3F6 cells were incubated with radiolabeled m67 probe in the presence or absence of 20 ng competitor DNA as indicated and resolved by EMSA.

not expressed in many T cell tumor lines (6); however, freshly isolated CD4⁺ T cells, other CD4⁺ T cell clones, the CD8⁺ T cell clone L3, and the NK cell line CRNK5 contain Stat4 and generate Stat4-containing, IL-12-induced complexes (data not shown).

Like many other Th1 clones, 3F6 responds to IL-12 stimulation with only a severalfold increase in IFN- γ production. While naive T cells exhibit a more dramatic developmental response to IL-12 by greatly increasing subsequent IFN- γ production, naive CD4⁺ cells are unable to produce significant amounts of IFN- γ without IL-12 treatment (2). Because Th1 clones retain responsiveness to IL-12 for augmentation of IFN- γ production and can be conveniently manipulated in vitro, they provided a suitable model for our initial characterization of IL-12 signaling.

Based on the reactivity with anti-STAT antisera (Fig. 5) and results from coimmunoprecipitations (Fig. 2B), it is likely that IL-12 induces at least two types of STAT-containing complexes (4). Our data support the presence of some Stat3/Stat4 heterodimers and imply that the Stat4 homodimers (or activated Stat4 in association with a currently unknown component) comprise most of the IL-12-induced complexes.

Among the well-characterized effects of IL-12 is the rapid induction of IFN- γ transcription (26, 27), although the mechanism of this action is currently unknown. In our analysis of the proximal IFN- γ promoter, a region important for transcription in T cells (40), we found no direct evidence of interaction between the IL-12-induced STAT factors and *cis*-acting elements. While we cannot rule out direct interactions of Stat3/Stat4 with *cis*-acting elements in other regions of the IFN- γ gene, these results may suggest an indirect mode of action of IL-12 on IFN- γ . This situation would be similar to the indirect action of IFN- γ for augmenting target genes, such as inducible nitric oxide synthase, through the intermediate induction of IRF-1 (45).

IL-12 is currently under evaluation as an immunotherapeutic and antitumor adjuvant, and it represents an important link between innate immunity and specific immune responses (46, 47). This study provides the first characterization of the IL-12 signal transduction pathway and the first identification of a physiologic activator of Stat4. These observations may provide a basis for understanding the development of cell-mediated immunity.

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