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*J Immunol* 1999; 162:7256-7262; http://www.jimmunol.org/content/162/12/7256

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Jérôme Galon,* Chitra Sudarshan,* Satoshi Ito, † David Finbloom, † and John J. O’Shea*

IL-12 is a critical immunoregulatory cytokine that promotes cell-mediated immune responses and the differentiation of naive CD4+ T cells to Th1 cells; however, relatively few IL-12 target genes have been identified. To better clarify the molecular basis of IL-12 action, we set out to characterize genes up-regulated by IL-12, first by contrasting IL-12- and IFN-α responsiveness of IRF-1 to the same extent as IFN-γ. This article must therefore be hereby marked for guest on December 23, 2017 http://www.jimmunol.org/ Downloaded from

We identified several genes up-regulated by IL-12, namely, MIP-1α, MIP-1β, IL-1RA, and IFN regulatory factor-1 (IRF-1). IRF-1 is a transcription factor regulated by IFNs that is also essential for Th1 responses. We demonstrated that IL-12 directly up-regulates IRF-1 to the same extent as IFN-α in normal human T cells and in NK cells. We showed that IL-12 had a direct effect on IRF-1, an effect not mediated indirectly by the induction of IFN-γ production. Furthermore, IL-2 and IL-12 synergistically induced IRF-1, whereas IFN-α and IL-12 did not. The participation of STAT4 in the regulation of IFN-γ expression was demonstrated in two ways. First, STAT4 was required for the IL-12-dependent transactivation of an IFN-1 reporter construct, and second, STAT4 binding to the IRF-1 promoter was shown using EMSA. In contrast to IL-12, no up-regulation of IRF-1 was found in IL-4-stimulated cells, and IL-4 did not block IL-12-dependent up-regulation of IRF-1. Therefore, IRF-1 may be an important contributor to IL-12 signaling, and we speculate that the defective IL-12 responses seen in IRF-1−/− mice might be attributable, in part, to the absence of this transcription factor. The Journal of Immunology, 1999, 162: 7256–7262.

Interleukin-12 is a critical immunoregulatory cytokine for cell-mediated immune responses and drives the differentiation of naive CD4+ T cells to Th1 cells (1). Its importance is exemplified by IL-12 knockout mice, which are deficient in their ability to generate a normal Th1 response and to produce IFN-γ (2). IL-12 induces tyrosine phosphorylation of JAK2 and TYK2 (3), which, in turn, phosphorylate other substrates, including the transcription factors STAT4, STAT1 (4), and STAT3 (5, 6); STAT4−/− mice, like IL-12−/− mice, have defective Th1 differentiation and impaired cell-mediated immunity. Despite the importance of IL-12, there is relatively little information pertaining to IL-12-inducible genes and genes known to be regulated by STAT4 (7). In contrast to IL-12, however, many IFN-inducible genes have been characterized (8, 9). While it is clear that STAT1 is essential for many IFN-α responses, IFN-α, like IL-12, activates STAT4. In addition, both IFN-α and IL-12 activate TYK2 (10, 11). This suggests that IFN-α and IL-12 may share a subset of target genes. The similarity in IFN-α and IL-12 signaling prompted us to define commonalities and differences in genes regulated by IFN-α and IL-12.

One well-characterized IFN-inducible gene is IFN regulatory factor-1 (IRF-1) (12). Interestingly, IRF-1 knockout mice have a broad range of defects. For instance, IRF-1-deficient mice have impaired ability to undergo DNA damage-induced cell cycle arrest (13, 14). These mice also have defects in inflammatory and innate immune responses (15) and disruption of NK and T cell development (16, 17). Importantly, IRF-1 knockout mice demonstrate the involvement of IRF-1 in multiple stages of the Th1 differentiation (18, 19). IRF-1−/− mice have defective IL-12 and IFN-γ production and exaggerated IL-4 secretion. The lack of NK cells in IRF-1−/− mice contributes, in part, to the impairment in IFN-γ production. In addition, there is defective IL-12 production, which has been attributed to the presence of a potential IRF-1-responsive element in the IL-12p40 gene (20). Most importantly, however, in addition to impaired IL-12 production, CD4+ T cells from IRF-1−/− mice have been shown to have impaired responsiveness to IL-12, as measured by IFN-γ production after IL-12 stimulation (18, 19).

Thus, although IRF-1 was initially described as an IFN-inducible transcription factor, the phenotype of IRF-1−/− deficient mice is dramatically different from that of IFN-γ or IFN-αβ receptor-deficient mice, which argues that IRF-1 serves functions other than simply transmitting IFN-mediated signals (reviewed in Ref. 21). This was the second issue that led us to investigate the potential regulation of IRF-1 by IL-12.

In the present study, we report that IFN-α and IL-12 up-regulate a number of genes in common, including macrophage-inflammatory protein (MIP)-1α, MIP-1β, and IL-1RA. Importantly, in addition to IFN-α, IL-12 and IL-2, but not IL-4, directly up-regulate IRF-1 expression. Thus, although IRF-1 was initially characterized as an IFN-inducible gene, IRF-1 can be viewed as a transcription factor that is also regulated by cytokines that participate in Th1 responses.

Materials and Methods

Materials

rhIL-12 and a human IFN-γ immunoassay kit were purchased from R&D Systems (Minneapolis, MN). Human IL-2 was provided by Dr. C. Reynolds (National Cancer Institute, Frederick, MD). Polyclonal rabbit anti-STAT4 Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-STAT1 Ab was provided by Dr. A. Larner.
(Cleveland Clinic Foundation, Cleveland, OH). An RNA extraction kit (RNAgent Total RNA Isolation System) was purchased from Promega (Madison, WI), and RiboQuant Multiprobe RNase Protection System was purchased from PharMingen (San Diego, CA). Cell culture supernatants of cell were collected at 4 h, and IFN-γ levels were analyzed using a commercial ELISA kit (R&D Systems) according to the manufacturer’s instructions. The plasmid p3xGAS-luc, containing the STAT binding site of the IRF-1 gene (22), was provided by Dr. R. Pine (The Public Health Research Institute, New York, NY), and pcDNA3-STAT4 was provided by Dr. J. Ihle (St. Jude Children’s Hospital, Memphis, TN). Plasmids containing the cDNA for IL-12Rβ1 and IL-12Rβ2 in expression vector pEF-BOS were provided by Dr. U. Gubler (Hoffmann-La Roche, Nutley, NJ). LipofectAMINE was purchased from Life Technologies (Gaithersburg, MD).

**Cells**

NK.3.3 cells were provided by Dr. J. Kornbluth (Arkansas Cancer Research Center, Little Rock, AR). PBMC from healthy donors were isolated by Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient centrifugation, activated with PHA (2 μg/ml) for 72 h, and cultured for an additional day in the presence of IL-2 (40 IU/ml), as described previously (3, 5).

**RNase protection assay and Northern analysis**

Total RNA was extracted from cytokine-activated cells using RNAgent (Promega). The RNase protection assay was conducted as follows: 32P-labeled RNA probe was synthesized using SP6 RNA polymerase for GAPDH, ISG15, ISG54, and DNA templates or with T7 RNA polymerase for granulocyte-macrophage colony-stimulating factor, IFN-γ, IFN-α, and the multiprobe template set (PharMingen). DNA was digested with DNase I (Boehringer Mannheim, Indianapolis, IN), and RNA probes were extracted with phenol and chloroform and precipitated with ethanol. Labeled RNA probes were hybridized overnight at 56°C to target RNA (5 μg) and were digested with T1 RNase (Life Technologies). The protected mRNA fragment was extracted with phenol and chloroform, precipitated with ethanol, resolved on a 6% denaturing polyacrylamide gel, and subjected to autoradiography. Gene transcripts were identified by the length of the protected fragments. Equal loading of RNA was estimated from the amounts of protected fragments of two housekeeping genes, namely, L32 and GAPDH.

For Northern blots, RNA samples (20 μg) were electrophoresed on 1% agarose gels containing formaldehyde (Ambion, Austin, TX), transferred to Hybond N+ membranes (Amerham, Arlington Heights, IL), and hybridized overnight at 56°C to radiolabeled probes. Membrane was washed at 62°C and exposed to film.

**Luciferase assay**

The NIH 3T3 cells stably transfected with the α, β, and γ chains of the IL-2R and JAK3 (NIH 3T3αβγ-JAK3) (23) and NIH 3T3 cells (3 × 10^5 cells per point) were transfected with 0.3 μg of constructs, and 1 μg of the IRF-1-promoter luciferase construct, pcDNA3-STAT4, pEF-BOS-IL-12Rβ1, and pEF-BOS-IL-12Rβ2 containing the luciferase reporter plasmid, the IRF-1-promoter luciferase construct, and pcDNA3-STAT4 were transfected using LipofectAMINE (Life Technologies). The luciferase reporter construct alone (pGL2-B) and that containing the IRF-1 promoter (1.3-kb 5' flanking region of the human IRF-1 gene) and the mutant STAT binding element (SBE) site at position −123 of the IRF-1 promoter (mutant sequence 5'-GATTCTCCTCCAT-3') were provided by Dr. Thomas A. Hamilton (Cleveland Clinic Foundation, Cleveland, OH) (24). Transfected cells were stimulated with cytokines, lyzed, and assayed for luciferase activity (Promega).

**EMSA**

Cell extracts were prepared from cytokine-stimulated NK3.3 cells, and EMSAs were performed as described (25) using a 1 μg of double-stranded oligonucleotide corresponding to the human IRF-1 promoter (5′-ACGTTCAGCTTATCCAGCCCCGCAATGGCGCTGAA-3′) and a control oligonucleotide (5′-CTGCAAGTAAACCGCCATTCCGAAGACGGATGA-3'). In supershift assays, complexes were incubated with 1 μl of STAT1 Ab, STAT4 Ab, or preimmune rabbit serum at 4°C for 30 min. The complexes were electrophoresed through a 5% non-denaturing acrylamide gel and subjected to autoradiography.

**Results**

**Identification of IFN-α- and IL-12-induced genes**

Since there appear to be some similarities in IFN-α and IL-12 signaling, we surveyed IFN-inducible genes to determine whether any of these were IL-12 inducible. IFN-α-inducible genes are relatively well characterized and include GBP, ISG15, and ISG54, as well as MIP-1α, MIP-1β, and IL-1RA. As is evident in this experiment shown in Fig. 1A, as is evident in this experiment, IL-12 up-regulated a subset of these genes. Specifically, as with IFN-α, we demonstrate that IL-12 up-regulated IL-1RA, MIP-1α, and MIP-1β. In contrast, IL-12 did not up-regulate GBP, ISG15, and ISG54. This is not unexpected, as the latter genes contain IFN-stimulation response element in their promoters, which bind STAT1/STAT2/p48 complexes, and IL-12 has not been shown to activate STAT1. IL-12 has previously been shown to up-regulate granzyme B (29). Interestingly, we found that IFN-α also up-regulated this gene, which demonstrates that another gene implicated in the apoptotic pathway, RVP1, was up-regulated by both IFN-α and IL-12. As a control, the expression of DAD1 is shown; neither IFN-α nor IL-12 affected expression of this gene or GAPDH.

**IL-12 up-regulates IRF-1 expression**

Because of the number of genes we found that were commonly regulated by IFN-α and IL-12, we next analyzed another gene typically induced by IFNs, namely IRF-1, a well-characterized transcription factor that is important in regulating a variety of genes. We therefore sought to determine whether IFR-1 was
induced expression of IRF-1. NK3.3 cells are a useful model system to study IL-12 signaling; we first tested inducibility in these cells. As shown in Fig. 1B, IFN-α induced expression of IRF-1 as expected. Interestingly, IL-12 also induced IRF-1 mRNA expression to the same extent as IFN-α (Fig. 1B, lane 3 vs lane 2); increased IRF-1 mRNA accumulation in IL-12-stimulated NK3.3 cells was reproducibly detected in 10 different experiments. Because IL-12 and IL-2 have overlapping effects on some genes (e.g., IFN-γ; data not shown), we next sought to determine whether IL-2 had similar or distinct effects relative to IL-12 and IFN-α. We found that IL-2 stimulation also induced IRF-1 mRNA (Fig. 1B, lane 4).

To confirm that IL-12 and IL-2 up-regulated IRF-1 in normal human T cells, we stimulated activated T cells with IFN-α, IL-12, and IL-2. As shown in Fig. 1C, stimulation with IFN-α, IL-12, and IL-2 induced IRF-1 mRNA up-regulation (lanes 2–4); increased IRF-1 mRNA accumulation in human T cells was reproducibly detected in five different experiments, using cells from five different donors. The basal level of IRF-1 was higher in T cells than in NK3.3 cells, presumably because it was necessary to preactivate T cells (see Materials and Methods) to induce IL-12R and IL-2R α-chain expression. Therefore, we used NK3.3 cells in subsequent experiments.

Since IRF-1 is regulated by IFNs, and since IL-12 and IL-2 are potent inducers of IFN-γ, we next considered the possibility that the IL-12 and IL-2 induction of IRF-1 was indirectly mediated by the production of IFN-γ. We used several approaches to address this issue. First, we directly examined the effect of IFN-γ on IRF-1, and under conditions tested, stimulation of the NK3.3 cells with IFN-γ did not induce IRF-1. The lack of effect of IFN-γ was due to an absence of the IFN-γ Rβ chain in this cell line, as determined by RNase protection assay. In addition, we attempted to inhibit the IRF-1 induction with Abs against IFN-γ. Anti-IFN-γ antibodies have no effect on IL-12-up-regulation, whereas anti-IL-12 Abs completely inhibited its induction of IRF-1. Finally, we also measured the amount of IFN-γ in the culture supernatants after 4 h of stimulation and found that no IFN-γ was produced at this time point (data not shown).

To confirm that the enhancement of expression of IRF-1 by IL-12 was a direct effect, we next added cycloheximide (CHX), a potent protein synthesis inhibitor, to our cultures. As shown in Fig. 2B, CHX did not inhibit the IL-12 induction of IRF-1 mRNA; in fact, CHX augmented the induction (lane 3 vs lane 2). This was not due to CHX alone, since CHX, in the absence of stimulation, did not alter IRF-1 mRNA levels as compared with the control (data not shown). This up-regulation was reproducibly detected in six different experiments and suggests the existence of a labile inhibitor.

We next examined the effect of stimulation with a combination of IL-12 with IL-2 or IFN-α. As shown in Fig. 3A, IL-12, IL-2, and IFN-α induced IRF-1 mRNA (lanes 2–4). The combination of IFN-α and IL-12 induced IRF-1 mRNA to the same extent as IL-12 alone (Fig. 3A, lane 6), whereas IL-12 and IL-2 had a synergistic effect (Fig. 3A, lane 5). As a control, the induction of ISG15 was also assessed; IL-2 and IL-12 either separately or in combination had no effect on this gene (Fig. 3A, lanes 2, 3, and 5). To confirm that the effect of the combination of IL-2 and IL-12 was direct and not mediated by the induction of another cytokine, the experiment was repeated using pretreatment with CHX. As shown in Fig. 3B, IL-12, IL-2, and IFN-α induced IRF-1 mRNA (lanes 2–4). When tested together, IL-12 and IL-2 again had a synergistic effect (lane 5) in the presence of CHX, whereas IL-12 and IFN-α did not (lane 6). These results determined by RNase protection assay were also confirmed by Northern analysis (Fig. 3C). Densitometry showed an 18-fold induction of IRF-1 with IL-12, a 15-fold induction with IL-2, and a 43-fold induction with the combination of both cytokines.

We next compared the kinetics of IRF-1 induction after IL-12 or IFN-α treatment, quantitating the fold induction of IRF-1 by densitometry. As shown in Fig. 4, CHX alone had no effect as compared with the untreated cells (Ctrl lanes). Both with or without CHX, IL-12 had little effect on IRF-1 expression after 30 min or 1 h, but the effect was more pronounced after 4 h (14- and 33-fold
induction, respectively). In contrast, IRF-1 induction by IFN-α is maximal after 1-h stimulation (23-fold induction) and declined by 4 h. Since both IL-12 and IFN-α exert their effect by similar mechanisms, there is no obvious explanation for this difference. One possible explanation would be that IL-12 activates IRF-1 through STAT4, whereas the major STAT induced by IFN-α is STAT1. Perhaps differential levels of expression of these STATs can explain the different kinetics of IRF-1 induction.

**FIGURE 4.** IL-12 and IFN-α induce IRF-1 with different kinetics. Shown is an RNase protection assay analysis of the expression of IRF-1 and GAPDH following IL-12 (10 ng/ml) and IFN-α (1000 IU/ml) stimulation. Stimulated NK3.3 cells were collected at times indicated. RNA was isolated, and the RNase protection assay was conducted. Fold induction as compared with untreated and unstimulated cells were calculated and normalized to GAPDH levels.

**Requirement of STAT4 in IL-12-mediated IRF-1 induction**

STAT4 serves an essential role in transmitting some but not all IL-12-dependent signals (30, 31). To determine whether STAT4 was involved in IL-12-induced expression of IRF-1, we assayed transactivation of a luciferase reporter gene driven by the STAT binding site from the IRF-1 gene (22). Since T cells and NK cells already express STAT4, confounding any conclusions, we performed these experiments in fibroblasts (NIH 3T3) specifically because they lack STAT4. NIH 3T3 cells were transiently transfected with this reporter gene construct along with cDNAs encoding STAT4 and IL-12R subunits. As seen in Fig. 5A, IL-12 induced transactivation of the reporter construct in cells transfected with receptor subunits and STAT4, but not in cells transfected with receptor subunits alone, suggesting that STAT4 is required for IL-12-induced activation of the IRF-1 gene. IL-2 also induced transactivation of the luciferase reporter gene in NIH 3T3αβγγJAK3 cells, but this did not require STAT4. This was expected, as IL-2 has been shown to activate STAT1, STAT3, STAT5α, and STAT5b. To confirm that IRF-1 transactivation was mediated by STAT4, we transfected NIH 3T3 cells with a plasmid construct in which a reporter gene was linked to a fragment of the IRF-1 promoter containing the transcription promoter-enhancer region (24). As shown in Fig. 5C, this promoter fragment was able to respond to IL-12, and this response was dependent on an intact SBE at position −123 to −113, as indicated by the loss of IL-12 sensitivity when the SBE was specifically altered by mutagenesis. Importantly, STAT4 was required for IL-12 induction of the IRF-1 promoter.

**FIGURE 5.** Involvement of STAT4 in IL-12 regulation of IRF-1. NIH 3T3 αβγ cells (A) and NIH 3T3 cells or NIH 3T3 cells stably transfected with IL-2R subunits (αβγ) and JAK3 (NIH3T3 αβγγJAK3) (B) were transiently transfected with 3xGAS-luc, IL-12Rβ1, IL-12Rβ2, and STAT4 and stimulated for 8 h with IL-12 (10 ng/ml), IL-2 (1000 IU/ml), or both as indicated. C, Wild-type IRF-1 promoter luciferase construct and a mutant construct in which the SBE site has been mutated. These luciferase reporter constructs and IL-12Rβ1, IL-12Rβ2, and STAT4 were transiently transfected into NIH 3T3 cells and stimulated with IL-12 (10 ng/ml) as indicated. D, NK3.3 cells were treated with medium alone (0), IL-12 (10 ng/ml), or IFN-α (100 U/ml) for 15 min. Whole-cell lysates were prepared and EMSA was conducted using 32P-labeled IRF-1 probe (lanes 1–3) or a control probe (lanes 4–6) (39). E, Supershift analysis was conducted with anti-STAT1, anti-STAT4, and preimmune serum, using the IRF-1 oligonucleotide probe.
We next sought to determine whether we could demonstrate binding of STAT4 to the human IRF-1 promoter using EMSA. As shown in Fig. 5D, stimulation of NK3.3 cells with IL-12 resulted in formation of DNA binding complexes with the human IRF-1 promoter. These complexes were specific because they were present in the stimulated cell only (Fig. 5D, lane 4 vs lane 3), were formed with the IRF-1 probe but not with the control probe (Ctl) (Fig. 5D, lanes 1 and 2), and were abolished with an excess of unlabeled IRF-1 probe (data not shown). Supershift experiments (Fig. 5E) with anti-STAT4 Abs showed that the EMSA signal detected after IL-12 stimulation contained STAT4 complexes (lane 3). A control Ab (Ctl) had no effect on the pattern of the complexes (Fig. 5E, lane 4), and none of the Abs had an effect on the unstimulated cells (data not shown).

IL-4 does not up-regulate IRF-1 expression and fails to inhibit IL-12-dependent up-regulation of IRF-1

The phenotype of IRF-1<sup>−/−</sup> mice indicates that IRF-1 is important for Th1 differentiation. In contrast to IL-12, IL-4 promotes Th2 responses; therefore, we next sought to determine whether IL-4 also regulated IRF-1 expression. In addition, IL-4 has been shown to inhibit IL-12-dependent induction of IFN-γ (32) and to inhibit IFN-γ-dependent up-regulation of IRF-1 (24). We therefore also analyzed whether IL-4 blocked IL-12-dependent IRF-1 induction. As shown in Fig. 6, whereas IL-12 up-regulated IRF-1 (lane 2), no up-regulation of IRF-1 was found in IL-4-stimulated cells (lane 3). Similar results were found using T cells (data not shown). IL-4 was active, since it induced the expression of IL-4R (data not shown). Similar results were found using T cells (data not shown). IL-4 was active, since it induced the expression of IL-4R (data not shown). Interestingly, IL-4 treatment did not block IL-12-dependent up-regulation of IRF-1 (Fig. 6, lane 4). These data are important because they not only indicate that a Th2 cytokine does not induce IRF-1 but also because they confirm that IL-12 directly up-regulates IRF-1. That is, although IL-4 inhibited IL-12-mediated IFN-γ production, IL-12-dependent up-regulation of IRF-1 was unaffected. This further demonstrates that the IL-12-mediated induction of IRF-1 is independent of IFN-γ.

Discussion

IL-12 is a critical immunoregulatory cytokine implicated in multiple steps of Th1 differentiation. However, the similarity in some aspects of IFN-α and IL-12 signaling suggested that these cytokines may regulate some genes in common. Moreover, the importance of IFN-α as an immunoregulatory cytokine has been suggested by several findings. First, IFN-α has been shown to increase the frequency of IFN-γ-secreting CD4 T cells and therefore favors the induction of Th1 cells (33). Second, IL-12R β2 subunit, which is expressed only on Th1 but not Th2 clones, is induced by IL-12 and IFN-α during differentiation of human naive cells along the Th1 but not Th2 pathway (34). For these reasons we thought it important to begin cataloging genes induced uniquely and commonly by IL-12 and IFN-α.

We show here that IL-12 up-regulated some genes (MIP-1α, MIP-1β, and IL-1RA) that had previously been shown to be up-regulated by IFN-α. Interestingly, it has been reported that Th1 but not Th2 cells produce MIP-1α, MIP-1β, and IL-1RA (36), and that these chemokines are efficient chemotactants specifically for Th1 cells. (37). Conversely, although granzyme B is recognized as an IL-12-inducible gene, IFN-α also up-regulated this gene. Additionally, although RVP1 was not previously recognized as being regulated by either IL-12 or IFN-α, its expression was regulated by both of these cytokines. In contrast, GBP, ISG15, and ISG54, each of which contains IFN-stimulation response element in its promoter region, were specific for IFN-α.

We also report that IL-12, as well as IL-2, induced IRF-1 gene expression. While IL-12 knockout mice have a profound defect in Th1 development and cell-mediated responses, the mechanisms underlying this defect are only partly understood. IL-12 is critical for cell-mediated immunity, and IFN-1 likely contributes to the regulation of the IL-12p40 gene. In addition, a major source of IFN-γ in the initial phase of the Th1 response is thought to be Nk cells (1), and IFN-1<sup>−/−</sup> mice have impaired Nk cell development. This may be because IFN-1 contributes to IL-15 gene regulation and IL-15 is important in Nk cell differentiation (16). Thus, the poor IFN-γ production in IL-1<sup>−/−</sup> is related in part to the absence of IL-12 and to the lack of Nk cells.

But does this completely explain the Th1 defect in IRF-1<sup>−/−</sup> mice? The answer appears to be no, because IRF-1<sup>−/−</sup> mice have also been shown to have impaired responses to IL-12 (18, 19). Stimulation of CD4<sup>+</sup> T cells from IRF-1<sup>−/−</sup> mice with IL-12 induces substantially less IFN-γ than wild type, even though expression of IL-12Rβ2 and IL-12Rβ1 is reportedly normal. Thus, it was suggested that impaired IL-12 production in IRF-1<sup>−/−</sup> mice does not account for the Th2-dominant response observed. Based on the present results, it can be proposed that the IL-12-up-regulation of IRF-1 is important for the function of this cytokine; the unresponsiveness of IRF-1<sup>−/−</sup> mice to IL-12 may be related in part to the lack of up-regulation of this transcription factor. It is important to emphasize, however, that our findings in an NK cell line and peripheral blood T cells may not directly relate to the process of Th1 development. In addition, while our results that IL-12 induces IRF-1 expression may shed light on the defective IL-12 responses in IRF-1<sup>−/−</sup> mice, IL-12 target genes are still poorly characterized, and it is not immediately clear how IRF-1 contributes to IL-12 signaling. Nonetheless, our results clearly indicate that IL-12 and IFN-α activate a number of genes in common, including the transcription factor IRF-1. CHX did not inhibit IL-12 (nor IL-2)-induced IRF-1 expression, which emphasize the direct
role of these cytokines in the up-regulation of IRF-1. Indeed, CHX enhanced the IRF-1 induction mediated by IL-12. What this labile inhibitor of IRF-1 expression is remains obscure. Whether members of the PIAS family or the CIS/SOCS/JAB/SFI family contribute to this inhibition is unknown but is presently under investigation. Another possible interpretation would be that IL-12 mediates its effect on the IRF-1 gene by message by inhibiting a degradative enzyme. Since there is no superinduction of IRF-1 by IL-2, it could postulated that IL-2 is acting on the transcriptional level, while IL-12 acts on the posttranscriptional level.

It has been shown that STAT4 is essential for many IL-12 effects and is required for the Th1 response (30, 31). We show here that STAT4 appears to be important for the IL-12-dependent regulation of IRF-1. We have demonstrated that IRF-1 transactivation by IL-12 was mediated by STAT4 binding and that the SBE present on the IRF-1 promoter-enhancer region was necessary (Fig. 5C) and sufficient (Fig. 5A) for the IRF-1 induction. We used fibroblasts in our studies because they lack STAT4 and thus provide a useful vehicle for testing STAT4 dependence of IL-12 transactivation of the IRF-1 promoter construct. Clearly, though, these results need to be verified in a more physiologic system. That STAT4 has the capacity to bind to the IRF-1 promoter has been recognized since the cloning of STAT4. In the initial studies, STAT4 was shown to bind the IFN-γ activation site (GAS) element of the murine IRF-1 promoter (38). Although the significance of this result was not pursued at that time, our data confirm that STAT4 binds the human promoter. Whether IRF-1 and STAT4 function in concert to effect gene regulation or in parallel by activating different sets of target genes has yet to be determined. Identification of target genes of IRF-1 and STAT4 after IL-12 stimulation will clarify this issue and help us to understand the gene-regulatory events mediating Th1/Th2 differentiation. Comparing and contrasting IL-12-dependent gene expression in IRF-1−/− and STAT4−/− mice will be of considerable interest in this regard. It should be noted that the IL-2-mediated induction of IRF-1 was, predictably, not dependent on STAT4. Similarly, although IFN-α can activate STAT4, we have no data indicating that STAT4 is important for IFN-α-dependent IRF-1 regulation; STAT1 is likely to be more relevant.

In contrast to IL-12, IL-4 promotes Th2 responses. IL-4 has been shown to inhibit IL-12-dependent induction of IFN-γ (32) and to inhibit IFN-γ-dependent up-regulation of IRF-1 (24). Based on the phenotype of IRF-1−/− mice, IRF-1 appears to participate in Th1 differentiation, and interestingly, no up-regulation of IRF-1 was found in IL-4-stimulated cells. Importantly, IL-4 treatment did not block IL-12-dependent up-regulation of IRF-1, and this contrasts with the induction of IFN-γ by IL-12. The experiment thus substantiates our claim that IL-12-dependent up-regulation of IRF-1 was not indirectly mediated by IFN-γ production, as IL-4 blocked IFN-γ induction but not IRF-1.

In conclusion, we have demonstrated that IL-12 and IFN-α up-regulate a number of genes in common. One important gene known to be regulated by IFN-α or IRF-1, but it too is directly regulated by IL-12 and IL-2, but not by IL-4. Thus, IRF-1 can also be viewed as a gene that is regulated by Th1 cytokines. The demonstration that IL-12 induces IRF-1 expression may help to clarify the defects found in IRF-1−/− mice; loss of this transcription factor not only interferes with IL-12 and IL-15 synthesis but may interfere with some of the actions of IL-12 and IL-2. To what extent IRF-1 serves as an IL-12-dependent transcription factor will be better appreciated as we learn more about the genes induced by these cytokines.

Acknowledgements

We thank Drs. Richard Pine, Ueli Gubler, and James Ihle for providing reagents. We also thank Dr. Dave Frucht and Keiko Ozato for reading the manuscript.

References


