Modulation of T Cell Cytokine Production by Interferon Regulatory Factor-4*

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Production of cytokines is one of the major mechanisms employed by CD4^{+} T cells to coordinate immune responses. Although the molecular mechanisms controlling T cell cytokine production have been extensively studied, the factors that endow T cells with their ability to produce unique sets of cytokines have not been fully characterized. Interferon regulatory factor (IRF)-4 is a lymphoid-restricted member of the interferon regulatory factor family of transcriptional regulators, whose deficiency leads to a profound impairment in the ability of mature CD4^{+} T cells to produce cytokines. In these studies, we have investigated the mechanisms employed by IRF-4 to control cytokine synthesis. We demonstrate that stable expression of IRF-4 in Jurkat T cells not only leads to a strong enhancement in the synthesis of interleukin (IL)-2, but also enables these cells to start producing considerable amounts of IL-4, IL-10, and IL-13. Transient transfection assays indicate that IRF-4 can transactivate luciferase reporter constructs driven by either the human IL-2 or the human IL-4 promoter. A detailed analysis of the effects of IRF-4 on the IL-4 promoter reveals that IRF-4 binds to a site adjacent to a functionally important NFAT binding element and that IRF-4 cooperates with NFATc1. These studies thus support the notion that IRF-4 represents one of the lymphoid-specific components that control the ability of T lymphocytes to produce a distinctive array of cytokines.

The coordination of an immune response is critically dependent on the ability of CD4^{+} T cells to perform a unique set of effector functions. Crucial among these effector functions is the capacity of CD4^{+} T cells to secrete a distinctive array of cytokines including IL[1]-2, IL-4, and IFN-γ. Although most antigen-specific CD4^{+} T cells have the potential to secrete all of these cytokines, CD4^{+} T cells exposed to specific microenvironments can differentiate into two distinct subsets, termed T helper 1 (TH1) and T helper 2 (TH2) cells. These two subsets are restricted in the pattern of cytokines that they can produce. Thus

TH1 cells secrete IL-2 and IFN-γ but not IL-4, while TH2 cells produce IL-4 (as well as IL-5, IL-6, IL-10, and IL-13) but not IL-2 or IFN-γ (1, 2).

One of the critical players responsible for transducing T cell activation signals into the acquisition of T cell effector functions is the NFAT family of transcriptional regulators (3–5). This family is comprised of four calcium-regulated members, NFAT1 (NFATc2, NFATp), NFAT2 (NFATc1, NFATc), NFAT3 (NFATc4), and NFAT4 (NFATc3, NFATx). Upon activation of T cells, these proteins are rapidly dephosphorylated and translocate to the nucleus. This process is mediated by calcineurin, a calcium-regulated phosphatase, which is a well known target of the immunosuppressive drugs cyclosporin A and FK506 (6). NFAT proteins have been shown to be involved in the regulation of several cytokine genes, including *IL-2* and *IL-4* (3, 5). The regulatory regions of cytokine genes usually contain multiple functionally important NFAT target sequences, for instance, the promoter of the *IL-4* gene contains four (to five) distinct NFAT binding sites, termed P0 through P4 (7). NFAT proteins bind DNA only weakly and optimal binding and NFAT-mediated transactivation requires their cooperation with additional transcription factors and the formation of "functional enhanceosomes" (8). Genetic studies have revealed a complex role for NFAT proteins in the regulation of cytokine production and have highlighted the fact that members of this family can exert not only positive but also inhibitory effects on the production of specific cytokine profiles (9–17). Despite the fact that NFAT proteins play a crucial role in the production of T cell cytokines, their expression can be detected in a wide variety of cells and deficiency of some NFAT proteins can lead to profound defects in the development of nonlymphoid cells (18). Many of the transcription factors, like AP-1, that have classically been shown to cooperate with NFAT proteins are also not restricted to lymphocytes. It is therefore unclear how lineage-specific expression of NFAT target genes is achieved.

IRF-4 is a recently discovered member of the interferon regulatory factor (IRF) family of transcription factors whose expression is primarily restricted to lymphocytes (19–22). IRF-4 expression in B and T cells is up-regulated by pathways known to drive their activation (19, 21, 23, 24), and genetic studies have demonstrated that IRF-4 is a critical effector of mature lymphocyte function (25). Studies of the mechanisms employed by IRF-4 to modulate lymphocyte activation have so far primarily focused on its role in B cells. In these cells, IRF-4 is involved in the regulation of genes that display B cell-specific expression/regulation, and that are normally induced in response to B cell activation stimuli (26). The ability of IRF-4 to target these genes requires the presence of DNA-bound PU.1, an Ets protein expressed in macrophages and B cells but not in T cells (19, 27). The interaction of IRF-4 with PU.1 is believed to cause a conformational change in IRF-4 that unmasks its DNA binding domain thus allowing it to target DNA sites

[1] The abbreviations used are: IL, interleukin; IRF-4, interferon regulatory factor 4; NFAT, nuclear factor of activated T cell; EMSA, electrophoretic mobility shift assay; GAS, interferon-γ-activated site(s); IRF, interferon regulatory factor; ICSBP, interferon consensus sequence binding protein; ISRE, interferon-stimulated regulatory element; ELISA, enzyme-linked immunosorbent assay; PMA, phorbol 12-myristate 13-acetate; TH, T helper; STAT, signal transducers and activators of transcription.

containing the core sequence for IRF binding (GAAA) (28, 29). As demonstrated by studies on CD23b, a gene synergistically induced by CD40 and IL-4, IRF-4 may also function in the integration of B cell activation pathways as a result of its ability to participate in the formation of "enhanceosome-like" complexes (23, 30). Genetic studies have revealed that IRF-4 plays a fundamental role in the T cell compartment as well (25). T cells from IRF-4-deficient mice can undergo early activation events but are unable to complete their activation program and display a profound block in their ability to produce cytokines like IL-2, IL-4, and IFN-γ. The mechanisms by which IRF-4 controls the acquisition of T cell effector function have, however, not been fully elucidated.

Here, we show that IRF-4 can modulate the expression of T cell cytokine genes by directly targeting their regulatory regions. Stable expression of IRF-4 in T cells lacking endogenous IRF-4 leads to a strong enhancement in the production of IL-2, IL-4, IL-10, and IL-13. Transient transfection assays employing reporter constructs driven by either the IL-2 or IL-4 promoters further demonstrate that the presence of IRF-4 leads to higher inducibility of these constructs. A detailed analysis of the human IL-4 promoter indicates that IRF-4 can bind to DNA elements situated next to well known NFAT binding sites. We furthermore show that IRF-4 can functionally cooperate with the NFATc1 (NFAT2) protein and that the effect of IRF-4 on cytokine production can be blocked by immunosuppressants known to interfere with NFAT activation. Taken together these data are consistent with the notion that IRF-4 can function as a lineage-specific partner for NFAT proteins. Thus, the induction of IRF-4 upon T cell activation is likely to represent one of the critical steps that can endow T cells with the ability to perform their unique set of biologic responses.

MATERIALS AND METHODS

Cell Lines and Cultures—The Jurkat (human T cell leukemia) cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The human T cell line HUT78 was obtained from Dr. Seth Lederman, Columbia University. All cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (Atlanta Biologicals, Inc.). For preparing IRF-4 stable transfectants, the Jurkat T cells were transfected by electroporation (960 μF, 260 V) using a BTX Electroporator with either a control vector (pIRES2-EGFP) or an IRF-4 expression vector (pIRES2-EGFP-myc-IRF-4). The transfectants were selected in Iscove's modified Dulbecco's medium containing 1.5 mg/ml G418 (Promega). Jurkat cells (1×10^6) or the IRF-4 stable transfectants were stimulated with PMA (50 ng/ml) and ionomycin (1 μM) in a final volume of 1 ml at 37 °C for 24 h. IRF-4 knockout mice on a C57BL6 background were obtained from Dr. T. Mak at the Departments of Immunology and Medical Biophysics, University of Toronto, and the Amgen Institute. C57/BL6 mice were used as controls. Mice were maintained under specific pathogen-free conditions.

Antibodies and Reagents—The rabbit polyclonal antiserum against IRF-4 has been previously described (23). The rabbit polyclonal antiserum against NFAT proteins (796) was a generous gift from Dr. Nancy Rice (NCI-Frederick Cancer Research and Development Center, Frederick, MD) (31). The monoclonal antibody against NFATc1 (7A6) and the rabbit polyclonal antisera against IRF-2, ICSBP, or β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phycoerythrin-labeled monoclonal antibodies against CD25 and CD69 were purchased from Pharmingen. Cyclosporin A and FK506 were purchased from Calbiochem.

DNA Constructs—Full-length human IRF-4 cDNA cloned into pBluescript vector (pBSK-myc-IRF-4) was a gift of Dr. Riccardo Dalla-Favera (Columbia University). The bicistronic IRF-4 expression plasmid (pIRES2-EGFP-myc-IRF-4) was constructed by cloning the entire coding region of the c-Myc epitope-tagged IRF-4 cDNA into the *Eco*RI and *Bam*HI sites of the pIRES2-EGFP mammalian expression vector (Clontech). The human IL-2 promoter luciferase reporter construct and the NFATc1 expression vector were a generous gift of Dr. Gerald Crabtree (Stanford University) (32). The human IL-4 promoter luciferase reporter construct (pLuc-IL-4-(−269/+11)) was a generous gift of Dr. M. Li-Weber and Dr. Peter Krammer (Tumor Immunology Program,

German Cancer Research Center, Heidelberg, Germany) (33). To prepare the P1-IRF and the P1-IRFM3 firefly luciferase reporter constructs, a trimer of the P1-IRF or the P1-IRFM3 element was synthesized with flanking *Bam*HI-*Bgl*II sites (Invitrogen), and then cloned into the *Bam*HI site (immediately upstream of minimal thymidine kinase promoter) of the TK200 luciferase reporter vector (a kind gift of Dr. Calame, Columbia University).

DNA Binding Assays, Cell Extracts, and Western Blot Analysis—The preparation and employment of DNA oligonucleotide probes for electrophoretic mobility shift assays (EMSAs) have been described previously (23). The oligonucleotides employed in these studies were as follows: P1-IRF wt (also referred to as P1 in Fig. 5A), 5′-gatcGTGTAACGAAA-ATTTCCAATGTAAA-3′; P1-IRFM1, 5′-gatcACACAACGAAAATTTCC-AATGTAAA-3′; P1-IRFM2, 5′-gatcGTGTCCTAAAAATTTCCAATGTA-AA-3′; P1-IRFM3, 5′-gatcGTGTAACGCCCCTTTCCAATGTAAA3′; P1-IRFM4, 5′-gatcGGTGTAACGAAAACTAGCAATGTAAA-3′; P4, 5′-gat-cTAGCAAATTATGGTGTAATTTCCTATGCTGAA-3′; CD23b GAS wt, 5′-gatcGGGTGAATTTCTAAGAAAGGGAC3′; GBP-ISRE, 5′-gatcCAA-GTACTTTCAGTTTCATATT-3′. Oligonucleotide competition and antibody interference assays were performed as previously described (34). Nuclear and whole cell extracts were prepared as previously described (23, 34). Western blotting was performed as described (23).

RNase Protection Assays—Total RNA was extracted by using the RNAqueous™ Kit (Ambion Inc., Austin, TX). RNase protection analysis was performed by using a human cytokine multiprobe RNase Protection Assay kit (Pharmingen). 10 μg of total RNA was hybridized simultaneously to antisense riboprobes of a set of human cytokines as well as of internal controls (L32 and glyceradehyde-3-phosphate dehydrogenase) transcribed by T7 RNA polymerase using [α-^{32}P]UTP. The annealed products were digested with a mixture of ribonuclease A and ribonuclease T1, then analyzed on a 6% polyacrylamide-urea denaturing gel.

Transient Transfections—For the transient transfection assays, 10 $\times$ 10^6 control or IRF-4 transfectants were cotransfected with 5 μg of the appropriate luciferase reporter plasmid by electroporation at 260 V and 960 microfarads with a BTX electroporator as described previously (23). In some experiments, the cells were also transfected with 5 μg of either an NFATc1 expression vector or an empty vector (PSH160C). 100–200 ng of the pRL-TK reporter plasmid expressing Renilla luciferase under the control of the thymidine kinase promoter was added to each transfection as a transfection efficiency control. The cells were allowed to recover for 16 h at 37 °C, 6% CO_2, spun, and resuspended in 3 ml of media and equally split into two 1.5-ml aliquots. The cells were cultured in the presence or absence of PMA (50 ng/ml) and ionomycin (1 μM) for 4 h. The transfected cells were then harvested, lysed, and assayed for luciferase activity with the Dual Luciferase Assay System (Promega) according to the manufacturer's instructions. The firefly luciferase activity was normalized on the basis of Renilla luciferase activity.

Measurement of Cytokine Levels by ELISA—Cytokine production by the Jurkat stable transfectants was assayed by using the human IL-2, IL-4, and IL-10 OptEIA™ ELISA kits (Pharmingen) according to the manufacturer's instructions. The human IL-5 and IL-13 ELISA kits were obtained from R & D Systems. Optical density was determined on a UV$_{max}$ kinetic microplate reader (Amersham Biosciences) at a wavelength of 450 nm. Data were analyzed using Softmax PRO version 3.0 software (Amersham Biosciences).

RESULTS

Early T Cell Activation Events Are Not Affected by Stable Expression of IRF-4—To start dissecting the mechanisms utilized by IRF-4 to control T cell effector functions, we proceeded to stably express IRF-4 in the human T cell line, Jurkat. Jurkat cells lack endogenous IRF-4 expression and are unable to upregulate IRF-4 upon mitogenic stimulation (Fig. 1A and data not shown). Independent sets of Jurkat stable transfectants were obtained utilizing either an IRF-4 expression vector or a control vector. As demonstrated by Western blot analysis, all the IRF-4 transfectants displayed comparable levels of IRF-4 expression, whereas no IRF-4 was detected in the cells transfected with the control vector (Fig. 1A) or in untransfected Jurkat cells. To ascertain whether expression of IRF-4 would globally affect the activation program of Jurkat T cells, fluorescence-activated cell sorter analysis was employed to determine the inducibility of CD69 and CD25, two well known surface markers whose expression is up-regulated upon T cell
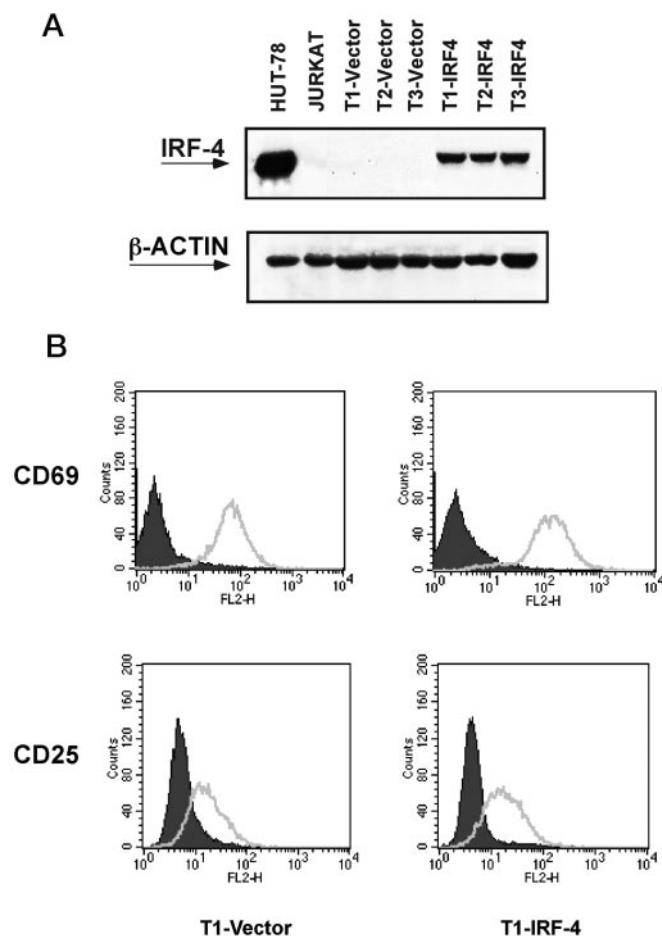
A

B

FIG. 1. **Early activation events in IRF-4-transfected cells.** *A*, whole cell extracts were prepared from Jurkat cells stably transfected with either a control or an IRF-4 expression vector, electrophoresed on a 7% SDS-polyacrylamide gel, and then analyzed by Western blotting using an anti-IRF-4 antibody (*upper panel*). The blot was later stripped and reprobed with a β-actin antibody (*lower panel*) to ensure for equal loading. Extracts from untransfected Jurkat cells and HUT 78 served, respectively, as negative and positive controls. *B*, Jurkat-transfected cells were either left unstimulated or were stimulated with PMA (50 ng/ml) and ionomycin (1 μM) for 24 h. The cells were then harvested and stained with either a phycoerythrin-labeled anti-CD69 (*upper panel*) or a phycoerythrin-labeled anti-CD25 antibody (*lower panel*) and analyzed by flow cytometry. *Filled* histograms represent unstimulated cells, whereas *empty* histograms represent cells stimulated with PMA and ionomycin. *Left panel*, vector transfectants; *right panel*, IRF-4 transfectants. Not shown is staining with an isotype-matched control, which did not reveal any significant differences between control and IRF-4 transfectants.

activation (Fig. 1*B*). Consistent with the fact that IRF-4-deficient mice do not display any significant disturbances in the expression/up-regulation of CD69 and CD25 (25), stable expression of IRF-4 did not significantly affect the basal levels and/or the inducibility of these T cell activation markers.

Enhancement of T Cell Cytokine Production by Stable Expression of IRF-4—Given the profound block in T cell cytokine synthesis exhibited by mice deficient in IRF-4 (25), we then examined the cytokine profile produced by the different sets of stable transfectants. Cells from the stable transfectants were either left unstimulated or were stimulated with PMA and ionomycin. After 24 h, the culture supernatants were collected and assayed for cytokine production by ELISA. Consistent with previous reports (35), control Jurkat transfectants produced moderate levels of IL-2 in the presence, but not in the absence, of stimulation with PMA and ionomycin (Fig. 2*A*). When compared with the control transfectants, the IRF-4 expressing cells

displayed markedly enhanced IL-2 production. Interestingly, the effect of IRF-4 on IL-2 synthesis could only be detected in cells that were concomitantly stimulated with PMA and ionomycin but not in unstimulated cells. In contrast to IL-2, expression of IRF-4 was unable to drive the production of another TH1-type cytokine, IFN-γ, regardless of the stimulation status of the cells (Fig. 2*B*). The synthesis of additional TH2-type cytokines, IL-4 (Fig. 2*C*), IL-10 (Fig. 2*D*), IL-5 (Fig. 2*E*), and IL-13 (Fig. 2*F*) was also examined. Remarkably, whereas control transfectants were unable to produce detectable levels of IL-4 and IL-10, expression of IRF-4 led to the production of considerable amounts of both of these two cytokines. Once again the effect of IRF-4 on IL-4 and IL-10 synthesis required the concomitant stimulation of the cells with PMA and ionomycin. This experiment furthermore revealed that the production of an additional TH2-type cytokine, IL-13, was also increased in the presence of IRF-4 (Fig. 2*F*). IRF-4 expression in Jurkat cells, however, was unable to direct synthesis of the full array of TH2 cytokines because the IRF-4-transfected cells did not display any production of IL-5 (Fig. 2*E*). All three sets of independent Jurkat transfectants displayed similar changes in their cytokine profiles. Taken together, these data thus indicate that the presence of IRF-4 leads to an enhanced ability of Jurkat cells to produce a TH1-type cytokine, IL-2. Furthermore, upon expression of IRF-4 these T cells become capable of producing measurable quantities of TH2-type cytokines like IL-4 and IL-10.

To determine whether the enhanced cytokine production exhibited by the IRF-4 transfectants was associated with increased transcription of these cytokine genes, we then performed an RNase protection assay designed to detect multiple cytokine mRNAs within a single sample. As shown in Fig. 3, this experiment confirmed that the presence of IRF-4 in Jurkat cells induces the expression of IL-2, IL-4, IL-10, and IL-13. Consistent with the ELISA results, no up-regulation of IFN-γ or IL-5 expression was detected. Therefore, expression of IRF-4 can exert profound effects on the ability of T cells to produce specific subsets of TH1 as well as of TH2-type cytokines.

IRF-4 Directly Targets Cytokine Promoters—The *IL-2* and *IL-4* regulatory regions have been extensively characterized (7, 36, 37). An examination of the promoters of these two cytokine genes revealed the presence of several functionally important DNA elements that contain GAAA, the core DNA sequence targeted by the IRFs (38). This finding thus suggested that the IRF-4-mediated enhancement of cytokine production could be due to a direct effect of IRF-4 on cytokine gene expression. To test this possibility, we proceeded to determine whether IRF-4 expression could increase the inducibility of luciferase reporter constructs driven either by the human IL-2 or by the human IL-4 promoter (32, 33). As shown in Fig. 4, the inducibility of both reporter constructs was indeed markedly enhanced in the presence of IRF-4. As in the case of the endogenous genes (Fig. 2, *A* and *C*), the IRF-4-mediated effect on these luciferase constructs required concomitant stimulation of the cells with PMA and ionomycin. These data thus suggest that IRF-4 can directly transactivate the human IL-2 and IL-4 promoters.

To further dissect the mechanisms by which IRF-4 can direct cytokine gene expression we focused our attention on the IL-4 promoter. This promoter has been shown to contain several functionally important NFAT binding sites termed P0 through P4 (5, 7). Because some of the potential IRF core sequences were located adjacent to these NFAT sites we first proceeded to determine whether oligonucleotides containing regions encompassing two of these elements (P1 and P4) could act as competitors of a known IRF-4 binding site from the CD23b promoter (the CD23b GAS) (23). For these experiments we utilized ex-
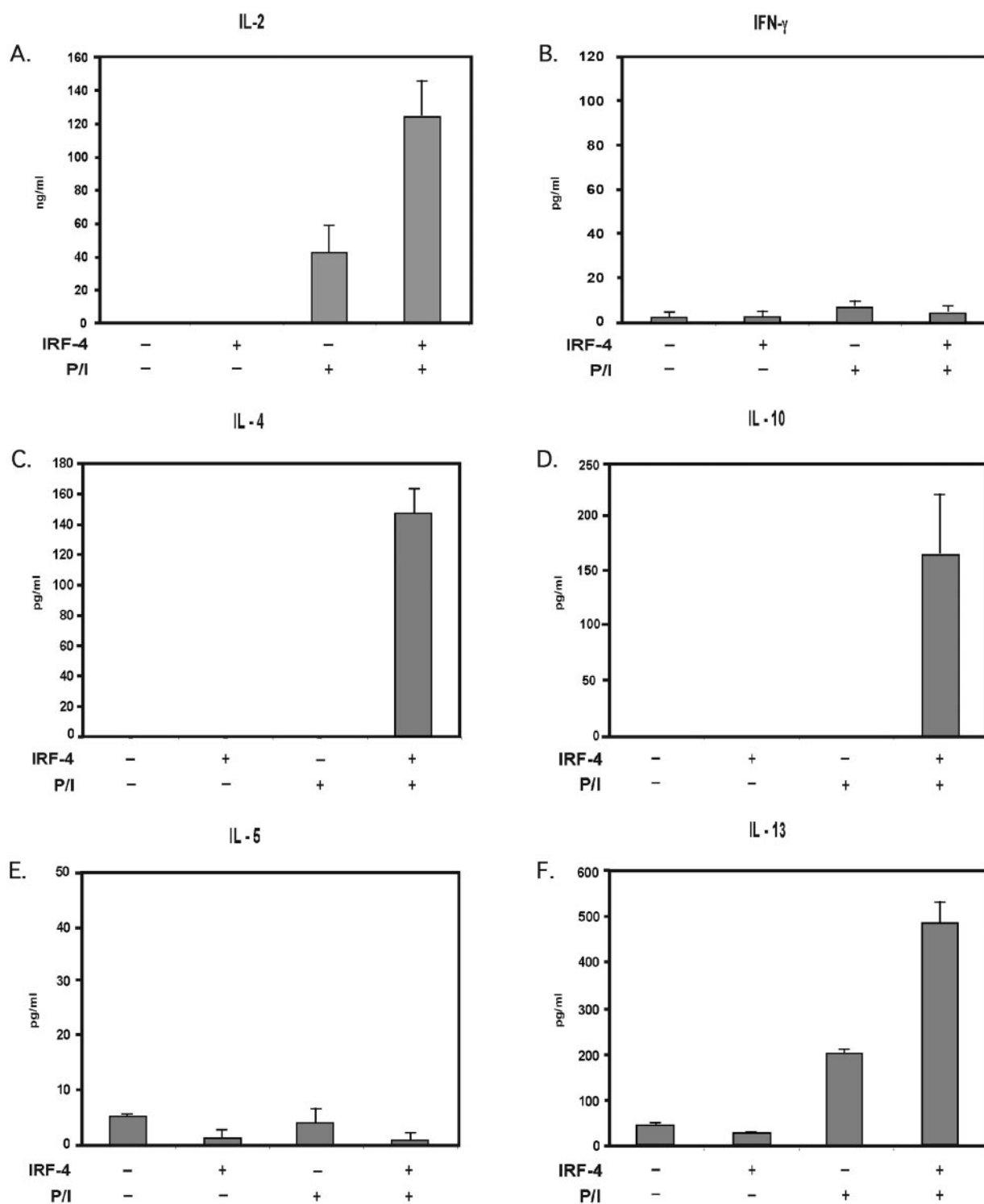
F_IG_. 2. **Modulation of cytokine production by IRF-4.** Control and IRF-4-transfected cells were either left unstimulated or were stimulated with PMA (50 ng/ml) and ionomycin (1 μM) for 24 h. Supernatants were then collected and analyzed for their cytokine content by ELISA. *A*, IL-2 production (ng/ml); *B*, IFN-γ production (pg/ml); *C*, IL-4 production (pg/ml); *D*, IL-10 production (pg/ml); *E*, IL-5 production (pg/ml); *F*, IL-13 production (pg/ml). Data shown are representative of six separate experiments performed on three independent sets of transfectants.

tracts from HUT78, a human T cell line derived from a cutaneous T cell lymphoma (39), which contains high levels of endogenous IRF-4 (Fig. 1*A*) as well as constitutive nuclear localization of NFAT proteins (data not shown). As shown in Fig. 5*A*, when extracts from HUT78 were subjected to electrophoretic mobility shift assays with a radiolabeled CD23b GAS probe, multiple DNA binding complexes could be detected. We have previously demonstrated that the slowest mobility com-

plex contains IRF-4 (23), whereas the faster mobility complexes include members of the NF-κB family of proteins.[2] When extracts from HUT78 cells were subjected to competition experiments with oligonucleotides containing either the P1 or P4 sites, binding of the IRF-4 containing complex to the CD23b

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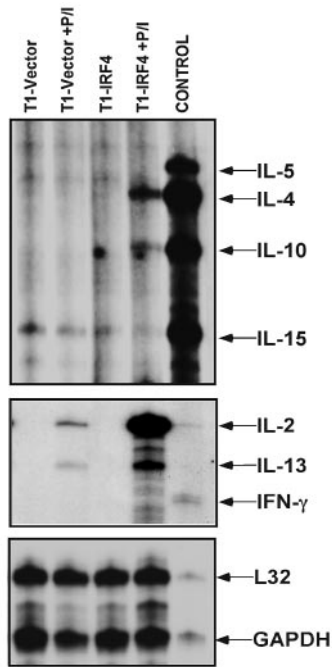[2] S. Gupta, unpublished observations.

FIG. 3. **RPA analysis of cytokine mRNA expression in control and IRF-4 transfectants.** Control and IRF-4-transfected cells were either unstimulated or stimulated with PMA and ionomycin as indicated in the legend to Fig. 2. Cells were then harvested and RNA was extracted. Cytokine transcript levels were then analyzed by RNase protection assay utilizing a human cytokine multiprobe template set (Pharmingen). The different panels represent different exposures of the same autoradiogram: *top panel*, 48 h exposure; *middle panel*, 24 h exposure; *lower panel*, 12 h exposure. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

GAS probe was completely abolished. As expected, competition with excess unlabeled CD23b GAS oligonucleotide also prevented binding of the IRF-4 complex to the probe. In contrast, no competition could be observed upon addition of an oligonucleotide containing a site (the CD23a GAS) that cannot be targeted by IRF-4 (30).

To directly assess whether IRF-4 can bind to functional elements within the IL-4 promoter we then performed EMSAs utilizing as a probe an oligonucleotide containing the P1 site from this promoter. This oligonucleotide also includes a potential IRF core sequence situated just upstream of the NFAT binding site and will be referred to as the P1-IRF wt (wild-type) probe. As shown in Fig. 5*B* (*left panel*), HUT78 cells contain multiple complexes that can bind to this probe, including a slow mobility complex whose mobility was very similar to that of the IRF-4 containing complex detected with a CD23b GAS probe (Fig. 5*B, middle panel*). Incubation of extracts from HUT78 cells with an anti-IRF-4 antiserum confirmed that this slow mobility complex contains IRF-4 (Fig. 5*B, left panel*). Additional antibody interference assays with antisera against other IRF family members, IRF-2 or ICSBP, failed to affect the appearance of any of the complexes binding to the P1-IRF probe despite appropriately supershifting IRF-2 or ICSBP-containing complexes bound to a GBP-ISRE probe (Fig. 5*B, right panel*). Consistent with previous studies, the additional complexes detected with the P1-IRF probe contain NFAT proteins because their appearance could be blocked by the addition of an antiserum that recognizes multiple NFAT family members (31). Interestingly, the anti-IRF-4, but not a control antiserum also affected the appearance of these NFAT-containing complexes suggesting that IRF-4 might be able to complex with NFAT proteins.

To more precisely define the exact nucleotides required for IRF-4 binding, we then carried out EMSA experiments utilizing a panel of mutated P1-IRF oligonucleotides as cold competitors of the radiolabeled P1-IRF wt probe (Table I). Because the P1-IRF wt probe contains a potential core sequence for IRF binding (GAAA) located immediately upstream of the known NFAT binding site (38, 40), we mutated each of these two sites (M3 and M4, respectively) as well as a region upstream of these two elements (M1 and M2), which has previously been shown to be targeted by AP-1 proteins (40–42). These competition experiments revealed that the P1-IRFM3 oligonucleotide, which contains a mutation within the potential IRF core sequence, is unable to compete the IRF-4 complex suggesting that this complex indeed targets the IRF recognition sequence (Fig. 5*C*). Consistent with previous results, mutating the NFAT binding site completely abolishes competition of the NFAT containing complexes by the P1-IRFM4 oligonucleotide (Fig. 5*C*). Interestingly, optimal IRF-4 binding may also require the NFAT binding site, because the P1-IRFM4 mutant could not fully compete the IRF-4 complex. Similarly, the IRF-4 binding site may also contribute to NFAT binding given that the P1-IRFM3 mutant was unable to completely block binding of the NFAT complex to the probe. Taken together these data thus suggest that an IRF-4 containing complex can bind to an IRF core sequence located just adjacent to a well known functional NFAT binding site within the IL-4 promoter.

To confirm that binding of IRF-4 to this critical regulatory region is not simply because of the transformed phenotype of the HUT78 cell line but also occurs during the activation of primary lymphocytes, we then proceeded to determine whether targeted disruption of IRF-4 would affect the pattern of proteins bound to the P1-IRF site. Splenocytes were thus harvested from wild-type C57/BL6 control mice as well as from IRF-4-deficient mice. Extracts from cells that were either unstimulated or were stimulated with PMA and ionomycin were obtained and assayed by EMSA utilizing a ^{32}P-radiolabeled P1-IRF probe. As shown in Fig. 5*D*, stimulation of splenocyte from wild-type mice resulted in the strong induction of a slow mobility P1-IRF binding complex. Strikingly, appearance of this inducible complex was abolished in splenocytes of IRF-4-deficient mice, suggesting that the presence of IRF-4 is critical for the proper assembly of this DNA binding complex.

Given the ability of IRF-4 to bind to a critical regulatory element within the IL-4 promoter, we next analyzed whether IRF-4 could function as a positive transactivator of this DNA element. We thus performed transient transfection assays with a luciferase reporter construct driven either by the P1-IRF wt site or by the P1-IRFM3 site in which the IRF-4 binding site is mutated. As shown in Fig. 6, the presence of IRF-4 led to a markedly increased inducibility of the P1-IRF wt reporter construct. In striking contrast, the presence of IRF-4 failed to significantly augment the inducibility of a reporter construct driven by the mutant P1-IRF element (P1-IRFM3), which does not bind IRF-4. The lower activity of this mutant construct in response to PMA and ionomycin even in the absence of IRF-4 is likely because of the fact that this mutation also affected binding of the NFAT proteins to the P1-IRF oligonucleotide as shown in the EMSA competition assays (Fig. 5*C*). Taken altogether, these data thus indicate that IRF-4 can indeed act as a positive transactivator of a functionally important element within the human IL-4 promoter.

Cooperation of IRF-4 with NFATc1—The previous experiments had indicated that IRF-4 requires costimulation with PMA and ionomycin to exert its enhancing effects on cytokine gene expression. This finding coupled with the ability of IRF-4 to target DNA elements adjacent to NFAT binding sites raised the possibility that IRF-4 might functionally cooperate with
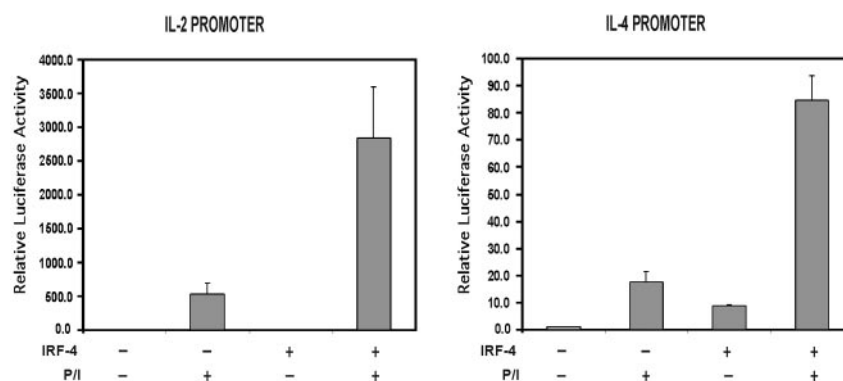
IL-2 PROMOTER

IL-4 PROMOTER

FIG. 4. **IRF-4 transactivates the human IL-2 and IL-4 promoters.** Control and IRF-4 Jurkat-transfected cells were transiently transfected with a luciferase reporter construct driven either by the human IL-2 promoter (*left panel*) or the human IL-4 promoter (*right panel*). The transfected cells were equally split into two 2-ml aliquots and then incubated for 4 h in the presence or absence of PMA (50 ng/ml) and ionomycin (1 μM). The data are presented relative to the activity of the reporter construct in unstimulated control cells, which was set to 1.0, as indicated in each experiment. Results show the mean $\pm$ S.E. of five (for the IL-2 promoter) and six (for the IL-4 promoter) independent experiments.

NFAT proteins. To explore this possibility in detail, we then assessed whether transfection of an NFATc1 expression vector would affect the ability of IRF-4 to transactivate a luciferase construct driven by the human IL-4 promoter (Fig. 7A). Consistent with previous results, transfection of the NFATc1 expression vector led to a moderate increase in the inducibility of this luciferase construct in the absence of IRF-4. However, expression of both IRF-4 and NFATc1 markedly augmented IL-4 luciferase reporter activity. These data thus indicate that IRF-4 can cooperate with NFATc1 in transactivating the IL-4 promoter.

To further confirm the requirement for NFAT proteins in the IRF-4-mediated effects on cytokine production, we then proceeded to determine whether addition of a well known inhibitor of NFAT activation, cyclosporin A, would affect the ability of IRF-4 to enhance the production of endogenous cytokines. As shown in Fig. 7B, pretreatment of the IRF-4 stable transfectants with cyclosporin A completely blocked IL-2 and IL-4 production by these cells in response to PMA and ionomycin. As expected, addition of cyclosporin A also inhibited the lower levels of IL-2 produced by the control transfectants. Pretreatment of the cells with FK506, another inhibitor of NFAT activation exerted similar effects on the IRF-4-mediated cytokine production (Fig. 7B). The inhibitory effects of cyclosporin A and FK506 were not due to any toxic effect on the cells because these inhibitors did not significantly affect the ability of the transfectants to up-regulate the surface expression of CD69 or CD25 upon mitogenic stimulation (data not shown). Taken together, these data are thus consistent with a model whereby IRF-4 can functionally cooperate with NFAT proteins in driving T cell cytokine gene expression.

DISCUSSION

The synthesis of a distinctive array of cytokines is one of the most characteristic and critical functions of CD4^{+} T cells (43). Although the mechanisms involved in T cell cytokine production have been extensively studied (2, 44), the factors that are responsible for the ability of lymphocytes to selectively produce specific cytokines have not been fully elucidated. It has previously been reported that mice deficient in IRF-4, a lymphoid restricted member of the IRF family of transcription factors, display striking disturbances in T cell cytokine production (25). In these studies, therefore, we set out to investigate the mechanisms by which IRF-4 controls T cell cytokine synthesis. Our results indicate that stable expression of IRF-4 exerts profound effects on the ability of human T cells to produce multiple cytokines, including IL-2 and IL-4. We furthermore show that IRF-4 directly targets the promoters of these cytokines and

that its effects require cooperation with NFATc1. Taken together with the information provided by the genetic studies, these data are thus consistent with the notion that IRF-4 represents one of the major lymphoid-restricted regulators of T cell cytokine synthesis.

We have shown that stable expression of IRF-4 in human T cells can activate the expression of TH2-type cytokines (IL-4, IL-10, and IL-13). This is in agreement with two recent reports, which found that IRF-4-deficient T cells are impaired in their ability to differentiate *in vitro* toward a TH2 phenotype (45, 46). This finding is furthermore supported by the fact that similarly to B cells (23), expression of IRF-4 in T cells can be up-regulated upon exposure to IL-4, the most potent TH2 differentiating stimulus.[3,4] Interestingly, no induction of IL-5 gene expression was noted in our system suggesting that additional factors may modulate the ability of IRF-4 to target different TH2-types cytokines.

Our observations, however, indicate that IRF-4 does not simply function as a TH2-specific factor but it may also participate in the control of TH1-type cytokines because presence of IRF-4 markedly enhanced the induction of IL-2, a cytokine normally associated with the TH1 phenotype. The effect of IRF-4 on human IL-2 production is consistent with the phenotype of T cells from IRF-4 deficient mice, which display a marked impairment in the synthesis of IL-2 (25). Interestingly, if supplied with exogenous IL-2, IRF-4-deficient T cells are able to produce moderate levels of IL-2 upon restimulation (45, 46). One possible scenario reconciling these findings is that the requirements for IRF-4 in the production of individual cytokines may be dynamically regulated as a T cell proceeds along a specific differentiation pathway. For instance, naive T cells may rely more heavily on the presence of IRF-4 for their initial "burst" of IL-2 production whereas differentiated TH1 cells may have evolved additional redundant mechanisms that render the IRF-4 requirement for IL-2 production less stringent. A role for IRF-4 in the control of TH1 cytokine production is further supported by the fact that recent studies have revealed that IRF-4-deficient T cells differentiated *in vitro* under TH1 conditions display moderate to severe defects in the ability to synthesize IFN-γ, another TH1-type cytokine (45, 46). Interestingly, preliminary results indicate that the up-regulation of IRF-4 is differentially controlled in the two TH subsets. Induction of IRF-4 expression in established TH1 cells can occur in response to TCR-mediated signals, but, consistent with the

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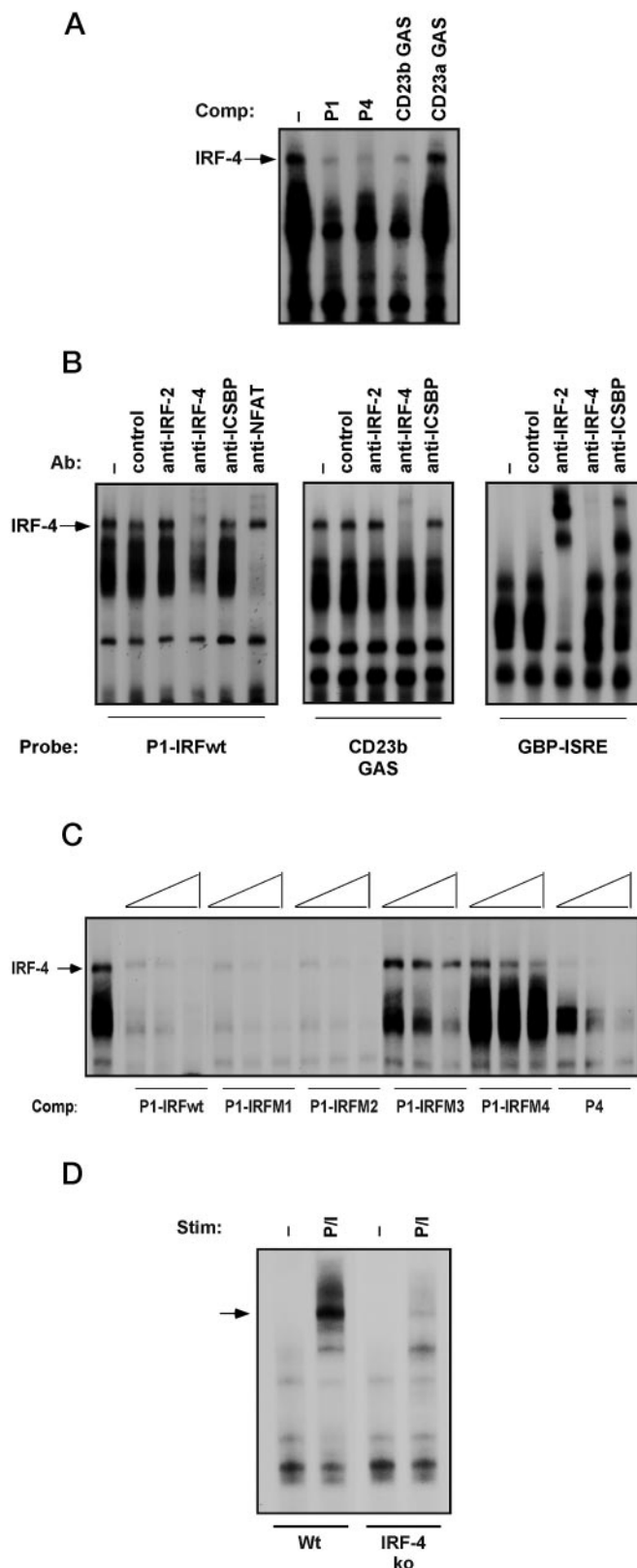[3] S. Jang, unpublished observations.

[4] A. Dent, personal communication.

TABLE I
Sequence comparison between the wild-type and mutant P1-IRF oligonucleotides

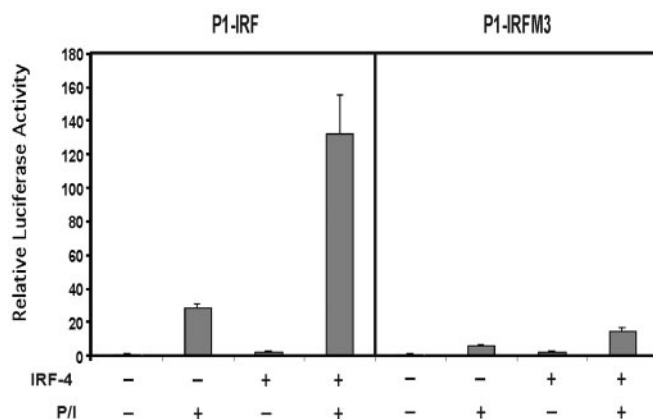|	AP-1 IRF-4 NFAT
P1-IRF WT	5′-GTGTAACGAAAATTTCCAATGTAAA-3′
P1-IRF M1	5′-**ACAC**AACGAAAATTTCCAATGTAAA-3′
P1-IRF M2	5′-GTGT**TCCT**AAAATTTCCAATGTAAA-3′
P1-IRF M3	5′-GTGTAACG**CCCC**TTTCCAATGTAAA-3′
P1-IRF M4	5′-GTGTAACGAAAA**CTAG**CAATGTAAA-3′

FIG. 6. **IRF-4 can act as a transactivator of the P1-IRF element.** Control and IRF-4 Jurkat cells were transfected with a luciferase reporter construct driven by either an oligomerized P1-IRF wt or an oligomerized P1-IRFM3 element. The transfected cells were equally split into two 2-ml aliquots and then incubated for 4 h in the presence or absence of PMA (50 ng/ml) and ionomycin (1 μM). The data are presented relative to the activity of the reporter construct in unstimulated control cells, which was set to 1.0, as indicated, in each experiment. Results show the mean $\pm$ S.E. of three independent experiments.

known extinction of IL-4 signaling in these cells (47), the IL-4-mediated up-regulation of IRF-4 is no longer detectable. Thus, although IRF-4 can be expressed in both TH1 and TH2 cells, its induction occurs in very distinct molecular milieus, and this, in turn, is likely to profoundly affect its functional capabilities.

Our studies indicate that IRF-4 can functionally interact with NFATc1, a member of a well known family of transcription factors known to play a key role in T cell cytokine production (3, 5). Although NFATc1 was originally identified as a critical regulator of *IL-2* gene expression in activated T cells (48), subsequent studies have uncovered a much broader biological role for this protein as demonstrated by the fact that lack of NFATc1 results not only in impaired T cell function but also in profound defects in the development of cardiac valves (49, 50). Given that NFATc1 expression is not solely confined to

FIG. 5. **IRF-4 containing complexes bind sequences flanking a known NFAT functional element within the human IL-4 promoter.** *A*, extracts from a human T cell line, HUT78, that constitutively expresses IRF-4 were prepared and analyzed by electrophoretic mobility shift assay utilizing a ^{32}P-labeled CD23bGAS wild-type probe. Oligonucleotide competition assays were performed either in the absence or presence of a 100-fold molar excess of cold oligonucleotides containing the P1 and P4 elements from the human IL-4 promoter, which were added to the shift reaction as indicated. Addition of cold oligonucleotides containing the CD23b GAS, or the CD23a GAS served, respec-

tively, as positive and negative controls. *B*, extracts from HUT78 cells were prepared and analyzed by electrophoretic mobility shift assay utilizing a ^{32}P-labeled P1-IRF wt probe (*left panel*). Antibody interference mobility shift assays were carried out by addition of antisera against IRF-2, IRF-4, ICSBP, NFAT, or control as indicated. All antisera were added at a final dilution of 1:20 for 30 min at 4 °C prior to incubation with the probe for 20 min at 25 °C. As a control for the IRF-2, IRF-4, and ICSBP antisera, antibody interference analysis utilizing either a ^{32}P-labeled CD23b GAS probe (*middle panel*) or a ^{32}P-labeled GBP ISRE probe (*right panel*) was simultaneously performed. *C*, extracts from HUT78 cells were obtained and assayed as described in *panel B*. Oligonucleotide competition assays were performed either in the absence or presence of an increasing molar excess (50-, 100-, and 200-fold) of cold P1-IRF wt, mutant P1-IRF, or P4 oligonucleotides added to the shift reaction as indicated. *D*, splenocytes from wild-type (*Wt*) or IRF-4-deficient mice (*IRF-4 ko*) were either left unstimulated or were stimulated with PMA (50 ng/ml) and ionomycin (1 μM) for 6 h. Cell extracts were obtained and assayed with a ^{32}P-labeled P1-IRF WT probe as described in *panel B*.

FIG. 7. **IRF-4 cooperates with NFAT in driving T cell cytokine production.** *A*, vector and IRF-4 Jurkat cells were co-transfected with a luciferase reporter construct driven by the human IL-4 promoter and either an NFATc1 expression vector or equivalent amounts of an empty vector. The transfected cells were equally split into two 2-ml aliquots and then incubated for 4 h in the presence or absence of PMA (50 ng/ml) and ionomycin (1 μM). The data are presented relative to the activity of the reporter construct in vector control cells, which was set to 1.0, as indicated, in each experiment. Results show the mean $\pm$ S.E. of four independent experiments. *B*, control and IRF-4-transfected cells were either left unstimulated or stimulated with PMA and ionomycin as indicated in the legend to Fig. 2. Stimulations were conducted in the presence or absence of cyclosporin A (1 μg/ml) or FK506 (10 ng/ml) as indicated. Supernatants were then collected and analyzed for their cytokine content by ELISA. Data shown are representative of four independent experiments and performed on three independent sets of transfectants.
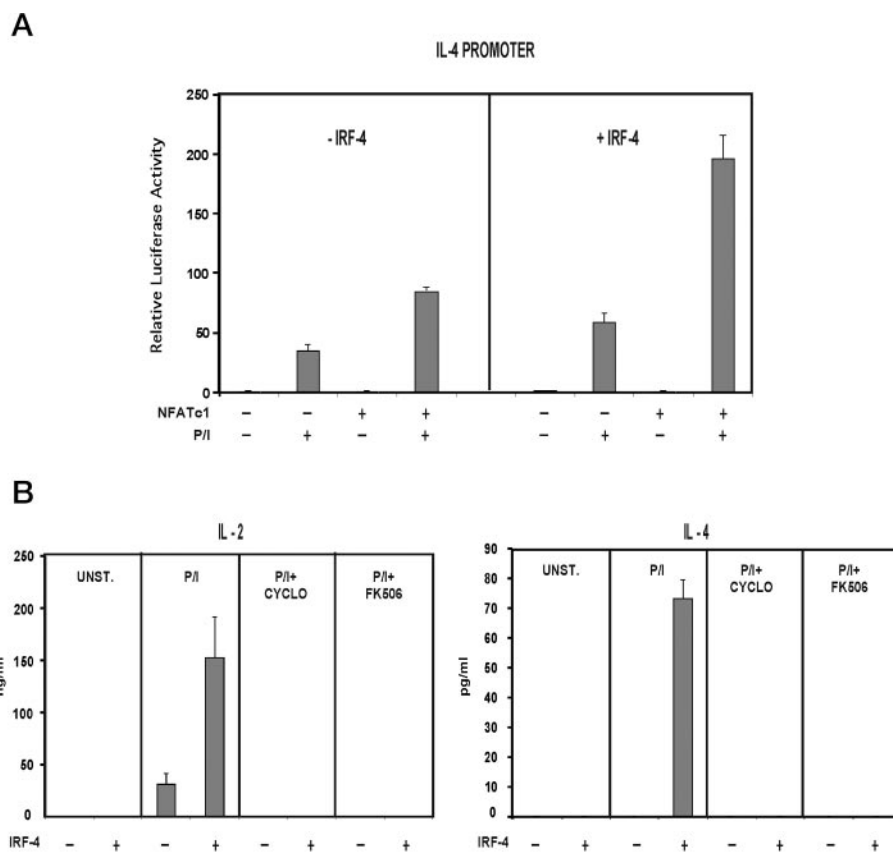
lymphocytes, the pairing of a lymphoid-restricted factor like IRF-4 with NFATc1 may thus enable NFATc1 to acquire the ability to exert its actions in a T cell-specific manner. Interestingly, T cell cytokine production is controlled not simply by NFATc1 but by a complex interplay among the different NFAT family members. This is evidenced by *in vivo* studies showing that lack of different combinations of NFAT proteins can result in either profound deficiencies or marked hyperactivation of T cell effector functions (15–17). Interestingly, during the course of these studies another group reported that murine IRF-4 can interact with a different NFAT family member, NFATc2 (45). It will thus be important to determine in *in vivo* settings whether distinct NFAT proteins can differentially modulate the ability of IRF-4 to drive cytokine production. An intricate association of IRF-4 with distinct members of the NFAT family may underlie the complex defects in TH differentiation observed in IRF-4-deficient mice (46).

Cooperation of IRF-4 and NFATc1 in IL-4 production is linked to the ability of IRF-4 to target the promoter of this gene at a site adjacent to a well characterized NFAT binding site, P1 (5, 7). Competition experiments furthermore suggest that IRF-4 complexes can similarly target additional NFAT binding sites present in the IL-4 promoter like P4. Interestingly, both P1 and P4 have been shown to be critical regulatory elements for *IL-4* gene expression in response to T cell stimulation and TH2 differentiation (33, 51, 52), further supporting a physiologic role for IRF-4 in the control of this cytokine. Given that both IRF-4 and NFATc1 have been reported to possess only weak DNA binding activity (3, 28), a likely scenario for their cooperation is that the interaction of NFAT with IRF-4 may facilitate IRF-4 binding to its DNA element and vice versa. This is indeed supported by our EMSA experiments, which demonstrate that addition of the anti-IRF-4 antibody can also affect DNA binding by NFAT (Fig. 5*B*) and that lack of IRF-4 blocks the appearance of all P1-IRF inducible complexes (Fig.

5*D*). We have furthermore found by glutathione *S*-transferase pull-down experiments that IRF-4 and NFATc1 can physically interact.[5] However, in contrast to what has been reported for the association between murine IRF-4 and NFATc2 (45), we have been unable to coimmunoprecipitate the endogenous proteins suggesting that ternary complex formation with DNA may be necessary to stabilize the IRF-4/NFATc1 interaction. Interestingly, the regions encompassing the P1 and P4 regulatory elements can be targeted by additional transcription factors like AP-1 and NF-κB/Rel proteins (40–42). It will thus be important to determine whether IRF-4 may interact with these additional factors as well. We furthermore suspect that, like the case of NFAT proteins (53, 54), IRF-4 may not simply target cytokine promoters but also additional enhancer elements that are critical for optimal and cell type-specific cytokine expression. The fact that deficiency of IRF-4 was also recently reported to be associated with defects in the up-regulation of GATA3 in TH2 cells (46) suggests that the mechanism employed by IRF-4 to modulate T cell cytokine production is likely to be multifaceted.

The ability of IRF-4 to cooperate with NFAT proteins may have important clinical implications. Indeed, addition of cyclosporin A and FK506, two well known NFAT inhibitors (6), completely blocked the ability of IRF-4 to drive cytokine synthesis. These findings suggest that in addition to exerting a direct inhibitory effect on NFAT proteins, these immunosuppressive drugs can also profoundly interfere with the function of tissue-restricted NFAT partners like IRF-4. Given that many of the side effects of cyclosporin A and FK506 have been attributed to inhibition of NFAT proteins in nonlymphoid tissues (6), targeting of the IRF-4/NFAT interaction may thus allow for the development of more selective immunosuppressants and min-

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[5] C. Hu, unpublished observations.

imize potentially deleterious side effects. The NFAT/IRF-4 interaction might also be a target for HTLVI, an oncogenic retrovirus known to usurp the activation program of T cells (55). The hallmark of HTLVI-mediated T cell transformation is the up-regulation of T cell cytokine production, and most notably of IL-2. Tax, the major HTLVI gene product involved in this effect has been shown to up-regulate the expression of IRF-4 in T cells via a pathway involving NF-κB and NFAT (56) as well as to induce the binding of NFAT-containing complexes to cytokine promoters (57). Such an elaborate effect of Tax on both IRF-4 and NFAT might represent a concerted effort by this virus to target both partners of this transcriptional complex and may potentially play a role in the pathophysiology of HTLVI-mediated T cell malignancies.

In summary, one of the major roles of IRF-4 in T cells may be to confer lineage specificity to their responses. In addition, given that IRF proteins are critical components of the IFN-β enhanceosome (58) and that IRF-4 participates in enhanceosome-like complexes in B cells (23), IRF-4 is likely to play a crucial role in the assembly of functional enhanceosomes in T cells as well. Interestingly, up-regulation of IRF-4 has been detected in response to distinct classes of activating stimuli and can be controlled by NF-κB (59), NFAT (56), as well as STAT6.[6] We thus favor a model whereby lymphocyte activation triggers a carefully programmed signaling cascade during which the rapid activation of powerful but broad early effectors, or "initiators" (NF-κB, NFAT, or STAT6), is followed by the induction/recruitment of a second wave of downstream lineage-restricted effectors (IRF-4). Early (NF-κB, NFAT, or STAT6) as well as downstream (IRF-4) effectors may then converge into the formation of enhanceosome-like complexes. Depending on the precise combination of activating stimuli, different IRF-4 containing multiprotein complexes may be assembled leading to markedly different gene expression patterns. IRF-4 may thus serve more as an "integrator" of lymphocyte responses rather than a "master regulator" of specific differentiation programs.

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