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CUTTING EDGE

Cutting Edge: IL-12 and IL-18 Differentially Regulate the Transcriptional Activity of the Human IFN-γ Promoter in Primary CD4$^+$ T Lymphocytes[1]

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We analyzed the molecular mechanisms by which IL-12 and IL-18 induce transcriptional activity of the IFN-γ promoter in primary human CD4$^+$ T cells. In transfection experiments, we found that IL-18 directly induces IFN-γ promoter activity, whereas significant activation with IL-12 required costimulation with αCD3/CD28. Furthermore, IL-12 caused in vivo protection of a STAT4 (−236) binding site, whereas stimulation with IL-18 or IL-12 plus αCD3/CD28 induced occupancy of a downstream AP-1 site. Mutation of this AP-1 site abrogated both IL-12- and IL-18-mediated promoter activation, whereas mutation of the STAT site inhibited IL-12-dependent activation. These data suggest that both AP-1 and STAT4 are required for IL-12-dependent IFN-γ promoter activity, whereas IL-18 causes direct activation via AP-1. This differential activation of the IFN-γ promoter gives further insights into molecular pathways governing Th1 T cell development and differentiation. ***The Journal of Immunology,* 1998, 160: 3642–3647.**

IL-12 is a major immunomodulatory cytokine that represents a functional bridge between the early innate resistance and subsequent Ag-specific adaptive immunity (1). It is produced mostly by phagocytic cells in response to bacteria, bacterial products, and intracellular parasites (2, 3). IL-12 induces the production of IFN-γ from T and NK cells by binding to its specific receptor. While the β2 chain of the IL-12 receptor is expressed only in Th1 T cells, the β1 chain is expressed in both Th1 and Th2 T cells. Thus, it is the expression of both the β1 and β2 chains that accounts for the responsiveness of T cells to IL-12 and mediates Th1 T cell differentiation (4–6). On binding to its receptor, IL-12 induces activation of specific members of the STAT family of transcription factors (STAT3 and STAT4), which then translocate to the nucleus and bind to genomic promoter regions, including that governing IFN-γ (7, 8). STAT4 is particularly important in this respect, as shown by the fact that STAT4-deficient T cells manifest impaired production of IFN-γ. In addition, the phenotype of the IL-12 p40 deficient mouse is similar to that of the STAT4-deficient mouse (8–10).

IL-18, designated also IFN-γ-inducing factor (IGIF),[3] is a recently cloned cytokine of 18.3 kDa the production of which is restricted to phagocytic cells (11). IL-18 strongly augments IFN-γ production by T cells, NK cell cytotoxicity, and T cell proliferation. Previous studies in T cell clones showed that recombinant IL-18 induces IFN-γ production more potently than does IL-12 (12). Interestingly, recent reports revealed that IL-12 and IL-18 exert a synergistic effect on IFN-γ production in T cells (13). It was suggested that one of the possible molecular mechanisms underlying this synergy consists of the induction of IGIF receptor by IL-12, thereby enhancing IL-18 responsiveness of T cells.

In this report, we examined the differential effects of IL-18 and IL-12 on IFN-γ production in primary T cells at the transcriptional level. We found that while IL-18 directly induces high IFN-γ promoter activity, IL-12 exhibits activating function only in the presence of costimulatory signals provided by αCD3/28 Abs. Furthermore, we identified differential molecular target sites at the IFN-γ promoter that were associated with IL-12 or IL-18 responsiveness of primary CD4$^+$ T cells.

Materials and Methods

Cell culture conditions

Freshly isolated CD4$^+$ T cells were cultured in RPMI 1640 supplemented with 10% FCS (PAA, Linz, Austria), 5% NCTC 135 medium (Life Technologies, Gaithersburg, MD), 20 mM HEPES buffer (Life Technologies),

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[3] Abbreviations used in this paper: IGIF, interferon-γ-inducing factor; LM-PCR, ligation-mediated PCR; DMS, dimethyl sulfate; EMSA, electrophoretic mobility shift assay.

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2 mM L-glutamine (Life Technologies), and 1000 U/ml penicillin/streptomycin (Biochrom, Berlin, Germany).

Isolation of CD4$^+$ T lymphocytes

Human PBMC were isolated from healthy volunteers using Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradients. Immunomagnetic beads specific for CD4$^+$ (obtained from Dynal, Oslo, Norway) were then used to isolate CD4$^+$ T cells (14). The beads were finally removed from the cells by treatment with Detachabead (Dynal). Only cell populations with a purity of >95% were used in the experiments described below. The cells were cultured in a humidified atmosphere with 5% CO_2 in a 37°C incubator.

Stimulation of CD4$^+$ T cells

Cells were stimulated with anti-human CD3 Ab (Cat. No. 30110D, PharMingen, San Diego, CA), anti-human CD28 Ab (Cat. No. 33740D, PharMingen), recombinant human IL-12 (p70, Cat. No. 19721 V, PharMingen), and IL-18 (Cat. No. 200–18, Peprotech, Rocky Hill, NJ). Anti-CD3 and CD28 Abs were directly added to the cells at a concentration of 1 μg/ml.

ELISA for IFN-γ

Primary CD4$^+$ T lymphocytes (10^6/well) were seeded out in triplicate in 48-well tissue culture plates and incubated at 37°C in the presence or absence of different stimuli as indicated in *Stimulation of CD4$^+$ T cells.* After 48 h, cell-free culture supernatants were removed and assayed for IFN-γ concentration by ELISA as described (14).

Dimethyl sulfate (DMS)-piperidine treatment of DNA for in vivo footprinting and ligation-mediated PCR (LM-PCR)

Cells were methylated with 0.1% DMS and lysed overnight in cell lysis buffer (14). After extraction of DNA with phenol/chloroform, the methylated DNA was precipitated, and the strand scission reaction was performed in 1 M piperidine. In vivo footprinting by LM-PCR was performed with 0.5 pmol of primer 1 for 1 μg of genomic DMS and piperidine-treated DNA (15). After primer extension with Sequenase 1.0 (United States Biochemical, Cleveland, OH), the linker ligation reaction was performed overnight at 15°C. Exponential PCR amplification was done with primer 2 and the linker primer for 15 to 22 cycles (94°C for 1 min, melting temperature + 1°C for 2 min, 76°C for 3 min). Finally, the ^{32}P-labeled third primer was added together with 2 U of *Taq* DNA polymerase and 2 μl of deoxynucleotide triphosphates (5 mM each), and a final PCR cycle was performed, followed by DNA analysis on a 5% denaturing urea/polyacrylamide gel. Primer sequences for LM-PCR: 1, 5′-gctgatcttcagatgatcag-3′; 2, 5′-aga acaatgtgctgcacctcctctg-3′; 3, 5′-atgtgctgcacctcctctggctgct-3′.

Reporter gene analysis

An IFN-γ promoter fragment (−572 to +7) was amplified by PCR from Jurkat T cell genomic DNA (upstream primer 5′-ccctttgtaaaggtttgagag gccc-3′, downstream primer 5′-atgtgctgcacctcctctggc-3′). The 397-bp IFN-γ promoter fragment was cloned into the pCRII vector (Invitrogen, Leek, the Netherlands) by TA cloning. The IFN-γ promoter DNA was then excised with *Eco*RI from the pCRII vector, treated with mung bean nuclease (Amersham, Amersham, Arlington Heights, IL), and cloned into the *Sma*I site of the promoterless pXP1 luciferase reporter gene vector. Ten micrograms of this pXP-IFN-γ vector were transfected into 1×10^7 CD4$^+$ T cells using the DEAE transfection method. After 24 h the cells were stimulated for 18 h as described in *Stimulation of CD4$^+$ T cells*, harvested, washed in PBS, and lysed in cell lysis buffer (Promega, Madison, WI). Luciferase activity was measured as light emission over a period of 10 s after addition of luciferase assay buffer (Promega) with a scintillation counter (Top Count, Packard, Meriden, CT). Data were normalized for transfection efficiency by galactosidase activity using the pSV-βgal reporter plasmid system (Cat. No. 6047-1, Clontech, Heidelberg, Germany).

Site-directed mutagenesis

Site-directed mutagenesis was performed with the Quickchange Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany, Cat. No. 200518) according to the manufacturer's instructions. Primer sequences were as follows (mutagenized nucleotides are underlined, and deletions are marked by an asterisk): AP-1 mut, 5′-ATG GGT CTG TAT CAT CGT CAA AGG A-3′; 3′-TAC CCA GAC ATA GTA GCA GTT TCC T-5′. STAT mut, 5′-AGT CCT TGA ATG GTG TGA AGT AAA AGT GCC *TCA AAC AAT CCC C-3′; 5′-TCA GGA ACT TAC CAC ACT TCA TTT TCA CGG *AGT TTG TTA GGG G-3′.

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were isolated as described (16). Oligonucleotides for EMSA were end-labeled with [γ-^{32}P]ATP (>5000 Ci/mmol, Amersham) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). For supershift assays, 2 μg of rabbit anti-human c-Jun/AP-1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) was used. The binding reaction was incubated at room temperature for 30 min. After electrophoresis, the gels were dried and exposed to Kodak MS films on intensifiying screens at −80°C. The sequences of the oligonucleotides for EMSA were as follows: AP-1, IFN-γ 5′-ATG GGT CTG TCT CAT CGT CAA AGG A-3′; 3′-TAC CCA GAC AGA GTA GCA GTT TCC T-3′. STAT, 5′-AGT CCT TGA ATG GTG TGA AGT AAA AGT GCC TTC AAA GAA T CC CC-3′; 5′-TCA GGA ACT TAC CAC ACT TCA TTT TCA CGG AAG T TT CTT AGG GG-3′.

EMSA gels were analyzed by densitometry using a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

Data from transfection experiments were analyzed by the Wilcoxon test using the program Statworks for MacIntosh.

Results

IL-18 but not IL-12 directly induces high IFN-γ promoter activity and IFN-γ protein production in primary human CD4$^+$ T cells

Previous studies demonstrated that whereas IL-18 (IGIF) exerts significant IFN-γ-inducing activity in human PBMC (11), IL-12 favors Th1 T cell differentiation by priming CD4$^+$ T cells for high IFN-γ production (1). To examine the specific effects of both cytokines on IFN-γ induction in human CD4$^+$ T cells, we performed ELISAs using supernatants from IL-12- and IL-18-stimulated primary CD4$^+$ T cells. rIL-18 (IGIF) markedly stimulated IFN-γ protein production by human CD4$^+$ T cells (Fig. 1*A*). In contrast, IFN-γ production of CD4+ T cells was not strongly induced by IL-12 treatment alone (Fig. 1*B*).

To determine the molecular mechanisms for the observed differences between IL-18 and IL-12 on IFN-γ induction in human T lymphocytes, we constructed a plasmid containing the wild-type IFN-γ promoter sequence (positions −572 to +7) upstream of a luciferase reporter gene. By transient transfection experiments with the resulting construct, denoted pXP-IFN-γ, we then determined IFN-γ promoter activity in primary T cells. In this experimental system, IL-18 alone was a potent inducer of IFN-γ promoter activity in primary CD4$^+$ T cells, while IL-12 alone did not have a significant effect on reporter gene activity (Fig. 2). However, in the presence of costimulatory signals provided by soluble Abs to CD3 and CD28, IL-12 strongly induced IFN-γ promoter activity in primary CD4$^+$ T cells.

In vivo footprinting of the IFN-γ promoter identifies different molecular targets elements for IL-12 and IL-18

In additional experiments, we determined potential molecular target sites for IL-12 and IL-18 at the IFN-γ promoter by in vivo DNA footprinting via LM-PCR. Accordingly, we isolated methylated DNA from unstimulated and stimulated CD4$^+$ T cells and subjected the DNA to the LM-PCR procedure (see *Materials and Methods*). An altered in vivo DMS reactivity in IL-18-stimulated CD4$^+$ T cells was found at an AP-1 binding site at −190 (Fig. 3) that was recently characterized in our laboratory as a critical element for IFN-γ promoter activity in primary CD4$^+$ T cells (14). In contrast, activation of CD4$^+$ T cells via IL-12 did not induce altered DMS reactivity over this AP-1 site but at a recently described STAT binding site (19) suggesting IL-12-dependent protein binding to this site in vivo. However, activation of T cells with IL-12 plus αCD3/28 led to an altered DMS reactivity over the downstream AP-1 binding site as observed with IL-18 alone.

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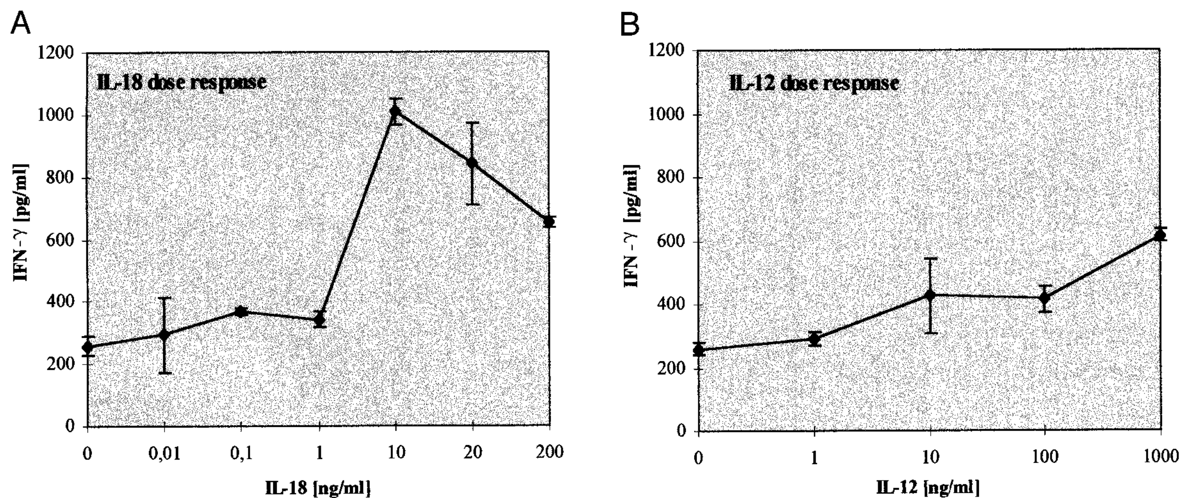

FIGURE 1. IFN-γ production of primary human CD4$^+$ T cells in response to IL-18 (*left*) and IL-12 (*right*). Freshly isolated human CD4$^+$ T cells were stimulated for 48 h in the presence of recombinant human IL-18 or IL-12 p70. Culture supernatants were taken after 48 h, and IFN-γ concentration was assessed by ELISA. Data represent mean ± SD. One representative experiment of three is shown.

IL-18 but not IL-12 directly induces AP-1 binding activity in CD4$^+$ T lymphocytes

In further studies, we assessed the inducibility of the AP-1-binding complex in nuclear extracts from primary human CD4$^+$ T cells in response to IL-12 and IL-18. As shown in Figure 4*A*, AP-1 binding activity was highly inducible by IL-18 (>20-fold) and αCD3/28 plus IL-12 treatment, but not by IL-12 application alone. Furthermore, IL-18 caused a dose-dependent increase of AP-1 binding activity and IFN-γ mRNA production by Jurkat T cells (data not shown). Interestingly, supershift experiments showed that c-jun Abs cause a supershift of the AP-1 band in IL-12 plus αCD3/28, but not in IL-12-stimulated CD4$^+$ T cells suggesting that induction of c-*jun* is important for IL-12 plus αCD3/28-dependent IFN-γ promoter activity (Fig. 4*B*). Finally, we found that stimulation of CD4$^+$ T cells with IL-12 or IL-12 plus αCD3/28 induced binding of STAT4 to the STAT site of the IFN-γ promoter (Fig. 4*C*). In contrast, this complex was not found after stimulation with IL-18 suggesting that IL-12 but not IL-18 induces STAT4 activation in primary CD4$^+$ T lymphocytes.

Site-directed mutagenesis reveals importance of the STAT- and AP-1 sites for inducible IFN-γ promoter activation

To determine the functional importance of the AP-1 and STAT sites for IL-12- and IL-18-dependent IFN-γ promoter activity, we performed transient transfection assays in CD4$^+$ T cells. Accordingly, we constructed mutagenized plasmids containing deletions or mutations of the AP-1 and STAT sites of the IFN-γ promoter that are known to prevent protein binding to these sites (14, 17). As shown in Figure 5, transfection of an AP-1 mutant IFN-γ promoter construct in primary CD4$^+$ T cells significantly ($p < 0.01$) reduced reporter gene activity in both IL-12- and IL-18-stimulated T cells compared with the wild-type construct. However, mutation of the STAT site caused only a slight inhibition of IL-18-induced promoter activity but a highly significant ($p < 0.01$) reduction in IL-12-dependent promoter activity.

Discussion

The ability to produce IFN-γ is a fundamental property of Th1 cells in various infectious and autoimmune diseases (1, 19). IL-18 and IL-12 strongly enhance IFN-γ production of CD4$^+$ T cells and are capable of acting in a synergistic fashion (11, 13). The data reported here show that that IL-12 and IL-18 act differentially to activate IFN-γ gene transcription in primary human CD4$^+$ T cells. Furthermore, they suggest that both AP-1 and STAT4 are required for IL-12-dependent IFN-γ promoter activation, whereas IL-18 causes direct promoter activation via AP-1. This differential activation of the IFN-γ promoter gives further insights into molecular pathways governing Th1 T cell development and differentiation.

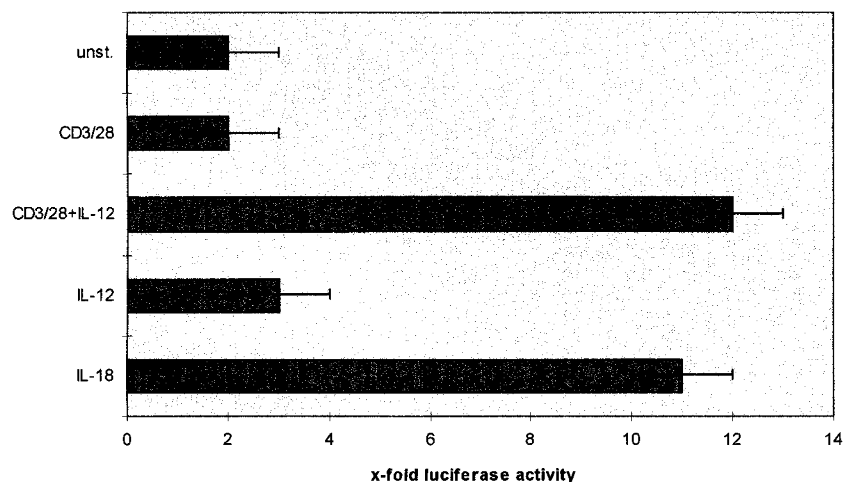

FIGURE 2. IFN-γ promoter activity in unstimulated (unst.) and stimulated primary human CD4$^+$ T cells. The pXP-IFN-γ reporter gene construct was transiently transfected into CD4$^+$ T cells. After 24 h, the cells were split and stimulated with IL-18 (10 ng/ml), IL-12 (10 ng/ml), αCD3/28 plus IL-12, or αCD3/28 alone. Results represent mean values ± SD from three independent experiments and are expressed as fold increase in luciferase activity compared with that seen with the pXP control vector after normalization for transfection efficiency. A suboptimal stimulation of T cells with soluble αCD3/CD28 Abs was used to analyze IL-12-dependent promoter activity, since stimulation with cross-linked αCD3/CD28 Abs alone strongly induced AP-1 binding activity (18) and IFN-γ promoter activation (data not shown).

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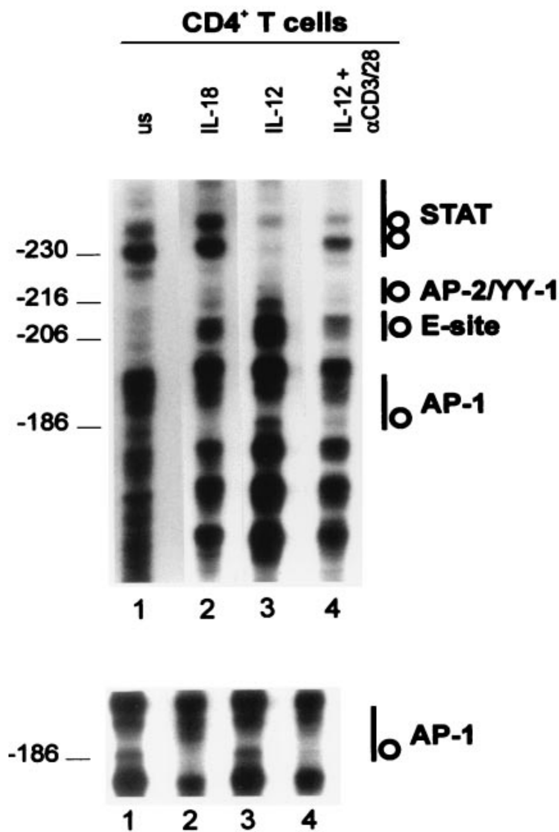

FIGURE 3. *Top*, Footprinting in vivo of the IFN-γ promoter from −170 to −260 on the coding strand in stimulated human CD4$^+$ T cells. Freshly isolated CD4$^+$ T cells were stimulated for 4 h in the presence or absence (*lane 1*) of 10 ng/ml IL-18 (*lane 2*), 10 ng/ml IL-12 (*lane 3*), or αCD3/CD28 plus IL-12 (*lane 4*). The DNA was then subjected to the LM-PCR procedure. The location of the AP-1 and STAT binding sites are indicated. ○, protected G residues. *Bottom,* Shorter exposure of the same gel as above in the AP-1 area. The E-site is an E-Box element of the IFN-γ promoter that has been identified by in vivo footpriting (14).

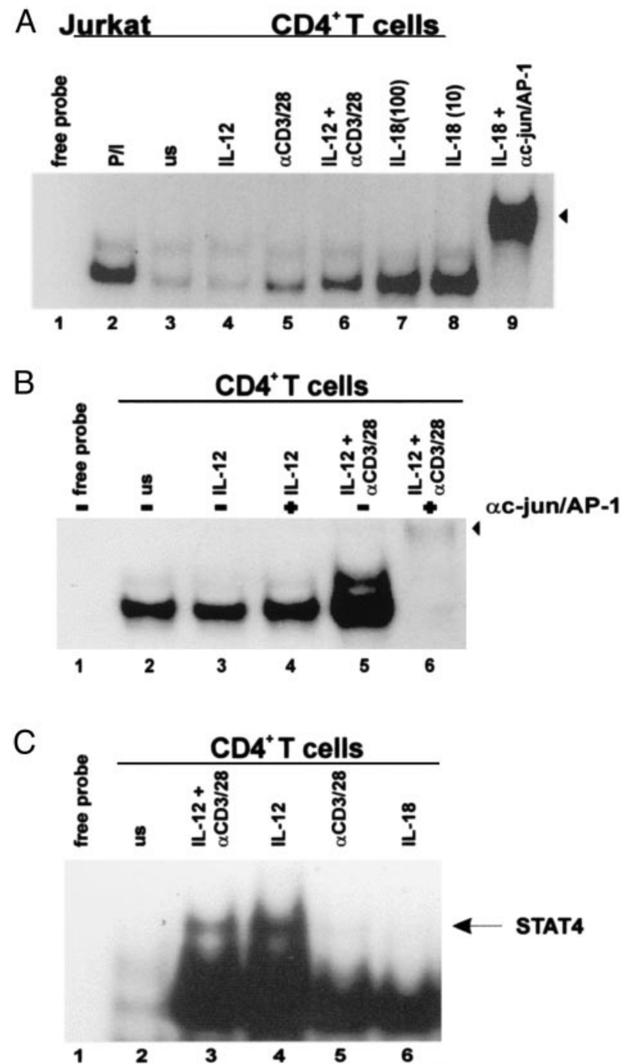

FIGURE 4. *A*, IL-18 but not IL-12 strongly induces AP-1 binding to its cognate sequence at −190 of the IFN-γ promoter. EMSA was performed with nuclear extracts from human CD4$^+$ T cells and the AP-1-binding oligonucleotide. The cells were activated for 2 h with indicated stimuli before extraction of nuclear proteins. *B*, Supershift assay at the AP-1 site using nuclear extracts from CD4$^+$ T cells. Abs to c-*jun* caused a supershift (arrow) of the AP-1 band using extracts from IL-12 plus αCD3/CD28, but not IL-12-stimulated cells, indicating the presence of c-*jun* in the former cells. *C*, IL-12 but not IL-18 induces binding of STAT4 (arrow) to the STAT site of the IFN-γ promoter. EMSA was performed with nuclear extracts from human CD4$^+$ T cells and the STAT-binding oligonucleotide. The STAT4 protein/DNA complex could be specifically competed with a STAT4 consensus oligonucleotide, and a supershift was observed after addition of Abs to STAT4 (data not shown).

In initial ELISA and transient transfection experiments, we showed that both IL-12 and IL-18 are capable of inducing IFN-γ promoter activity. However, whereas IL-18 stimulation alone strongly enhanced IFN-γ promoter activity, promoter activation via IL-12 required costimulatory signals provided by soluble αCD3/28 Abs. These data in primary T cells are consistent with data from the work of Jacobson et al. (7) showing that IL-12 alone does not activate IFN-γ promoter activity in murine EL-4 T cell lines. In further studies, we identified various *cis*-responsive elements for IL-12 and IL-18 at the IFN-γ promoter. We found an intense footprint in IL-18-stimulated T cells over an AP-1 sequence element at −190 that plays a critical role in inducible activity of the IFN-γ promoter in primary T cells (14). Mutagenesis of this AP-1 element abrogated IFN-γ promoter activation by IL-18 and IL-12 plus αCD3/CD28, indicating that this site is absolutely required for both IL-12- and IL-18-mediated promoter activation. Thus, although there are various other important *cis*-regulatory elements at the IFN-γ promoter that are involved in the regulation of its activity (20–25), these data suggest that AP-1 plays a crucial role in mediating both IL-12- and IL-18-dependent IFN-γ promoter activity in primary human CD4$^+$ T-cells.

Further studies of the IFN-γ promoter regulation in primary T cells showed in vivo occupation of a recently characterized STAT binding site (24) at position −236 of the transcriptional start site. Whereas the identification of this STAT binding site was based on in vitro binding assays, our genomic footprinting and transfection data indicate for the first time functional importance of this STAT binding site for mediating IL-12 effects in primary CD4$^+$ T cells in vivo. Previous studies have shown that IL-12 induces STAT3/4 tyrosine phosphorylation and finally nuclear STAT-binding activity in T cells (7). Consistent with this model, our results indicate that IL-12 stimulation of CD4$^+$ T cells induces STAT binding to its target DNA sequence at −236 in the IFN-γ promoter in vivo. Interestingly, however, such binding seems to be required but not sufficient to induce high IFN-γ promoter activity in primary CD4$^+$ T cells. However, after IL-12 plus αCD3/28 stimulation a footprint over the downstream AP-1 site (−190) of the IFN-γ promoter was

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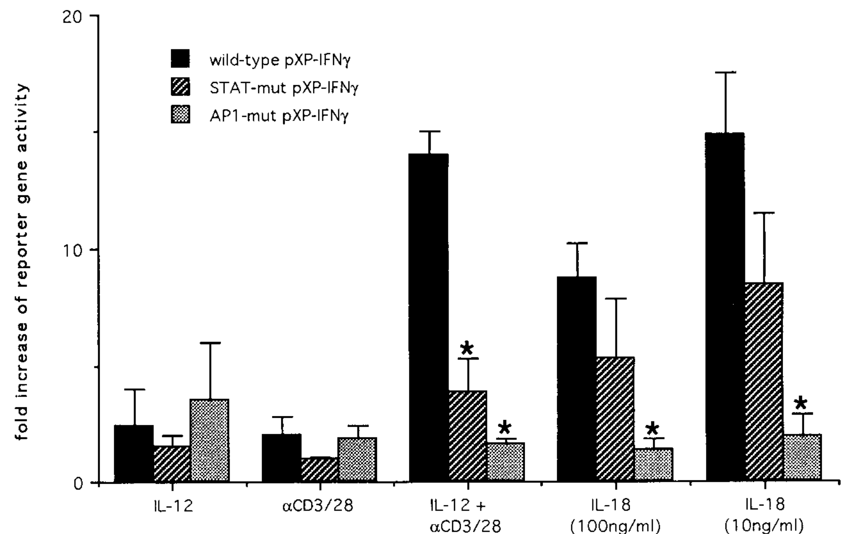

FIGURE 5. Transfection analysis of IFN-γ promoter constructs carrying mutations of the AP-1- and STAT-binding elements. Wild-type and mutagenized luciferase reporter gene constructs were transfected into primary human CD4$^+$ T cells. Data represent mean values ± SE of four independent experiments after normalization for transfection efficiency. Luciferase activity is reported as fold induction compared with unstimulated transfected T cells.

found the appearance of which strongly correlated with high IFN-γ promoter activity and IFN-γ protein production. Interestingly, mutation of either the STAT or the AP-1 site abrogated IL-12 plus αCD3/28 effects on IFN-γ promoter activity, suggesting that both sites are absolutely required for this stimulation condition.

The data reported here provide evidence that IFN-γ gene transcription in primary CD4$^+$ T cells is differentially modulated by IL-12 and IL-18 (Fig. 6). Whereas IL-18 activates directly the IFN-γ promoter by recruitment of AP-1 binding, IL-12 stimulation induces STAT4 binding but requires costimulatory signals provided by αCD3/28 Abs to activate IFN-γ promoter activity via STAT/AP-1. Thus, regulation of AP-1 and STAT binding activities emerges as a key event in controlling the transcriptional activity of the human IFN-γ promoter in CD4$^+$ T lymphocytes.

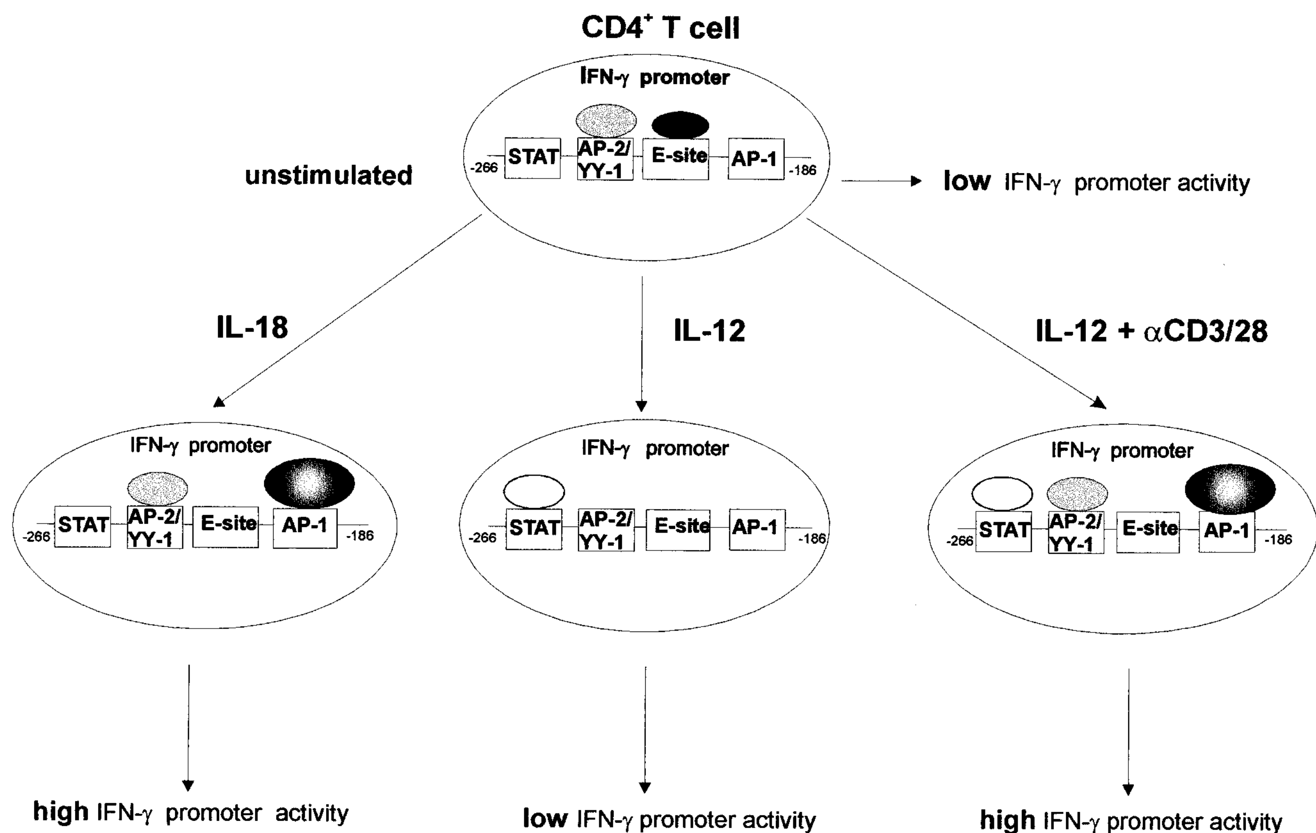

FIGURE 6. Hypothetical model of the molecular activation pathways of the IFN-γ promoter in primary human CD4$^+$ T cells in response to IL-18, IL-12, and IL-12 plus αCD3/CD28.

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