Interferon Regulatory Factor 4 (IRF4) Interacts with NFATc2 to Modulate Interleukin 4 Gene Expression

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Abstract

Proteins of the nuclear factor of activated T cells (NFAT) family of transcription factors are critical for lymphocyte activation in the immune system. In particular, NFATs are important regulators of inducible IL-4 gene expression. Interferon regulatory factor 4 (IRF4) is an immune system–restricted interferon regulatory factor that is required for lymphocyte activation, but its molecular functions in the T lineage remain to be elucidated. We demonstrate that IRF4 potently synergizes with NFATc2 to specifically enhance NFATc2-driven transcriptional activation of the IL-4 promoter. This function is dependent on the physical interaction of IRF4 with NFATc2. IRF4 synergizes with NFATc2 and the IL-4–inducing transcription factor, c-maf, to augment IL-4 promoter activity as well as to elicit significant levels of endogenous IL-4 production. Furthermore, naïve T helper cells from mice lacking IRF4 are compromised severely for the production of IL-4 and other Th2 cytokines. The identification of IRF4 as a partner for NFATc2 in IL-4 gene regulation provides an important molecular function for IRF4 in T helper cell differentiation.

Key words: IRF4 • NFAT • IL-4 • transcriptional regulation • interaction

Introduction

The immune response to foreign antigens is initiated by signals transmitted from the cell surface to the signaling network in the cytoplasm and culminates in the nucleus with the transcription of genes that mediate the effector response. Proteins of the nuclear factor of activated T cells (NFAT)* family of transcription factors are central to the transcriptional regulation of several immune-response genes, including those encoding cytokines and cell-surface receptors (1). The calcium-regulated NFAT proteins reside in the cytoplasm within resting cells. After antigenic stimulation, NFATs are dephosphorylated by the phosphatase calcineurin and are rapidly translocated into the nucleus, where they interact with other transcription factors to induce target gene expression. When intracellular calcium levels decline, NFAT proteins are rephosphorylated by specific kinases and exported back into the cytoplasm. This cycle of activation can be inhibited by the immunosuppressive drugs cyclosporin A (CsA) and FK506 (1, 2).

The importance of NFAT proteins in the regulation of IL-4 gene transcription has been well established. Sequences within the proximal IL-4 promoter that are specific for NFAT binding have been identified and shown to be critical for inducible IL-4 gene expression (3–6). In addition, a CsA-sensitive enhancer region has been identified 3′ of the IL-4 gene and has been demonstrated to bind NFATc2 in stimulated Th2, but not Th1, cells (7). Evidence from mice deficient in NFAT family members suggests that the in vivo role of NFATs in IL-4 transcription is complex and that NFAT proteins likely regulate IL-4 gene expression at multiple stages of IL-4 expression within the immune system. NFATc1 (NFATc, NFAT2)-deficient mice produce decreased levels of IL-4, whereas mice that lack NFATc2 (NFATp, NFAT1) exhibit increased IL-4 production (8–11). Although T cells lacking both NFATc1 and NFATc2 produce little IL-4, mice doubly deficient in NFATc2 and NFATc3 (NFAT4) produce extremely elevated levels of IL-4, suggesting a negative regulatory role for NFATs in IL-4 production; this may not be directly at

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**Abbreviations used in this paper:* CsA, cyclosporin A; HA, hemagglutinin; HRP, horseradish peroxidase; IRF4, interferon regulatory factor 4; NFAT, nuclear factor of activated T cells; NIP45, NFAT-interacting protein 45.

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the level of transcription of the IL-4 gene (12). Additional proteins controlled by NFAT or that modulate NFAT function may determine the outcome of NFAT-mediated gene expression.

NFAT proteins are known to engage in protein–protein interactions in regulating the expression of target genes. NFATs cooperatively interact with activator protein 1 (AP-1) transcription factors at composite AP-1/NFAT sites within the IL-4 promoter (6). The NFAT-interacting protein 45 (NIP45) physically associates with NFATc2 and enhances NFAT-driven IL-4 promoter activity (13). NFATc2 also functionally synergizes with the Th2-restricted transcription factor c-maf to greatly enhance IL-4 transcriptional activity (14). We hypothesized that interactions between NFAT proteins and additional, hitherto unidentified transcription factors might play a role in IL-4 gene transcription. The presence of putative binding sequences for interferon regulatory factor (IRF) proteins within the proximal IL-4 promoter, some of which overlap with NFAT binding sites, led us to investigate whether members of this family influenced IL-4 transcriptional activity.

IRF4 (PIP, LSIRF, and ICSAT) is a lymphoid- and myeloid-restricted member of the IRF family of transcription factors that binds to interferon sequence response elements (consensus **AA**NN**GAAA**) present within the promoters of IRF-regulated genes like the type 1 interferons (15–18). Although IRF4 has weak affinity for ISREs and represses their function, it is known to participate in gene activation via interactions with different transcription factor partners. One such well-characterized complex is formed by IRF4 and the Ets family member PU.1 on composite elements present within the immunoglobulin light chain (Igκ/λ) gene enhancers (15, 19). IRF4 is stably recruited to these enhancer elements via protein–protein and DNA–protein interactions, and functions synergistically with PU.1 to regulate Ig light chain gene expression in B cells (15, 19, 20). IRF4 also enhances DNA binding by the E2A-encoded gene product E47 and functions synergistically to activate transcription from the Igκ 3′ enhancer (21). Whereas IRF4 has been extensively studied in B cells, comparatively little is known about the function of IRF4 in T cells. IRF4 is clearly critical for lymphocyte function since IRF4-deficient mice exhibit cell intrinsic defects in both B and T cells (22). Significantly, IRF4 is rapidly induced in Th cells after activation via the T cell receptor, suggesting a role for IRF4 in the regulation of gene expression in Th cells (16, 17). However, no interaction partner for IRF4, involved in T cell effector functions, has been identified to date.

We have uncovered a role for IRF4 in the regulation of IL-4 gene expression in conjunction with NFATc2. We demonstrate that IRF4 potently synergizes with NFATc2 to enhance NFATc2-driven activation of the IL-4 promoter and endogenous IL-4 production. This function is dependent on the physical association of IRF4 and NFATc2. Furthermore, naïve Th cells lacking IRF4 are unable to differentiate into IL-4–producing Th2 effector cells in vitro. Thus, we have identified NFATc2 and IRF4 as transcriptional partners that together regulate inducible IL-4 gene expression upon T cell activation.

Materials and Methods

Plasmids and Cell Lines. The M12 B cell lymphoma lines and EL4 thymoma cell lines were maintained in complete medium containing RPMI 1640 supplemented with 10% FCS (HyClone Laboratories), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 mM Hepes, and 50 μM β-ME. 293T cells were maintained in complete DMEM as above. The Flag-NFATc2 construct was obtained from Jun O. Liu (Massachusetts Institute of Technology, Cambridge, MA). The HA-IRF8 and HA-IRF4 expression plasmids and details on the various mutant IRF4 constructs have been published previously (19, 20). c-maf and NIP45 expression plasmids have been described previously (13, 14). The luciferase reporter constructs IL-4-luc, IL-2-luc, and Egr3-luc have been described previously (3, 23, 24). IRF4$^{-/-}$ mice were obtained from Taconic Laboratories, and C57BL6 mice were used as controls.

Transient Transfection Assays and ELISA. M12 cells were transfected using a 280-V electroporator (975 μF; Bio-Rad Laboratories) using 5×10^6 cells in 0.4 ml RPMI per transfection using 2.5 μg reporter plasmid and 5–10 μg expression plasmid or as indicated in the figure legends to Figs. 1 and 2. Each transfection was cultured in 5 ml complete medium, luciferase assays were performed after 24 h, and the luciferase activity in 20% of each was measured as per instructions (Promega). Values are reported as fold increases over the luciferase reporter alone. EL4 cells were transfected as above except using 20% RPMI. 293T cells were transfected with 2 μg of each expression plasmid by Effectene transfection reagent (QIAGEN) as per instructions.

Endogenous IL-4 from the supernatant of transfected M12 cells was assayed 72 h after transfection by ELISA as per instructions (reagents from BD PharMingen).

Coimmunoprecipitation Assays and Immunoblotting. Transfected 293T cells from a 10-cm^2 petri dish were stimulated with 50 ng/ml PMA and 1 μM ionomycin for 2 h. Cells were washed in 1× PBS, and were harvested and lysed in 1 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5% NP-40, 5 mM EDTA, and 50 mM NaCl) with the addition of protease inhibitor cocktail tablets (Roche). After lysis at 4°C, lysates were precleared using A/G beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C and incubated with either agarose-conjugated anti-HA antibody (Santa Cruz Biotechnology, Inc.) or anti-Flag antibody (Sigma-Aldrich) for 1.5 h. The agarose beads were washed five times in cold lysis buffer, and the immunoprecipitates were subjected to SDS-PAGE and transferred onto Nytran membranes. These were probed with horseradish peroxidase (HRP)–conjugated anti-HA (Santa Cruz Biotechnology, Inc.) or anti-Flag (Sigma-Aldrich) antibodies.

Day 5 Th1 or Th2 cultures (from BALB/c mice) were stimulated for 24 h with plate-bound anti-CD3. Cells were harvested and lysed in IP buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.5% NP-40, 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 1 mM sodium vanadate, and 1 mM PMSF plus protease inhibitor cocktail; Roche). Immunoprecipitations were performed using 1 mg of cell extracts. Extracts were precleared with protein G–Sepharose (Amersham Pharmacia Biotech) for 30 min followed by overnight incubation at 4°C with an anti-NFATc2 polyclonal antibody (Upstate Biotechnology) or preimmune serum and protein G. Beads were washed five times with IP buffer and with vortexing. Proteins were eluted in SDS sample buffer, separated on a 4–20% gradient SDS-PAGE (Bio-Rad Laboratories), and

transferred to polyvinylidene difluoride membrane. IRF4 antisera was conjugated to HRP using EZ-Link activated peroxidase antibody labeling kit (Pierce Chemical Co.) as per the manufacturer's instructions, except labeled antibody was isolated using a Microcon 100 filter device (Millipore) as instructed. Blots were probed with a 1:1,000 dilution of HRP-conjugated IRF4 antisera in 1% ovalbumin and developed by chemiluminescence (Amersham Pharmacia Biotech). Blots were reprobed with a monoclonal NFATc2 antibody (Santa Cruz Biotechnology, Inc.) followed by an incubation with a rabbit anti–mouse secondary antibody (Zymed Laboratories).

Cell Culture and Analysis of Cytokine Production. Naïve Th cells were isolated from lymph nodes of wild-type (C57B6) and IRF4$^{-/-}$ mice and sorted to 95–98% purity by FACS (MoFlo; Becton Dickinson) for CD4^{+} CD62L^{high} populations. For Th differentiation assays in vitro, cells were resuspended at 10^6 cells/ml in complete medium containing RPMI 1640 supplemented with 10% FCS (HyClone Laboratories), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 mM Hepes, and 50 μM β-ME at 37°C in 5% CO_2 and stimulated with plate-bound anti-CD3 (1 μg/ml) plus anti-CD28 (2 μg/ml). Cells were split 1:4 with fresh medium plus 200 U/ml IL-2 on day 3 after primary stimulation and then rested. On day 7, cells were washed, counted, and restimulated with 1 μg/ml anti-CD3 at 10^6 cells/ml in complete medium. For Th1-skewing, 2 ng/ml IL-12 and 10 μg/ml anti–IL-4 were added to the primary culture. For Th2-skewing, 10 ng/ml IL-4 and 10 μg/ml anti–IFN-γ and 10 μg/ml anti–IL-12 were included. Cell-free supernatants were analyzed for cytokine production by ELISA 24 h after secondary stimulation or by assessing intracellular cytokine production by FACS analysis as per instructions (BD PharMingen).

Results

IRF4 Synergizes with NFATc2 to Specifically Enhance NFAT-mediated Activation of the IL-4 Promoter. We tested the ability of IRF4 to transactivate the IL-4 promoter, either on its own or in conjunction with NFATc2. M12 B lymphoma cells and EL4 thymoma cells were each transfected with an IL-4 luciferase reporter construct (IL-4-luc; Fig. 1, a and b, respectively). Cotransfection with NFATc2 increased the luciferase activity of IL-4-luc by 10–15-fold compared with that of the vector control. IRF4 by itself did not significantly increase IL-4 promoter activity in either cell type, consistent with its known weak DNA-binding properties. Interestingly, however, IRF4 was able to synergize with NFATc2 to substantially augment the ability of NFATc2 to transactivate the IL-4-luc reporter by ~100-fold (Fig. 1, a and b).

We next sought to determine whether IRF4 functions to enhance NFAT-mediated activation of the promoters of other genes for which NFAT proteins are known to be critical regulators. The IL-2 proximal promoter contains well-characterized NFAT-binding sites and NFATc2 has been shown to transactivate the IL-2 promoter in vitro (1). The luciferase activity of an IL-2 luciferase reporter construct (IL-2-luc) transfected into M12 cells was increased in the presence of NFATc2 but was not affected by IRF4 (Fig. 1 c). In addition, cotransfection of both IRF4 and NFATc2 did not further enhance IL-2-luc activity (Fig. 1 c). The immediate early gene, Egr3, is regulated by NFATc2 and its promoter contains DNA sequences that are bound by NFAT proteins (23, 25). The activity of the Egr3 promoter luciferase construct (Egr3-luc) was increased by overexpression of NFATc2, however, IRF4 did not modulate this transactivation (Fig. 1 d). Thus, the transcriptional synergy between IRF4 and NFATc2 is not generalizable to other NFAT-regulated genes such as IL-2 and Egr3 but has particular relevance to IL-4 gene transcription. Furthermore, this is not due to the binding of IRF4 to NFAT recognition sites within the IL-4 promoter since IRF4 did not transactivate a luciferase reporter construct containing three tandem NFAT consensus sequences, nor did it increase NFAT-driven transactivation of the reporter (Fig. 1 e).

IRF4 Physically Associates with NFATc2. Functional and Physical Interaction between NFATc2 and IRF4 Does Not Extend to IRF8. To assess whether the functional interaction between IRF4 and NFATc2 was the consequence of a physical association between the two proteins, we performed coimmunoprecipitation assays using IRF4 fused to the hemagglutinin (HA) epitope (HA-IRF4) and Flag-tagged NFATc2 (Flag-NFATc2). The two proteins were ectopically coexpressed in 293T cells and total protein lysates prepared after stimulation with PMA and ionomycin. Immunoprecipitation of HA-IRF4 using anti-HA antibody resulted in the coimmunoprecipitation of Flag-NFATc2 as detected by immunoblotting with anti-Flag antibodies (Fig. 2 a). The association of IRF4 and NFATc2 was corroborated by the reverse coimmunoprecipitation experiment using anti-Flag antibody to immunoprecipitate NFATc2 and anti-HA to detect the associated HA-IRF4 by Western blot (Fig. 2 b). Thus, the potent enhancement of IL-4 promoter activity seen in T and B cell lines is reflected by the protein–protein interaction between IRF4 and NFATc2 within cells.

To establish the physiological significance of this interaction, we performed coimmunoprecipitation assays using primary CD4^{+} Th cells. Protein levels of IRF4 were determined to be induced strongly within 24 h after restimulation of differentiated Th1- or Th2-cytokine producing cells in vitro (unpublished data). Therefore, we chose this time point to assess endogenous association of NFATc2 and IRF4 within primary Th1 and Th2 subsets (see Materials and Methods). Precleared Th1 and Th2 cell lysates were subjected to immunoprecipitation using control preimmune sera (Fig. 2 c, lane 1) or anti-NFATc2 antibody (Fig. 2 c, lanes 2 and 3) and immunoblotted using HRP-conjugated IRF4 antisera to detect the associated IRF4 protein (Fig. 2 c). Blots were reprobed with anti-NFATc2 antibody to detect the levels of NFATc2 present in the immunoprecipitates. Thus endogenous NFATc2 and IRF4 proteins associate with each other within primary Th cells.

To further study the specificity of the IRF4–NFATc2 interaction, we asked whether IRF8 (ICSBP), the IRF family member with the greatest homology to IRF4, would associate with NFATc2. Interestingly, IRF8 does not interact with NFATc2 under identical conditions (Fig.

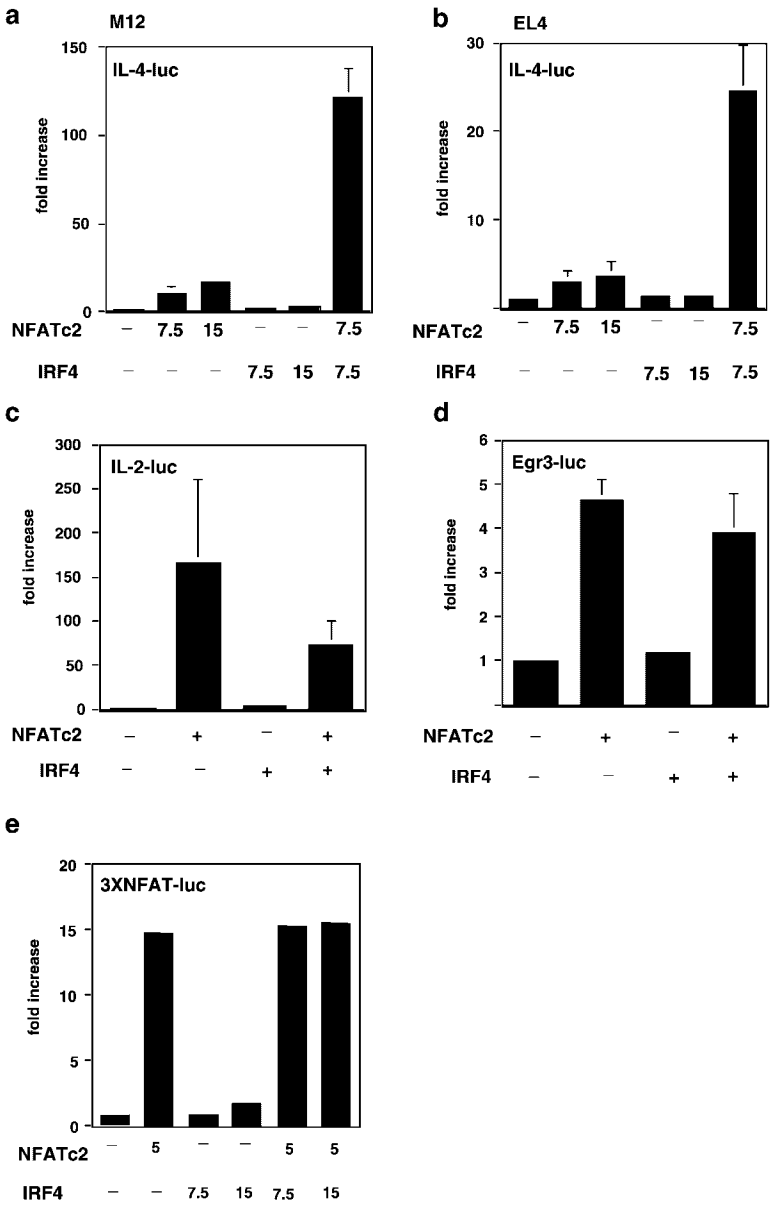

Figure 1. IRF4 synergizes with NFATc2 to enhance NFATc2-driven IL-4 transcriptional activity but does not regulate the IL-2 and Egr3 promoters. M12 B lymphoma cells (a) and EL4 thymoma cells (b) were transiently transfected with the IL-4 luciferase reporter and cotransfected with the indicated microgram amounts of IRF4 and/or NFATc2 expression plasmids. M12 cells were transiently transfected with (c) IL-2 luciferase reporter or (d) Egr3 luciferase reporter and cotransfected with control vectors (15 μg), NFATc2 (15 μg) or IRF4 plasmids (15 μg) individually or together (7.5 μg each). (e) M12 cells transfected with the 3×NFAT luciferase reporter and the indicated microgram amounts of IRF4 and/or NFATc2. All results represent the mean of three to five independent experiments. Luciferase activity is reported as a fold increase relative to reporter alone ± SEM.

2 d). We went on to ask whether IRF8 was capable of synergizing with NFATc2 to activate IL-4 transcriptional activity. Ectopic expression of IRF8 alone, as well as in combination with NFATc2, failed to modulate NFAT-driven induction of IL-4-luc promoter activity in M12 cell lines (Fig. 2 e). Thus, the functional synergy between IRF4 and NFATc2, leading to an enhanced IL-4 transcriptional activity, is not applicable to the closely related IRF protein IRF8. This is consistent with the inability of IRF8 to physically interact with NFATc2.

Domains within IRF4 Required for Interaction and Transcriptional Synergy with NFATc2. To identify the regions within the IRF4 protein that are important for its interaction with NFATc2, we performed coimmunoprecipitation experiments using a series of HA-tagged mutant IRF4 constructs (Fig. 3 a) each coexpressed in 293T cells in conjunction with Flag-NFATc2. Compared with the full-length IRF4 (1–450), COOH-terminal deletion mutants 1–439 and 1–419 exhibited reduced interactions with NFATc2, whereas deleting 40 amino acids in the COOH terminus of IRF4 (1–410) completely abolished interaction with NFATc2. Amino acid residues 399–413 within this COOH-terminal region of IRF4 were predicted to form an α-helix that was shown to be critical for its interaction with PU.1 (20). Full-length IRF4 with the putative α-helix deleted (Δ H) also was unable to interact with NFATc2, indicating the importance of the α-helix in IRF4-NFATc2 protein–protein interactions. In addition, regions of IRF4 between amino acids 150 and 340 of the protein likely contribute to the association of IRF4 and NFATc2 as seen by the weak associations between NFATc2 and each of the internal deletion mutants of

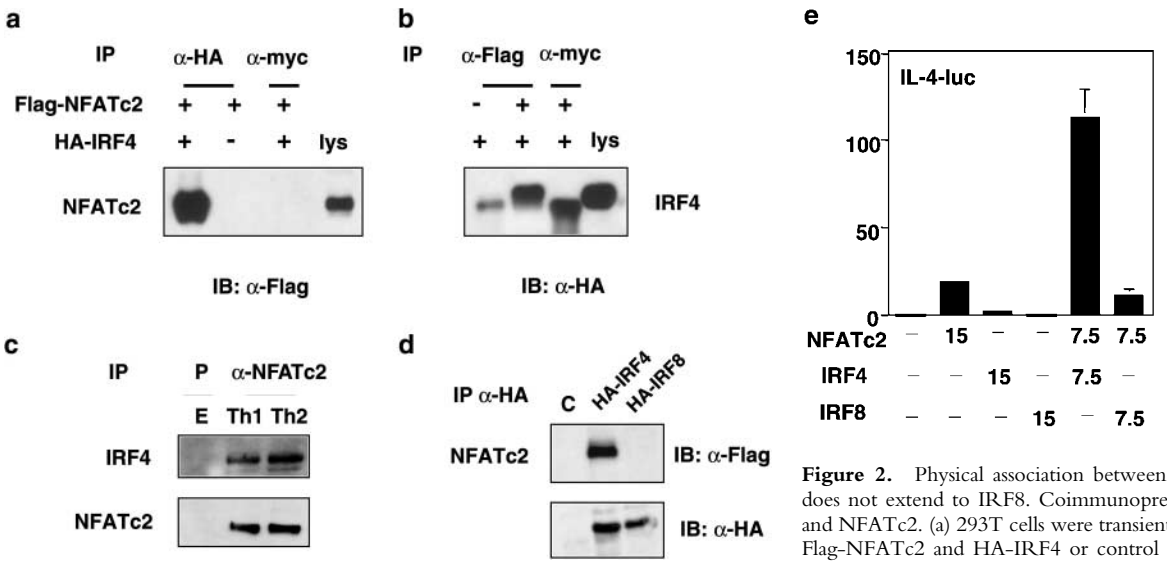

Figure 2. Physical association between NFAT and IRF4 does not extend to IRF8. Coimmunoprecipitation of IRF4 and NFATc2. (a) 293T cells were transiently transfected with Flag-NFATc2 and HA-IRF4 or control vector and immunoprecipitated using anti-HA or control anti-myc antibodies. Proteins were run on SDS-PAGE and immunoblotted with anti-Flag antibody to detect Flag-NFATc2 in the immunoprecipitate and lysates (lys) (b) 293T cells were transiently transfected with HA-IRF4 and Flag-NFATc2 or control vector. Immunoprecipitation was performed using anti-Flag or control anti-myc antibodies and immunoblotted with anti-HA antibody to detect HA-IRF4 in the immunoprecipitate or lysate (lys). (c) Extracts from Th1 or Th2 cells were immunoprecipitated with preimmune serum (P) or anti-NFATc2 antibody and probed with anti-IRF4-HRP or reprobed with anti-NFATc2. (d) M12 cells were transiently transfected with Flag-NFATc2 and HA-IRF4 or HA-IRF8. Immunoprecipitation with anti-HA antibody and immunoblotting with anti-Flag antibody to detect immunoprecipitate. Anti-HA antibody was used to immunoblot input lysates to detect IRF4 and IRF8. (e) Comparison of IRF4 and IRF8 transcriptional synergy with NFATc2 in M12 cells. Results represent the mean of three to five independent experiments. Luciferase activity is reported as a fold increase relative to reporter alone ±SEM. Control plasmid DNA was used to normalize for DNA concentration.

IRF4, Δ 200–260, Δ 260–340, Δ 150–260, Δ 150–340 (Fig. 3 a).

We next sought to assess whether the domains within IRF4 that were important for its interaction with NFATc2 also were critical for its transcriptional synergy with NFATc2 on the IL-4 promoter. Various IRF4 mutants were transiently transfected into M12 cells along with NFATc2 and their effect on IL-4 promoter activity assessed (Fig. 3 b). Consistent with its essential role in interacting with NFATc2, the COOH-terminal region of IRF4 also is important for transcriptional synergy. However, residues 419–439 may play an inhibitory role in transcriptional activation of the IL-4 promoter since the mutant 1–419 exhibited greater transcriptional activity in conjunction with NFATc2 compared with that of IRF4 mutants 1–439 and 1–410 (Fig. 3 b). The putative interaction helix (399–413) is critical for the ability of IRF4 to synergize with NFATc2 since deleting the α-helix (Δ H) severely abrogated IL-4 promoter activity. In addition, amino acid residues between 150 and 340 also appear to be important for transcriptional synergy, as evidenced by the inability of internal deletion mutants Δ 200–260, Δ 260–340, Δ 150–260, and Δ 150–340 to synergize with NFATc2 and transactivate the IL-4 promoter (Fig. 3 b). Thus, the COOH-terminal region and the amino acids 150–340 of the IRF4 protein are important for transcriptional synergy as well its interaction with NFATc2.

IRF4 Synergizes with c-maf and NFATc2 to Enhance IL-4 Production. We wished to examine the effect of IRF4 and NFATc2 on endogenous IL-4 gene expression. Therefore, we attempted to reconstitute IL-4 gene expression in an established M12 B cell system. Although M12 cells do not normally express IL-4, we have demonstrated previously that coexpression of the Th2-specific transcription factor, c-maf, NFATc2, and the NFAT-interacting protein, NIP45 leads to the induction of endogenous IL-4 production in M12 cells (13). Our data suggested that IRF4 contributes to IL-4 induction through its interaction with NFATc2, and so we sought to examine whether IRF4 would function in combination with NFATc2 and c-maf to modulate endogenous IL-4 production in this system.

Consistent with previously reported results, c-maf by itself induced IL-4 promoter activity in M12 B cells by ~100-fold over baseline controls (Fig. 4 a). c-maf and NFATc2 together augmented IL-4 promoter activity and also induced detectable levels (100 pg/ml) of endogenous IL-4 in M12 B cells (Fig. 4, a and b). Coexpression of IRF4 and NFATc2 increased IL-4 activity by >300-fold compared with controls. Interestingly, IRF4 potently synergized with both NFATc2 and c-maf to greatly enhance IL-4 transcriptional activity by ~900-fold above baseline (Fig. 4 a). Importantly, this synergy at the IL-4 promoter was accompanied by the induction of substantial levels (700 pg/ml) of endogenous IL-4 production in M12 B cells (Fig. 4 b).

Compromised Th2 Differentiation in IRF4$^{-/-}$ Naïve T Helper Cells. It has been shown previously that IRF4$^{-/-}$ T cells display reduced proliferation and overall cytokine production in response to stimulation compared with wild-type cells. To ascertain whether IL-4 expression requires

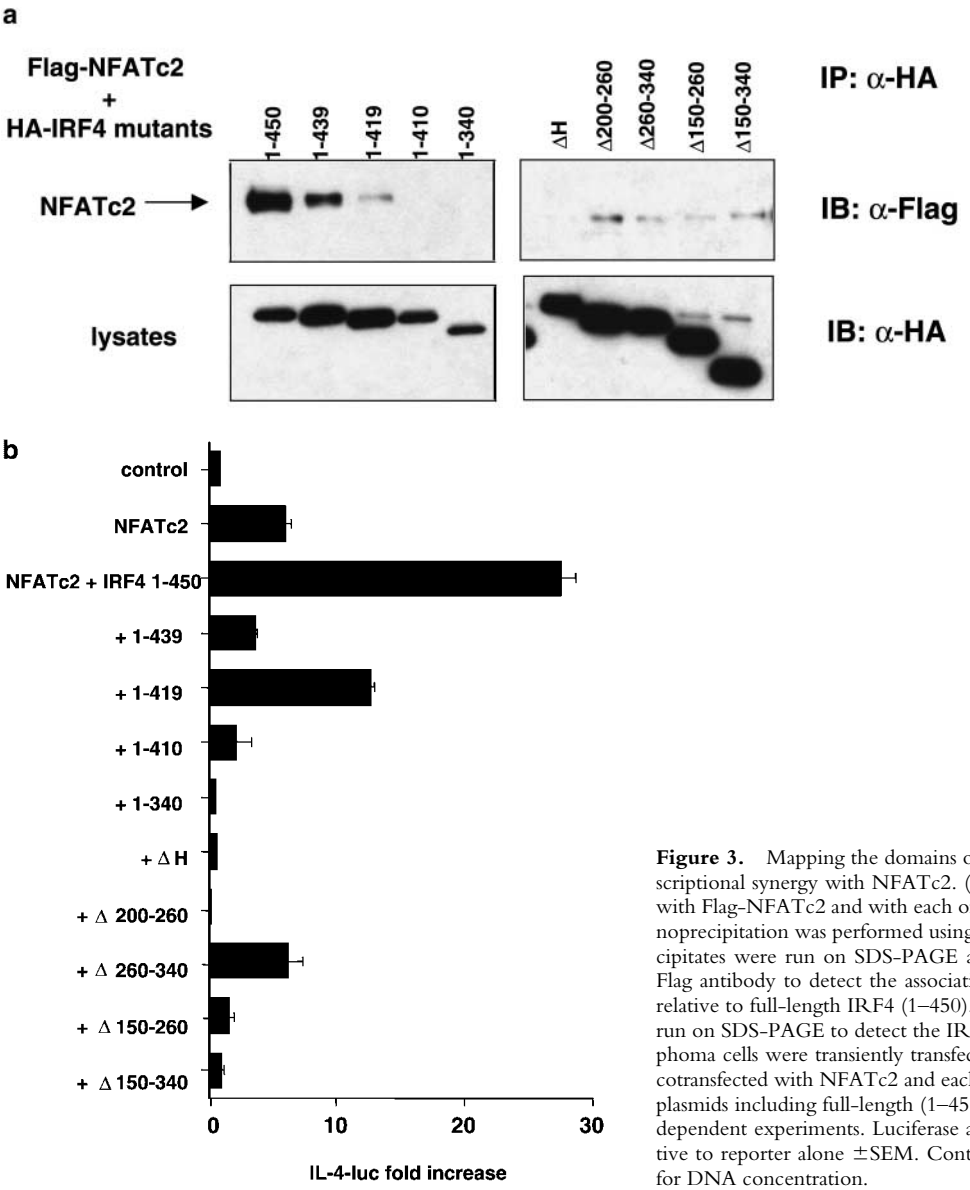

Figure 3. Mapping the domains of IRF4 required for interaction and transcriptional synergy with NFATc2. (a) 293T cells were transiently transfected with Flag-NFATc2 and with each of the different HA-IRF4 mutants. Immunoprecipitation was performed using anti-HA antibody, and the immunoprecipitates were run on SDS-PAGE and detected by immunoblot using anti-Flag antibody to detect the association of each IRF4 mutant with NFATc2 relative to full-length IRF4 (1–450). Lysates from each transfection also were run on SDS-PAGE to detect the IRF4 proteins in the input. (b) M12 B lymphoma cells were transiently transfected with the IL-4 luciferase reporter and cotransfected with NFATc2 and each of the different IRF4 mutant expression plasmids including full-length (1–450). Results represent the mean of five independent experiments. Luciferase activity is reported as a fold increase relative to reporter alone ±SEM. Control plasmid DNA was used to normalize for DNA concentration.

the presence of IRF4, we assessed the ability of naïve T helper precursors (Thp) from IRF4$^{-/-}$ mice to differentiate into cytokine-producing Th1 and Th2 effector subsets in vitro. Naïve Thp were isolated from wild-type or IRF4$^{-/-}$ lymph nodes and spleens by FACS (see Materials and Methods) and stimulated with anti-CD3 and anti-CD28 under unskewed conditions (U) and conditions that would skew them to differentiate into Th1- or Th2-polarized cells. On day 7 after primary stimulation, the percentage of U, Th1, and Th2 cells that secreted IL-4 and IFN-γ were determined by intracellular cytokine staining (Fig. 5 a). Under unskewed conditions, both wild-type and IRF4$^{-/-}$ cells produced little IL-4, whereas 51% of IRF4$^{-/-}$ cells secreted IFN-γ compared with 30% in the wild type. Under Th1 conditions, both genotypes showed increased IFN-γ, although the percent increase in cells secreting IFN-γ relative to unskewed cultures was greater for wild type (30–80%) compared with IRF4$^{-/-}$ (51–69%; Fig. 5 a). Importantly, whereas wild-type cells were able to differentiate into Th2 cells secreting IL-4 (31%) and negligible IFN-γ, IRF4$^{-/-}$-deficient cells were unable to develop into the Th2 subset even when provided with exogenous IL-4, as evidenced by the very small number (4%) of cells producing IL-4 (Fig. 5 a).

To more closely analyze the inability of IRF4$^{-/-}$ Th cells to develop into the Th2 subset, cells from each genotype and condition also were restimulated with anti-CD3 and the supernatants were analyzed for Th1- (IL-2 and IFN-γ) and Th2 (IL-4, IL-5, and IL-13)-cytokine production (Fig. 5 b). The levels of IFN-γ produced by wild-type and IRF4$^{-/-}$ Th cells under U, Th1, and Th2 conditions paralleled that of the results from intracellular cytokine staining (Fig. 5 b). IL-2 production by IRF4-deficient cells was somewhat diminished relative to wild type under all

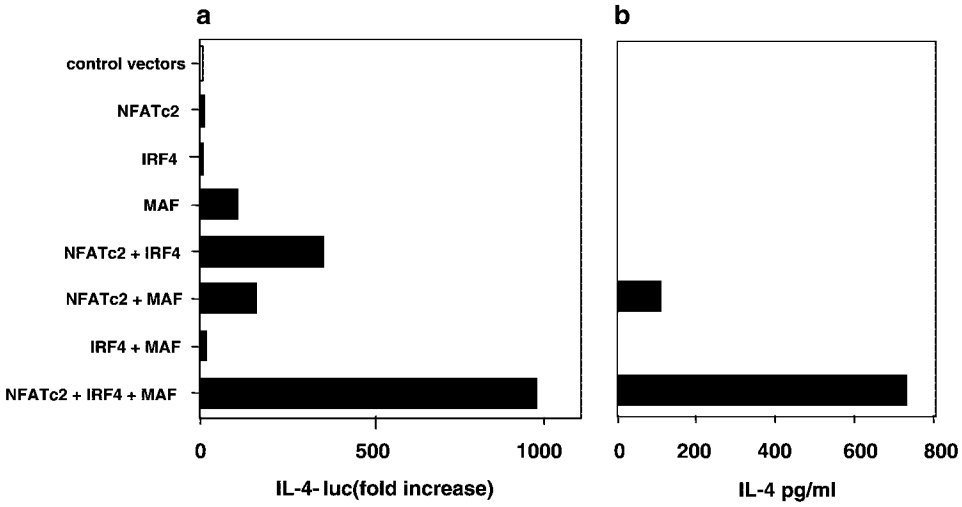

Figure 4. IRF4 synergizes with NFATc2 and c-maf to enhance IL-4 transcriptional activity and endogenous IL-4 production. M12 cells were transiently transfected with the indicated expression plasmids along with IL-4 luciferase reporter. (a) After transfection, half of the transfected cells were cultured for 24 h and assayed for luciferase activity. (b) Cells from the other half were cultured for 72 h, and the supernatants were assayed for IL-4. Results are representatives of five independent experiments. Luciferase activity is reported as a fold increase relative to reporter alone ±SEM. IL-4 was quantified by ELISA.

three conditions. Strikingly, naïve Thp from IRF4$^{-/-}$ mice were severely deficient in producing IL-4, IL-5, and IL-13 under Th2-differentiating conditions that included exogenous IL-4 (Fig. 5 b). Thus, naïve Thp from IRF4$^{-/-}$ mice are unable to differentiate into Th2 cells, but retain the ability to secrete substantial levels of IFN-γ and IL-2.

Discussion

We have identified IRF4, an immune system–restricted member of the IRF family of transcription factors, as a partner for NFATc2. Whereas IRF4 is known to activate the transcription of genes expressed in B cells via its interaction with transcription factor protein partners, no such partner has been described in T cells until this report. The striking ability of IRF4 to enhance NFAT-driven IL-4 transcriptional activity (Fig. 1, a and b) is consistent with the known function of IRF4 in transcriptional synergy. The functional synergy between NFATc2 and IRF4 is not generalizable to other NFAT-regulated promoters of genes such as IL-2 and Egr3 (Fig. 1, c and d). The role of IRF4 in IL-4 production is underscored by the inability of IRF4-deficient Th cells to differentiate into Th2 cells. Thus, IRF4 constitutes a new protein partner with which NFATc2 interacts to regulate context-specific gene transcription. Interestingly, the closely related family member, IRF8, neither synergizes nor interacts with NFATc2, conferring some specificity to the NFATc2-IRF4 association (Fig. 2, c and d).

What Might Be the Reasons for Differential Involvement of IRF4 and IRF8 with NFATc2? Although IRF4 and IRF8 show significant homology (80% identity) in their NH_2-terminal domains, particularly in the conserved IRF DNA binding domains, they are only 48% homologous over a 160–amino acid region of the IRF4 protein (254–413). Thus, the interaction surfaces required for protein–protein contact with NFATc2 may not exist in IRF8. Unlike IRF4, IRF8 does not appear to possess an activation domain (19). Therefore, while it complexes with PU.1 on λB DNA, it does not augment transcription of the λB reporter (19). Moreover, IRF8 is important in regulating IL-12 expression in macrophages and, thus, may not be involved in IL-4 gene regulation (26, 27).

The NFATc2-IRF4 Interaction. The interaction between IRF4 and PU.1 has been well characterized at the molecular level. Many of the functional correlates of IRF4 protein structure have been elucidated with respect to the ternary complex formed between IRF4, PU.1, and DNA. The IRF4 protein (full-length 1–450) possesses an NH_2-terminal (1–134) DNA binding domain with high sequence homology to other IRF proteins, followed by a region termed the activation domain that includes a proline-rich segment. The COOH-terminal contains the regulatory domain (15, 19). Residues 150–340 are important for ternary complex formation. Amino acids 410–439 are necessary for complex formation as well autoinhibition of IRF4 binding to DNA (19). A predicted α-helical structure (399–413) was shown to be critical for interaction as well as transcriptional synergy with PU.1 (20). The interaction of IRF4 with NFATc2 shows some parallels but also some differences compared with that of PU.1. Like PU.1, the COOH-terminal residues 410–450, particularly amino acids 410–439, appear to be required for interaction with NFATc2 and transcriptional synergy (Fig. 3, a and b). In addition, deleting residues 399–413 (Δ H), which comprise the putative α-helix, eliminates both interaction of IRF4 with NFATc2 and synergistic activation of the IL-4 promoter. Residues 150–340 also appear to be important for association and synergy with NFATc2 (Fig. 3, a and b).

The interaction of PU.1 and IRF4 has been shown to be dependent on DNA for the formation of a stable ternary complex (20). Our results indicate that DNA was not necessary for NFATc2 and IRF4 to interact as we were able to coimmunoprecipitate NFATc2 and IRF4 when transiently coexpressed in cell lines (Fig. 3, a and b), as well as within primary Th1 and Th2 cells. Furthermore, using recombinant or in vitro-translated NFATc2 and IRF4 proteins in EMSAs with regions of the IL-4 promoter containing

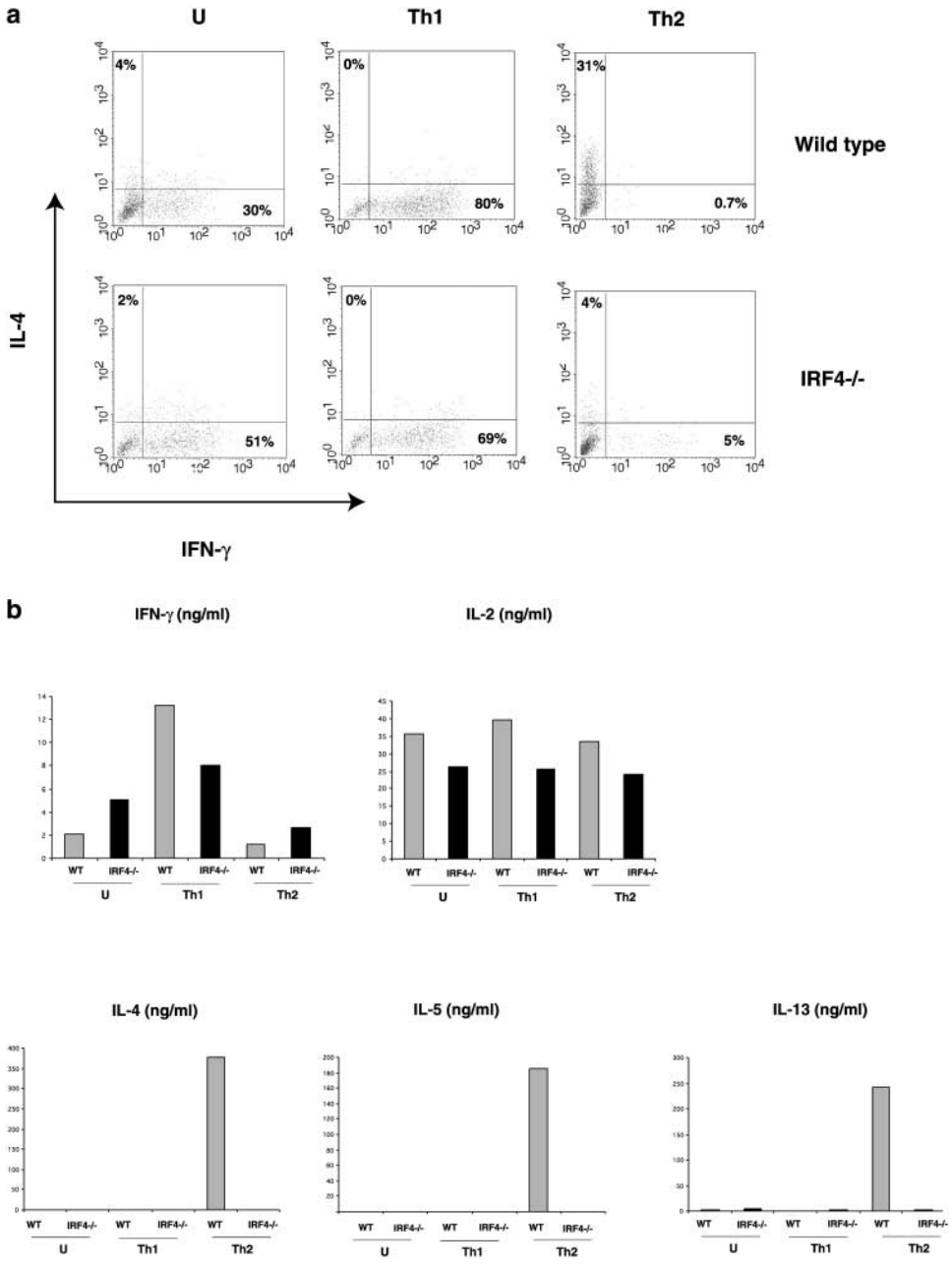

Figure 5. Compromised Th2 differentiation in IRF4$^{-/-}$ naïve Th cells. Naïve Thp were isolated from wild-type and IRF4$^{-/-}$ mice and differentiated in vitro under unskewed (U) Th1 or Th2 conditions (see Materials and Methods). (a) Intracellular cytokine staining for IL-4 and IFN-γ production on day 7 upon stimulation with PMA and ionomycin. Percentage of cells that secrete each cytokine is indicated. (b) On day 7, U, Th1, and Th2 cells from wild-type (WT) and IRF4$^{-/-}$ mice were stimulated with anti-CD3, and supernatants were analyzed for cytokine production 24 h later.

NFAT sites and putative IRF recognition sequences, we were unable to detect enhanced binding of NFATc2 to DNA, nor the formation of a ternary complex (unpublished data). Notably, the interaction between IRF4 and E2A also does not manifest in a ternary complex, although IRF4 augments the binding of E2A to DNA. We suggest two possible ways in which NFATc2 and IRF4 may associate with one another. The first invokes the formation of a ternary complex at the IL-4 promoter. IRF4 may cobind with NFAT to a novel composite element. The functional synergy between IRF4 and NFATc2 displays some specificity for the IL-4 promoter (Fig. 1, c and d) and the consensus NFAT site alone (Fig. 1 e) is not sufficient for IRF4 to enhance NFATc2-mediated induction of IL-4 promoter activity. These data suggest that an IRF4–NFATc2 complex may form but dissociate under conditions used for gel electrophoresis. IRF4 is known to possess very weak affinity for DNA and, thus, the in vitro conditions of the EMSAs may not reconstitute the in vivo conditions in which IRF4 may be able to bind DNA in conjunction with NFATc2. Moreover, additional proteins may be required for IRF4 and NFATc2 to associate on DNA. Alternatively, IRF4 may be tethered to the IL-4 promoter through protein–protein contacts with NFATc2. Additional proteins, specific to the IL-4 promoter, may be necessary to maintain this association. Thus, such interactions would confer specificity to the ability of IRF4 to modulate NFAT-driven gene expression. The Th2-specific protein c-maf, with

which IRF4 potently synergizes in conjunction with NFATc2 to induce endogenous IL-4 production (Fig. 4, a and b), may play a role in facilitating the interactions proposed in this model. The critical function for IRF4 in Th2-cytokine production that we have demonstrated in IRF4-deficient mice supports a role for IRF4 in modulating NFAT-dependent pathways via protein–protein interaction with NFATc2 in vivo.

IRF4 and T Helper Cell Function. The specific interaction between NFATc2 and IRF4 within primary Th1 and Th2 cells (Fig. 2 c) suggests that the association of these two proteins within Th cells plays a role in regulating Th cell function. Previous studies indicated that IRF4-deficient T cells showed reduced proliferation and cytokine production (22). In this report, we specifically examined naïve Th precursor cells from IRF4$^{-/-}$ mice and observed a slight reduction in IFN-γ and IL-2 production, which may reflect decreased proliferative capacity relative to wild type. Importantly, IRF4$^{-/-}$ mice are compromised severely for the production of IL-4, IL-5, and IL-13 and are unable to differentiate into Th2 cells (Fig. 5). This profound reduction in Th2 cytokine production, even with the provision of exogenous IL-4, cannot be fully explained by the modest reduction in IL-2 production (Fig. 5 b). Thus, these data imply that the presence of IRF4 is necessary for IL-4 production and for generating the Th2 compartment. What might be the mechanism for the regulation of IL-4 by IRF4? IRF4 mRNA is expressed equally in Th1- and Th2-polarized subsets (unpublished data) and IRF4–NFATc2 protein complexes were equally present in both subsets (Fig. 2 c). However, the specific protein complexes in which NFATc2 and IRF4 participate may be different in the Th1 versus the Th2 context. There is evidence that the chromatin conformation at the IL-4 locus is different between Th1 and Th2 cells (28). Moreover, both NFATc2 and the Th2-specific transcription factor GATA3 have been shown to bind to a CsA-sensitive enhancer region at the IL-4 gene locus in Th2, but not in Th1, cells (7). IRF4 may be a component of such a Th2-specific protein complex and serve to enhance the function of NFATc2 via its physical interaction with NFATc2 at the IL-4 locus. Indeed, the production of other Th2 cytokines such as IL-5 and IL-13 also was severely abrogated in IRF4$^{-/-}$ mice. Genes encoding IL-4, IL-5 and IL-13 are clustered in the genome, suggesting their coordinate regulation. Thus, IRF4 may influence the expression of the IL-4/IL-5/IL-13 locus through protein–protein contact with NFATc2.

The interplay between NFATc2 and IRF4 may extend to additional aspects of NFAT function in T cells. We have observed, similar to recently published results, that the induction of IRF4 mRNA is CsA-sensitive (unpublished data; 29). We have evidence that IRF4 may be induced, in part, by NFATc2 since Th cells from mice doubly deficient in NFATc2 and NFATc3 exhibit reduced IRF4 mRNA induction (unpublished data). Thus, it is intriguing to consider that NFATs may induce as well as interact with IRF4 in regulating target gene expression.

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