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Down-Regulation of IL-4 Gene Transcription and Control of Th2 Cell Differentiation by a Mechanism Involving NFAT1

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Summary

Transcription factors of the NFAT family play a critical role in the immune response by activating the expression of cytokines and other inducible genes in antigen-stimulated cells. Here we show that a member of this family, NFAT1, is involved in down-regulating the late phase of IL-4 gene transcription, thus inhibiting T helper 2 responses. Whereas stimulated T cells from wild-type mice show a transient increase and then a rapid decline in the steady-state levels of IL-4 mRNA in vitro, the levels of IL-4 gene transcripts in NFAT1-deficient T cells are maintained at high levels under the same conditions. Consistent with this observation, NFAT1$^{-/-}$ mice are more susceptible to infection with Leishmania major. This report provides evidence that NFAT proteins regulate not only the initiation but also the termination of gene transcription.

Introduction

In response to antigen stimulation, naive T helper cells differentiate into at least two types of effector cells, characterized by their distinct patterns of cytokine expression as well as their disparate effects on the ongoing immune response (Mosmann and Coffman, 1989; Paul and Seder, 1994; Abbas et al., 1996). The hallmark of T helper 1 (Th1) cells is the secretion of interferon-γ (IFNγ), which promotes a predominantly cell-mediated immunity, whereas Th2 cells produce interleukin-4 (IL-4), IL-5, IL-10, and IL-13 and activate immunoglobulin E (IgE) and eosinophilic responses. The initial description of this dichotomy of T helper cell differentiation was quickly followed by the recognition of its fundamental pathophysiological effect on many clinical syndromes such as infection, autoimmune disease, and allergy (Abbas et al., 1996; Romagnani, 1997).

Several factors have been shown to influence the phenotype that a T cell acquires following its activation. In addition to the dose and route of administration of the activating antigen and the engagement of costimulatory molecules, the local cytokine environment is of the utmost importance, since IL-12 and IFNγ favor Th1 development whereas IL-4 promotes the generation of Th2 cells (Paul and Seder, 1994; Abbas et al., 1996; Constant and Bottomly, 1997). Concomitantly, the transcription factors STAT4 and STAT6 (signal transducers and activators of transcription–4 and –6), which are selectively activated by the polarizing cytokines IL-12 and IL-4 (Hou et al., 1994; Jacobson et al., 1995), are critical for the differentiation of Th1 and Th2 cells, respectively (Kaplan et al., 1996a, 1996b; Takeda et al., 1996). Th1/Th2 development is also markedly dependent on genetic background: upon stimulation, T cells from BALB/c mice tend to differentiate into Th2 cells, rendering the mice incapable of controlling infection with the intracellular pathogen Leishmania major, while T cells from C57BL/6, 129/SvJ, and B10.D2 mice tend to differentiate into Th1 cells, thus enabling these mice to control the disease (Hsieh et al., 1995; Reiner and Locksley, 1995).

A region on chromosome 11 containing a cluster of genes including those encoding granulocyte-macrophage colony-stimulating factor, IL-3, IL-4, IL-5, and IL-13 has been identified as a candidate locus for the genetic control of Th1/Th2 development (Gorham et al., 1996); this region is syntenic with a locus on human chromosome 5, which had previously been linked to elevated serum IgE levels in atopic individuals (Marsh et al., 1994). IL-4 itself, because of its strong ability to drive T helper cell differentiation along the Th2 pathway, is considered a candidate regulatory gene in this locus (Casolaro et al., 1996). However, other genes, directly or through control of that locus, also might regulate Th1/Th2 development, as demonstrated by the recent finding that Bcl-6–deficient mice show increased expression of Th2 cytokines, possibly through dysregulation of STAT-responsive genes (Dent et al., 1997).

Activation of T lymphocytes in response to antigen stimulation requires the synthesis and activation of multiple transcription factors, which determine the subsequent program of gene expression within the cell (Hill and Treisman, 1995). Two transcription factors, c-Maf and GATA-3, are reported to be selectively expressed in Th2 but not Th1 cells (Ho et al., 1996; Zheng and Flavell, 1997). When exogenously expressed, both c-Maf and GATA-3 independently enable the production of Th2 cytokines in Th1 or B cells, suggesting a specific role for these factors in regulating Th2 cytokine synthesis. In contrast, nuclear factors of activated T cells (NFAT) are present in both Th1 and Th2 cells (Rooney et al., 1994; Rooney et al., 1995) and activate the transcription of cytokines characteristic for both cell types (Rooney et al., 1995; Rao et al., 1997). Furthermore, NFAT transcriptional activity is similarly regulated during Th1/Th2 development in NFAT reporter–transgenic mice (Rincón and Flavell, 1997). In the same system, however, NFAT transcriptional activity is enhanced in differentiated Th2 compared to Th1 cells, perhaps because these effector subsets differ in their expression of Fos/Jun family members, which cooperate with NFAT proteins (Rincón et al., 1997b).

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Numerous studies have indicated that NFAT proteins are indispensable activators of several cytokine genes (reviewed by Rao et al., 1997), a concept supported by the finding that patients who are unable to generate functional NFAT complexes have severely impaired cytokine synthesis (Castigli et al., 1993; Feske et al., 1996). It was therefore unexpected that mice lacking the transcription factor NFAT1 (NFATp), the first family member to be isolated and molecularly cloned, display not a compromised but rather an enhanced immune response in various experimental systems (Hodge et al., 1996b; Xanthoudakis et al., 1996). The four different NFAT proteins identified to date show overlapping patterns of tissue expression (Hoey et al., 1995; Lyakh et al., 1996; Rao et al., 1997), suggesting that other NFAT family members might compensate for the lack of NFAT1 in maintaining the immunocompetent status of NFAT1-deficient mice. Conversely, the enhancement of secondary immune responses (proliferation and eosinophil recruitment) observed in NFAT1-deficient mice (Hodge et al., 1996b; Xanthoudakis et al., 1996) indicates that this NFAT family member might selectively activate genes the products of which exert an overall inhibitory effect on the immune response.

Here we have further analyzed the immune responses of NFAT1$^{-/-}$ mice. We show that NFAT1-deficient and wild-type T cells differ strikingly in the kinetics of IL-4 gene transcription. T cells expressing or lacking NFAT1 are identical in the early kinetics of IL-4 gene transcription and in the peak steady-state levels of IL-4 gene transcripts; however, IL-4 gene transcription is rapidly extinguished in NFAT1$^{+/+}$ cells but maintained at high levels in NFAT1$^{-/-}$ cells. The maintained production of IL-4 in NFAT1$^{-/-}$ cells drives their differentiation into Th2 effector cells, producing high levels of the type 2 cytokines IL-4, IL-5, and IL-13. Concomitantly, NFAT1-deficient mice show increased susceptibility to infection with L. major. We conclude that within the NFAT family, NFAT1 plays a unique role in regulating T helper cell differentiation by selectively modulating the late phase of IL-4 gene transcription during the immune response.

Results

NFAT1-Deficient T Cells Preferentially Differentiate into Type 2 Effector Cells In Vitro

We determined the patterns of cytokine mRNA expression in NFAT1$^{+/+}$ and NFAT1$^{-/-}$ T cells differentiated and restimulated with anti-CD3 in vitro. Spleen and lymph node cells from NFAT1-deficient or wild-type mice were cultured for 4 days in the presence of immobilized anti-CD3, rested for 2 days, and then restimulated for 6 hr with anti-CD3. Total RNA from these cells was simultaneously analyzed for the presence of multiple cytokine transcripts using a quantitative RNase protection assay. Under default conditions, with no cytokines or antibodies other than anti-CD3 added during the primary stimulation, NFAT1$^{-/-}$ cells showed a marked increase, relative to NFAT1$^{+/+}$ cells, in the levels of mRNAs encoding the type 2 cytokines IL-4, IL-5, IL-10, and IL-13 (Figure 1A, left). Although the effect was less pronounced in other experiments, the steady-state levels of IL-2 transcripts were also enhanced in restimulated NFAT1$^{-/-}$ compared to NFAT1$^{+/+}$ cells. Both cell populations expressed very high levels of transcripts encoding the type 1 cytokine IFNγ, causing saturation of the probe in our assay (Figure 1A). We addressed this problem by reducing the input RNA amounts of the same samples by 20-fold; under these conditions, IFNγ transcript levels in NFAT1$^{-/-}$ cells were at least 2- to 3-fold lower than those in NFAT1$^{+/+}$ cells (data not shown). No difference was found in the expression of transcripts for tumor necrosis factor-α (TNFα), TNFβ, and transforming growth factor-β, and none of the cytokine transcripts was visible in cells that had not been restimulated with anti-CD3 (data not shown).

The increase in type 2 cytokine transcripts in restimulated NFAT1$^{-/-}$ spleen cells was almost completely abolished by inclusion of anti-IL-4 (or IL-12 and anti-IL-4) during the primary stimulation (Figure 1A, middle). Conversely and as expected, the addition of IL-4 skewed the differentiation of NFAT1 wild-type cells toward the Th2 pathway (Figure 1A, right). Measurement of cytokine levels by enzyme-linked immunosorbent assay (ELISA) indicated that supernatants of restimulated NFAT1$^{-/-}$ spleen cells contained higher levels of IL-4 and lower levels of IFNγ than supernatants of restimulated NFAT1$^{+/+}$ splenocytes, when differentiated under default conditions or in the presence of IL-4 but not when primed in the presence of anti-IL-4 (Figure 1B). These results indicate that in the course of prolonged activation with the polyclonal stimulus anti-CD3, NFAT1$^{-/-}$ spleen and lymph node cells developed a phenotype characterized by enhanced expression of type 2 cytokines as well as IL-2. Moreover, the development of this phenotype was dependent on IL-4, since it was abolished by the addition of anti-IL-4 during the primary differentiation phase.

As the initial source of IL-12, antigen-presenting cells (APCs) are thought to be critical for the priming of Th1 cells in response to infection (Hsieh et al., 1993). It has been recently proposed that APCs play a comparable role in Th2 cell development by inducing IL-4 in naive T cells via an IL-6–dependent mechanism (Rincón et al., 1997a). Conversely, the genetic background of the T cells themselves may determine the outcome of T cell differentiation under default conditions (Hsieh et al., 1995). To separate effects intrinsic to T helper cells from those due to APCs and other cell types, we separated CD4^{+} and CD4^{-} spleen and lymph node cells from NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice and differentiated and restimulated them independently in the absence of added cytokines with immobilized anti-CD3 alone. Under these conditions, the difference between NFAT1$^{+/+}$ and NFAT1$^{-/-}$ cells in the levels of type 2 cytokines was as pronounced in CD4^{+} T cells as in unseparated splenocytes (Figure 2A, compare middle and left). In contrast, NFAT1-deficient splenocytes depleted of CD4^{+} T cells showed a moderate increase only in IL-13 and IL-2 transcript levels, relative to comparably depleted NFAT1-expressing cells (Figure 2A, right).

IL-4 produced by Mel-14^{low} CD4^{+} ("preactivated") T cells has been shown to be capable of driving Mel-14^{high} CD4^{+} ("naive") T cells into Th2 cells in vitro (Bradley et al., 1991; Gollob and Coffman, 1994). We therefore

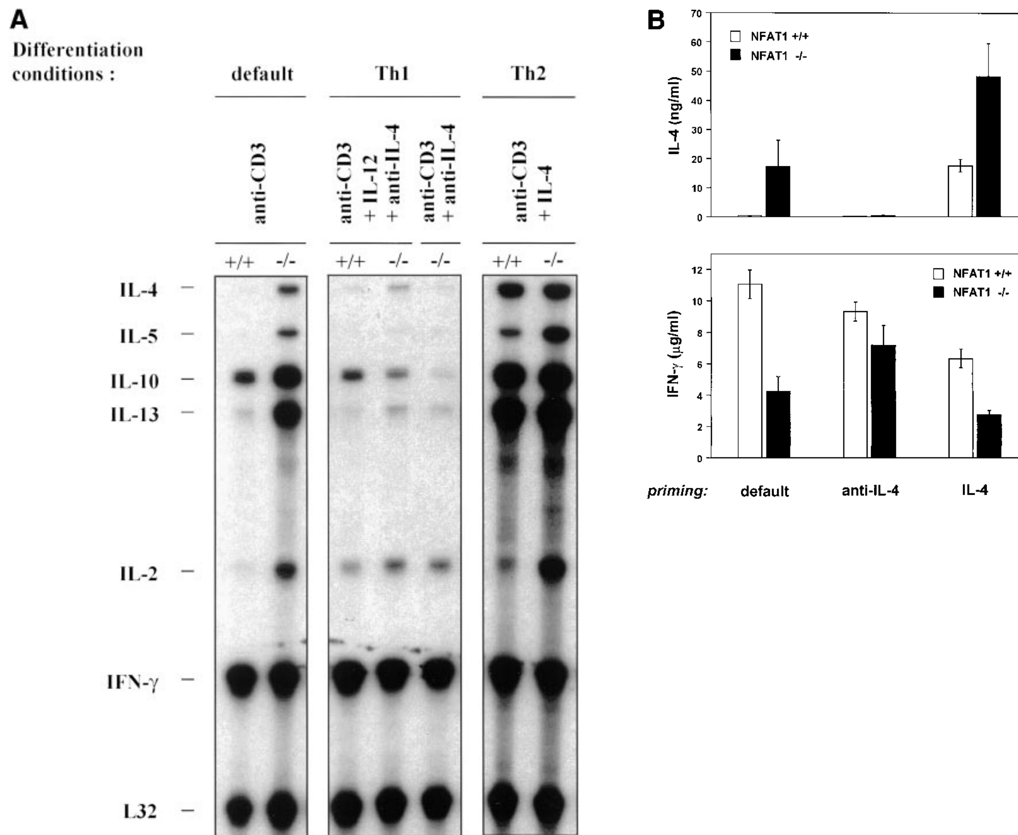

Figure 1. NFAT1-Deficient T Cells Preferentially Differentiate into Type 2 Effector Cells In Vitro

(A) Spleen and lymph node cells of NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice were cultured for 4 days with plate-bound anti-CD3 alone (default conditions, left) or in conditions favoring Th1 (middle) or Th2 cell development (right), rested for 48 hr, and restimulated with plate-bound anti-CD3. Six hours after restimulation, total cellular RNA was isolated and analyzed by RNase protection assay for transcript levels of the indicated cytokines. Protected fragments were separated on a 6% denaturing polyacrylamide gel and visualized by autoradiography. A fragment of the housekeeping gene encoding L32 included in the multitemplate set served as an internal control. Lower exposure of the gel confirmed that comparable amounts of RNA were loaded in each lane.

(B) Spleen and lymph node cells of NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice were cultured for 4 days with plate-bound anti-CD3 alone (left bars), anti-CD3 plus anti-IL-4 (middle bars) or anti-CD3 plus IL-4 (right bars), rested for 48 hr, and restimulated with plate-bound anti-CD3. Supernatants were collected 24 hr after restimulation, and IL-4 and IFNγ levels determined by ELISA. Values are expressed as the means ± SEM of three independent experiments.

examined the cytokine profile of CD4^{+} NFAT1$^{-/-}$ T cells that had been sorted for high expression of Mel-14 and then differentiated and restimulated with immobilized anti-CD3 alone. These cells also showed skewing toward Th2 development relative to wild-type cells (Figure 2B). We conclude that NFAT1-deficient T cells preferentially differentiate into Th2 cells after stimulation with immobilized anti-CD3, and that Mel-14^{high} CD4^{+} T cells are sufficient for this selective differentiation.

NFAT1-Deficient Spleen Cells Do Not Effectively Down-Regulate IL-4 mRNA Expression

T helper cell development is known to be strikingly influenced by the local cytokine environment (Paul and Seder, 1994; Abbas et al., 1996). To determine whether the skewing toward Th2 differentiation of NFAT1$^{-/-}$ cells can be explained by a difference in the early production of IL-4 or other cytokines, we examined the kinetics of cytokine mRNA expression during primary stimulation. NFAT1$^{+/+}$ and NFAT1$^{-/-}$ spleen and lymph node cells were stimulated for various times with anti-CD3, and total RNA was analyzed for the levels of cytokine transcripts. As shown in Figure 3A, IL-4 mRNA reached comparable peak levels in NFAT1$^{+/+}$ and NFAT1$^{-/-}$ cells 2–4 hr after stimulation. However, when the cytokine kinetics were followed for longer times of primary stimulation, it became apparent that NFAT1$^{+/+}$ and NFAT1$^{-/-}$ cells differed in the late phase of IL-4 mRNA expression (Figure 3B). In NFAT1-expressing cells, the early increase in IL-4 mRNA was followed by a rapid decrease, starting at 4–8 hr and approaching baseline levels by 20–24 hr after stimulation (Figure 3B, lanes 1–4). Strikingly, this was not the case in NFAT1-deficient cells, where the levels of IL-4 transcripts peaked at 2–4 hr as in wild-type cells but then declined only slightly and reached a plateau that was maintained throughout the stimulation (Figure 3B, lanes 5–8). A pattern similar to that for IL-4 was seen for IL-5, IL-10, and IL-13 transcripts, although the overall levels of these cytokine mRNAs were much lower than that of IL-4 (and too

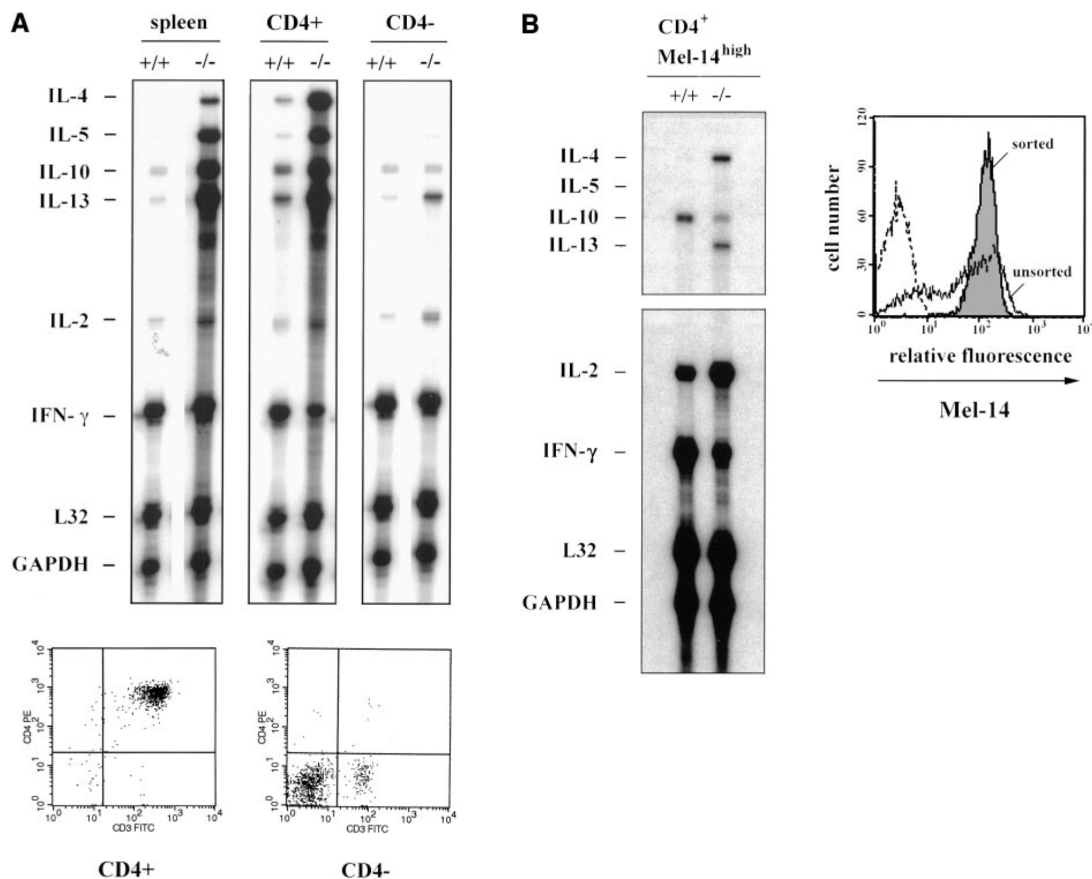

Figure 2. $CD4^+$ T Cells Are Sufficient and Required for the Development of $NFAT1^{-/-}$ Type 2 Effector Cells

(A) Spleen and lymph node cells of $NFAT1^{+/+}$ and $NFAT1^{-/-}$ mice were left untreated or were separated into $CD4^+$ and $CD4^-$ cell populations. Unseparated spleen and lymph node cells (left), purified $CD4^+$ T cells (middle), and the remaining $CD4^-$ cell population (right) were cultured independently for 4 days with plate-bound anti-CD3 alone (default conditions) and then rested and restimulated, as described for Figure 1A. Top, cytokine transcript levels analyzed by RNase protection assay; bottom, fluorescence-activated cell sorter analysis of separated cell populations for CD4 and CD3 expression.

(B) $CD4^+$ T cells were purified from spleen and lymph node cells of $NFAT1^{+/+}$ and $NFAT1^{-/-}$ mice and further sorted on a flow cytometer for high expression of Mel-14 (CD62L). The resulting Mel-14^{high} $CD4^+$ $NFAT1^{+/+}$ or $NFAT1^{-/-}$ T cell populations were cultured for 4 days with plate-bound anti-CD3 alone (default conditions) and then rested and restimulated as described for Figure 1A. Left, cytokine transcript levels analyzed by RNase protection assay. The top and bottom panels belong to the same autoradiogram: top, longer exposure (12 hr); bottom, shorter exposure (4 hr). Right, fluorescence-activated cell sorter analysis for Mel-14 expression of the unsorted (solid line, open) or sorted (solid line, filled) $CD4^+$ T cell populations. Dotted line, isotype control.

low for accurate quantitation). Presumably reflecting the continuous high expression of IL-4 mRNA, $NFAT1^{-/-}$ cells showed increased expression of the Th2 cytokines IL-4, IL-5, IL-10, and IL-13 as early as 48 hr after primary stimulation (Figure 3B, compare lanes 4 and 8). In some experiments, a slight delay in the kinetics of induction of IL-2 and IFNγ transcripts was observed in $NFAT1^{-/-}$ compared to $NFAT1^{+/+}$ cells (Figure 3A), although comparable levels were attained by 8–20 hr in each case (Figure 3B).

As summarized in Figure 3C, the results from several experiments were quantitated by densitometry, measuring IL-4 mRNA levels in $NFAT1^{+/+}$ and $NFAT1^{-/-}$ spleen and lymph node cells during the course of primary stimulation with anti-CD3. Wild-type and NFAT1-deficient cells were identical in the early kinetics of IL-4 mRNA induction and in the peak levels attained approximately 3 hr following stimulation. This early phase was followed in wild-type cells by a rapid and very consistent decline, which reduced IL-4 transcript levels to 20%–25% of peak levels by 6 hr and to 10%–15% of peak levels by 12 hr. The early phase of this decline was also manifest in $NFAT1^{-/-}$ cells, although to varying degrees (see points at 4–6 hr); however, by 12 hr IL-4 transcript levels stabilized and were maintained at about 50%–70% of peak level until 48 hr following stimulation. Similar kinetics were observed in purified $CD4^+$ T cells (data not shown). ELISA confirmed that supernatants of $NFAT1^{-/-}$ spleen and lymph node cells cells stimulated for 48 hr with anti-CD3 contained increased levels of IL-4 protein (Figure 3D). These results indicate that $NFAT1^{-/-}$ cells indeed show increased production of IL-4 during the

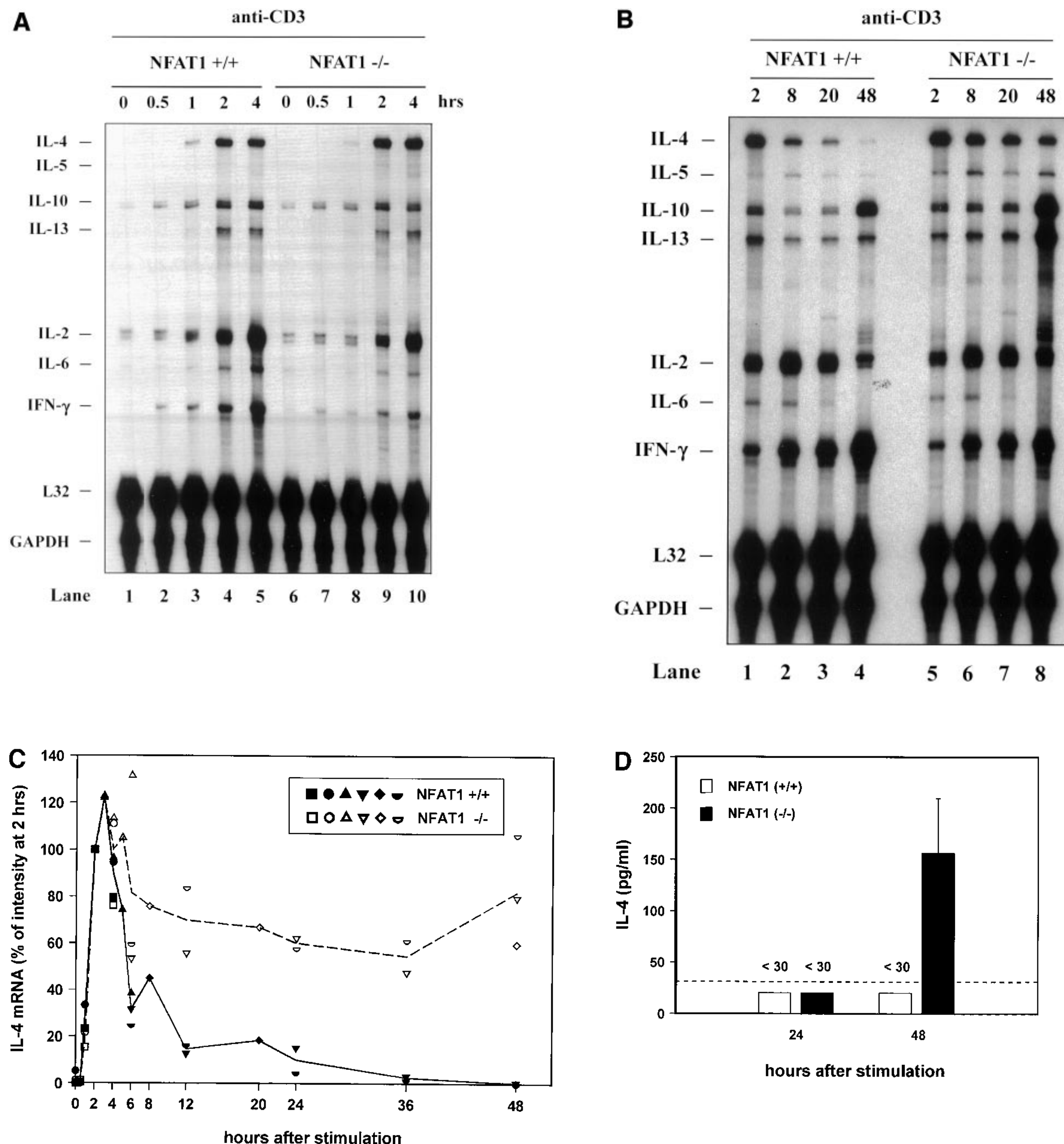

Figure 3. Kinetics of Induction of Cytokine mRNAs during Primary In Vitro Stimulation of NFAT1$^{+/+}$ and NFAT1$^{-/-}$ Spleen and Lymph Node Cells

(A) and (B) Spleen and lymph node cells isolated from NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice were stimulated for 0.5–4 hr (A) or 2–48 hr (B) with plate-bound anti-CD3. At the indicated time points, cells were harvested and the total cellular RNA analyzed for cytokine transcript levels by RNase protection assay. Analysis of the bands representing the housekeeping genes encoding L32 and GAPDH on a lower exposure of the gel showed that comparable amounts of RNA were loaded in each lane.

(C) In six independent experiments similar to those shown in (A) and (B), spleen and lymph node cells isolated from NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice were stimulated for varying times ranging from 0.5–48 hr with plate-bound anti-CD3. At the indicated time points, cells were harvested and the total cellular RNA analyzed for cytokine transcript levels by RNase protection assay. IL-4 mRNA levels were quantified by densitometric analysis and expressed relative to the level at 2 hr in the same experiment. The level of IL-4 mRNA induction at 2 hr was always comparable between NFAT1$^{+/+}$ and NFAT1$^{-/-}$ cells. The mean values at each time point are connected by solid (NFAT1$^{+/+}$) or dashed (NFAT1$^{-/-}$) lines. Each symbol represents one experiment.

(D) Spleen and lymph node cells isolated from NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice were stimulated for 24 or 48 hr with plate-bound anti-CD3. Supernatants were collected at the indicated time points and IL-4 levels determined by ELISA. Values are expressed as the means ± SEM of four independent experiments.

first in vitro stimulation. Strikingly, the major difference between NFAT1-deficient and wild-type T cells was not in the peak levels of IL-4 mRNA produced 2–4 hr after stimulation, but rather in the ability of NFAT1$^{-/-}$ T cells to maintain IL-4 transcripts at high levels for prolonged times.

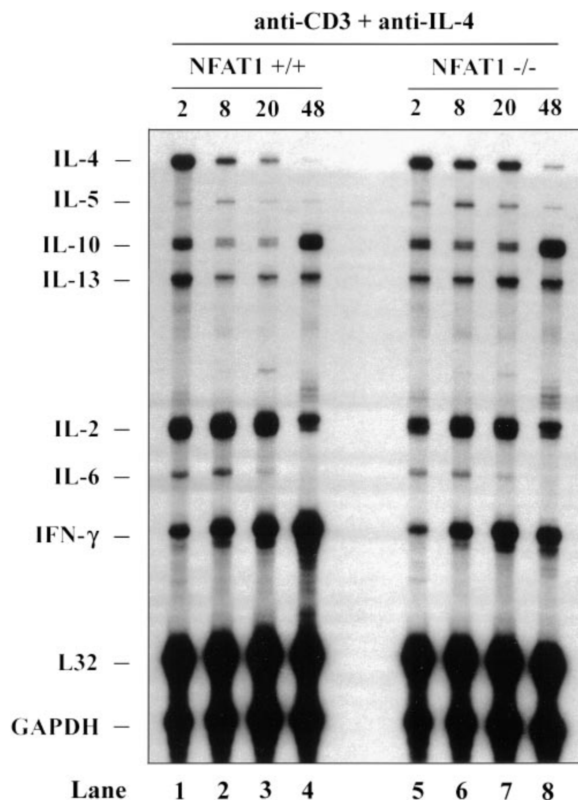

Figure 4. The Maintained Expression of IL-4 mRNA in $NFAT1^{-/-}$ Spleen and Lymph Node Cells Is Not Explained by Hyperresponsiveness to IL-4

Spleen and lymph node cells isolated from $NFAT1^{+/+}$ and $NFAT1^{-/-}$ mice were stimulated for 2–48 hr with plate-bound anti-CD3 in the presence of anti-IL-4. At the indicated time points, cells were harvested and the total cellular RNA analyzed for cytokine transcript levels by RNase protection assay. Analysis of the bands representing the housekeeping genes encoding L32 and GAPDH on a lower exposure of the gel showed that comparable amounts of RNA were loaded in each lane.

The Maintained Expression of IL-4 mRNA in NFAT1-Deficient Spleen Cells Is Regulated at the Level of Gene Transcription and Cannot Be Explained by Hyperresponsiveness to IL-4

IL-4 is known to induce its own production by promoting the differentiation of IL-4–producing Th2 cells (Paul and Seder, 1994; Abbas et al., 1996). Therefore the maintained expression of IL-4 transcripts by $NFAT1^{-/-}$ T cells might result from a hyperresponsiveness of these cells to IL-4 produced early in the primary response. To address this point, we evaluated the kinetics of cytokine production in the presence of anti-IL-4, to block the secondary Th2-promoting effect of IL-4. Anti-IL-4 was capable of efficiently neutralizing endogenous IL-4, since it blocked the up-regulation of Th2 cytokines in $NFAT1^{-/-}$ spleen cells stimulated for 48 hr (Figure 4). However, the presence of anti-IL-4 did not affect the ability of $NFAT1^{-/-}$ spleen cells to maintain high levels of IL-4 transcripts for as long as 20 hr after primary stimulation, nor did it affect the rapid decline of IL-4 transcripts in spleen cells from $NFAT1^{+/+}$ mice (Figure 4). Thus, the maintained expression of IL-4 mRNA by $NFAT1^{-/-}$ cells cannot be due solely to hyperresponsiveness to IL-4.

The steady-state levels of mRNA transcripts are determined by the balance between transcription and degradation. It has been established that both mechanisms have a significant effect on cytokine mRNA levels in stimulated cells (Lindsten et al., 1989; Dokter et al., 1993; Umlauf et al., 1995). The maintained level of IL-4 transcripts in $NFAT1^{-/-}$ cells could thus be related either to continuous transcription of the IL-4 gene or to increased posttranscriptional processing or stabilization of the IL-4 mRNA. To distinguish between these possibilities, we used the immunosuppressive drug cyclosporin A (CsA) to block IL-4 gene transcription at defined time points after activation of the cells. CsA was used in preference to the RNA polymerase inhibitor actinomycin D, which has been shown to affect the turnover rate of IL-2 mRNA as well as block transcription of the gene (Umlauf et al., 1995). CsA, by inhibiting the calcium-dependent phosphatase calcineurin, promotes the rapid nuclear export of NFAT proteins (Loh et al., 1996b; Shibasaki et al., 1996; Timmerman et al., 1996), thereby inhibiting within 15 min the transcription of NFAT-dependent genes, as previously shown for IL-2 (Shaw et al., 1988; Umlauf et al., 1995; Timmerman et al., 1996). Since differences in the steady-state levels of IL-4 mRNA between $NFAT1^{+/+}$ and $NFAT1^{-/-}$ cells began to be apparent by 4–6 hr after stimulation (Figure 3), we reasoned that a 4 hr activation period would be sufficient for synthesis of any inducible proteins needed for degradation or stabilization of IL-4 mRNA.

We first confirmed that the transcription of IL-4 and other cytokines in NFAT1-deficient cells was still sensitive to CsA. As demonstrated in Figure 5A, neither $NFAT1^{+/+}$ nor $NFAT1^{-/-}$ spleen cells stimulated with anti-CD3 in the continuous presence of CsA showed detectable levels of IL-4 transcripts. We then used CsA to assess the half-life of IL-4 transcripts in $NFAT1^{+/+}$ and $NFAT1^{-/-}$ cells (Figure 5B). The cells were stimulated for 4 hr so that equivalent peak levels of IL-4 would be attained and so that any inducible proteins needed for mRNA degradation or stabilization would be synthesized. CsA was then added to the cultures; aliquots of cells were removed at hourly intervals thereafter; and RNA was prepared and analyzed for cytokine transcripts by RNase protection assay. Under these conditions we anticipated that CsA would rapidly block any ongoing transcription of the IL-4 gene, so that the resulting decline in IL-4 transcript levels would reflect only the degradation rate of preexisting transcripts.

The results indicated that IL-4 mRNA declines with a half-life of less than 1 hr under these conditions, in both $NFAT1^{+/+}$ and $NFAT1^{-/-}$ cells (Figure 5B, bottom). Identical results were obtained when CsA was added at 2 hr, instead of 4 hr, after stimulation of the cells (data not shown). These results suggest that $NFAT1^{-/-}$ spleen cells maintain IL-4 transcripts at high levels because of an ability to maintain a high rate of IL-4 gene transcription and not because of an ability to stabilize IL-4 transcripts. Since we used CsA, however, we cannot rule out posttranscriptional mechanisms that are sensitive to this drug. Of note, the measured half-life of IL-4 mRNA

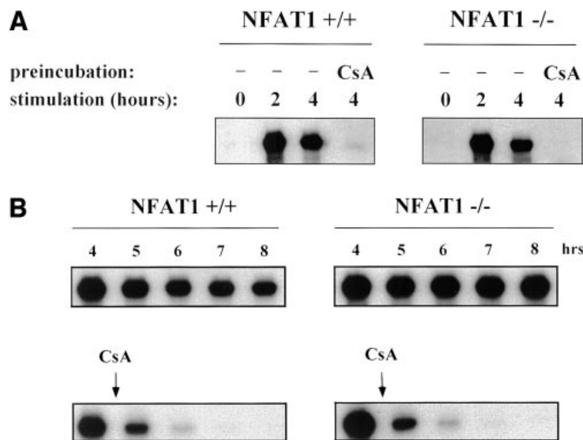

Figure 5. The Maintained Expression of IL-4 mRNA in NFAT1-Deficient Cells Is Regulated at the Transcriptional Level

(A) Spleen and lymph node cells isolated from NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice were stimulated for the indicated times with plate-bound anti-CD3 alone or in the continuous presence of CsA, added 30 min prior to stimulation. At the indicated time points, cells were harvested and the total cellular RNA analyzed for IL-4 transcript levels by RNase protection assay, as described in Figure 1.

(B) Spleen and lymph node cells isolated from NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice were stimulated for 4 hr with plate-bound anti-CD3, and then either they were left untreated (top) or CsA was added to the culture (bottom). Cells were harvested at the indicated time points and the total cellular RNA analyzed for IL-4 transcript levels by RNase protection assay. Analysis of the bands representing the housekeeping genes encoding L32 and GAPDH in (A) and (B) showed that comparable amounts of RNA were loaded in each lane.

was very short ($t_{1/2}$ <1 hr), suggesting that posttranscriptional mechanisms may indeed have a major effect on the steady-state levels of IL-4 mRNA.

NFAT1-Deficient Mice Are More Susceptible than Wild-Type Mice to Infection with L. major

The in vitro differentiation experiments described above suggested that the maintained transcription of the IL-4 gene and consequent increase in IL-4 production by NFAT1$^{-/-}$ cells altered the direction of T helper cell differentiation toward the generation of type 2 cytokine-producing cells. To determine whether this biased differentiation has pathophysiological consequences in vivo, we used the well-studied system of infection with the intracellular pathogen L. major. NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice, both of mixed C57BL/6 and 129/SvJ genetic backgrounds, were injected with L. major in one hind footpad and the course of the disease was followed by measuring the lesion size. As expected from the fact that both C57BL/6 and 129/SvJ strains are resistant to L. major infection (Reiner and Locksley, 1995), NFAT1$^{+/+}$ mice were capable of controlling the infection: the initial slight swelling of their injected footpads was followed by healing within 3–4 weeks (Figure 6A). In contrast, NFAT1$^{-/-}$ mice developed progressive enlargement of their footpads that continued for 6–7 weeks, indicating that they were not able to restrict the infection. Neutralization of endogenously produced IL-4 completely restored the ability to control the disease in NFAT1$^{-/-}$ mice and, as described, in control mice of the highly susceptible BALB/c strain (Sadick et al., 1990; Reiner and Locksley, 1995).

It is noteworthy that the course of infection differed between NFAT1$^{-/-}$ mice and BALB/c mice. Whereas the footpads of all of the BALB/c mice steadily increased in size until they ulcerated, NFAT1$^{-/-}$ mice displayed first a rapid and then a slower increase in their footpad sizes, which approached steady state 6–7 weeks after infection. When followed for a longer period of time, one of the five NFAT1$^{-/-}$ mice in this group developed a progressive infection, with its footpad ulcerating between 5 and 6 weeks after injection of the pathogen. The four remaining mice, however, eventually seemed able to control the infection, since the size of their footpads slowly decreased, although this occurred several weeks later than in NFAT1$^{+/+}$ mice and had not yet reached normal levels even 12 weeks after infection.

To confirm that the increased susceptibility of NFAT1$^{-/-}$ mice to L. major was reflected by increased expression of IL-4, we analyzed cytokine transcripts present in cells of the draining lymph nodes of NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice 4 weeks after infection with L. major. As shown in Figure 6B, lymph node cells isolated from NFAT1$^{-/-}$ mice contained higher levels of IL-4 mRNA compared to cells from NFAT1$^{+/+}$ mice, whether or not they had been treated with anti-IL-4 (Figure 6B, top). This finding was consistent with the in vitro observation that the maintenance of IL-4 transcription in NFAT1$^{-/-}$ cells was not affected by addition of anti-IL-4 to the cultures (Figure 4). Of note, no difference in mRNA levels for IFNγ was detected between the three groups (Figure 6B, bottom). We conclude that NFAT1$^{-/-}$ mice are more susceptible to infection with L. major than are NFAT1$^{+/+}$ mice and that this effect is potentially mediated by continuous expression of IL-4 during the course of the infection.

Discussion

NFAT proteins are well established as activators of gene transcription during the immune response (Rao et al., 1997). Our results provide evidence that a member of this family, NFAT1, plays an essential down-regulatory role in gene transcription. We show that unlike normal mice of the same genetic background, NFAT1$^{-/-}$ mice are disposed to produce cytokines of the type 2 category during the immune response. This phenotype is observed both in vitro, in T cells stimulated with anti-CD3, and in vivo, in cells taken from the draining lymph nodes of mice injected several weeks previously with L. major (Figures 1 and 6B). Mechanistically, the phenotype correlates with a pronounced difference between NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice in the late kinetics of IL-4 gene transcription: spleen cells of NFAT1$^{-/-}$ mice do not show the characteristic rapid down-regulation of IL-4 mRNA expression observed in NFAT1$^{+/+}$ cells, but rather maintain a high level of IL-4 transcripts for up to 24 hr following stimulation. The resulting increase in IL-4 production by NFAT1$^{-/-}$ relative to NFAT1$^{+/+}$ cells during the initial stimulation is reinforced by the well-known positive feedback effect of IL-4 (Paul and Seder, 1994; Abbas et al.,

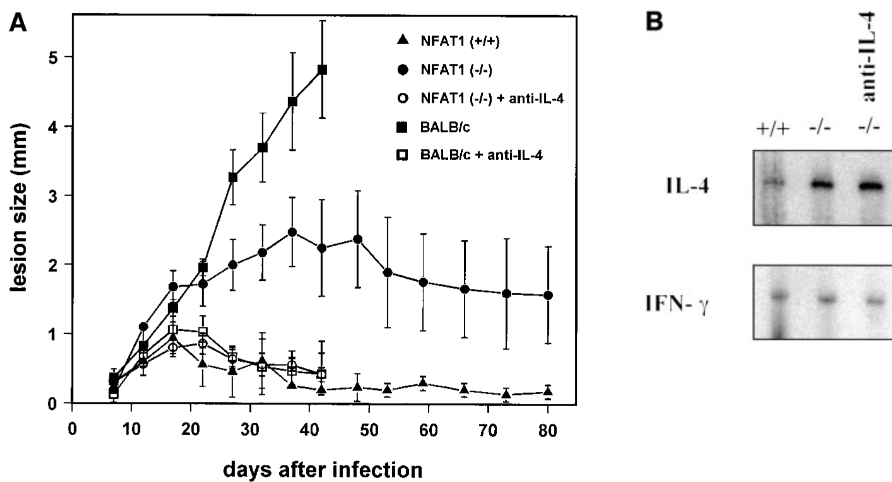

Figure 6. NFAT1-Deficient Mice Are More Susceptible than NFAT1 Wild-Type Mice to Infection with L. major

(A) NFAT1$^{+/+}$ mice and NFAT1$^{-/-}$ mice (five mice per group) were infected with promastigotes of L. major in one hind footpad. One group of NFAT1$^{-/-}$ mice was injected with the 11B11 MAb against IL-4 24 hr prior to the infection and weekly thereafter. Two groups of BALB/c mice, treated and not treated with anti-IL-4 (three per group), were infected as controls. The sizes of both footpads of each mouse were measured every fifth day with a metric caliper and the lesion size calculated as the difference between the injected and the uninjected footpad.

(B) NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice, treated or not treated with anti-IL-4 (three mice per group), were infected with promastigotes of L. major in both hind footpads. Four weeks after infection, mice were sacrificed and the cells of the popliteal lymph nodes draining the lesions were isolated and pooled within groups. Total cellular RNA was extracted immediately and analyzed for cytokine mRNA levels. The panel for IL-4 shows an exposure of the autoradiogram to a film with higher sensitivity than the panel for IFNγ. Comparable amounts of RNA were loaded in each lane, as confirmed by analysis of the bands representing the housekeeping genes encoding L32 and GAPDH.

1996), giving rise to a striking bias toward type 2 cytokine production in the secondary immune response. These results extend an independent report that NFAT1$^{-/-}$ spleen cells show enhanced Th2 differentiation in vitro (Hodge et al., 1996b). They may also explain our previous observation that NFAT1$^{-/-}$ mice show increased eosinophil recruitment in the pleural cavity in an in vivo model of allergic inflammation (Xanthoudakis et al., 1996; J. P. B. V. et al., unpublished data). In another biological consequence of this bias, NFAT1$^{-/-}$ mice show increased susceptibility to infection with L. major, approaching the susceptibility of BALB/c mice despite their mixed C57BL/6 and 129/SvJ genetic backgrounds.

It is unlikely that the observed differences in IL-4 mRNA expression are due merely to an increase in IL-4–producing cell populations, such as memory T cells (Gollob and Coffman, 1994) or NK1.1^{+} T cells (Yoshimoto and Paul, 1994), in NFAT1$^{-/-}$ versus wild-type mice. We did not detect any significant skewing of these T cell subpopulations in NFAT1$^{-/-}$ mice, as judged by expression of the cell surface markers Mel-14, CD44, CD45RB, CD25, CD69, and NK1.1 (A. K., unpublished data). In particular, both memory cells and NK1.1^{+} T cells are reported to have a Mel-14^{low} ("preactivated") phenotype (Bradley et al., 1991; Gollob and Coffman, 1994; Vicari and Zlotnik, 1996); however, CD4^{+} Mel-14^{high} ("naive") splenic T cells purified by cell sorting from NFAT1$^{-/-}$ mice showed the same striking increase in type 2 cytokines relative to wild-type cells, as observed for unseparated spleen cells and purified CD4^{+} T cells (Figure 2). Moreover, the Th2 skewing of NFAT1$^{-/-}$ T cells is clearly cell autonomous, since it is observed in CD4^{+} T cells stimulated with immobilized anti-CD3 in the absence of APCs.

It is also unlikely that the maintained expression of IL-4 transcripts, which is manifest as early as 6 hr after stimulation (Figure 3C), results from overproduction or down-regulation of known cytokines by NFAT1$^{-/-}$ cells. In particular, IL-4, IL-12, and IFNγ are known to influence T helper cell differentiation (Paul and Seder, 1994; Abbas et al., 1996). IL-4 acts positively to enhance IL-4 production; however, anti-IL-4 did not affect the maintained expression of IL-4 gene transcripts in NFAT1$^{-/-}$ cells, ruling out the possibility that NFAT1$^{-/-}$ cells were hyperresponsive to the low concentrations of IL-4 produced during the initial stimulation in vitro (Figure 4). Conversely, IFNγ exerts most of its down-regulatory effects on Th2 differentiation by inducing APCs to produce IL-12 and by maintaining IL-12 responsiveness in T cells (Trinchieri, 1995; Abbas et al., 1996; Szabo et al., 1997). However, purified CD4^{+} populations of NFAT1$^{-/-}$ cells, stimulated with plate-bound anti-CD3 in the absence of APCs, resembled unseparated spleen cells in their ability to differentiate preferentially into type 2 cells, suggesting that IL-12–dependent mechanisms were not involved (Figure 2). Furthermore, the Th2 skewing of NFAT1$^{-/-}$ relative to NFAT1$^{+/+}$ T cells was maintained even when IFNγ or anti-IFNγ were added to cultures of CD4^{+} T cells during the primary differentiation phase (A. K., unpublished data). Based on these results, we conclude that there is an intrinsic, cell-autonomous difference in the kinetics of IL-4 gene transcription in T cells that express or lack NFAT1. However, our results do not rule out the possibility that distinct, APC-dependent mechanisms also contribute to the Th2 phenotype of NFAT1$^{-/-}$ mice.

Is NFAT1 involved in the early phase of IL-4 gene transcription? NFAT1 clearly does not influence the peak

levels or early kinetics of IL-4 gene transcription, which appear identical in NFAT1$^{-/-}$ and wild-type mice. However, it is unlikely that NFAT1 plays no role whatsoever in activating IL-4 gene transcription: NFAT1 in nuclear extracts binds to each of the identified NFAT sites of the IL-4 promoter (Chuvpilo et al., 1993; Szabo et al., 1993; Ho et al., 1995; Rooney et al., 1995); NFAT1 is capable of driving IL-4 promoter transcription in transient assays, alone or in synergy with c-Maf and NIP-45 (Ho et al., 1996; Hodge et al., 1996a; Luo et al., 1996); and an early impairment of IL-4 mRNA expression has been reported in an independent strain of NFAT1-deficient mice (Hodge et al., 1996b). Taken together, these results indicate that NFAT1 indeed participates in IL-4 gene transcription, and this is particularly apparent under conditions where NFAT1 is the predominant or limiting family member in the nucleus. A second possibility is that NFAT1 competes with more effective NFAT proteins for binding to IL-4 promoter sites or for cooperation with other essential transcription factors such as GATA-3, STAT-6, or c-Maf. This hypothesis of early competition predicts that the peak levels of IL-4 mRNA attained 2–4 hr after stimulation would be much lower in wild-type than in NFAT1$^{-/-}$ cells, since NFAT1 is the predominant family member in both resting and stimulated T cells (Loh et al., 1996a; Xanthoudakis et al., 1996). However, it is clear that there is no difference in the peak levels of IL-4 mRNA in NFAT1$^{-/-}$ versus NFAT1$^{+/+}$ cells (Figure 3). Furthermore, NFAT1$^{-/-}$ mice do not compensate for the lack of NFAT1 by up-regulating other NFAT proteins (Hodge et al., 1996b; Xanthoudakis et al., 1996). The third, most intriguing possibility is that NFAT1 is equivalent to the other family members in its ability to induce the early phase of IL-4 gene transcription, but unique in its ability to induce the late down-regulatory phase.

The molecular mechanism by which NFAT1 exerts its down-regulatory effects remains unknown and is the focus of present investigations. When IL-4 gene transcription was acutely blocked by adding CsA, the degradation rates of IL-4 mRNA were identical in NFAT1$^{+/+}$ and NFAT1$^{-/-}$ cells (Figure 5B), suggesting that the selective down-regulation seen in wild-type cells occurred at the transcriptional level. An intriguing possibility is that transcription of the entire IL-4/IL-5/IL-13 gene cluster might be coordinately regulated by NFAT1, as suggested by the finding that IL-5 and IL-13 transcripts behave similarly to IL-4 transcripts (the levels of these transcripts were too low for accurate quantitation). Potentially, NFAT1 itself might shut off transcription at the IL-4 locus by undergoing a functional switch from an activator to a repressor. For instance, NFAT1 might be the unique target (within the NFAT family) of a kinase, phosphatase, or other modifying enzyme that was induced late after T cell receptor stimulation, and the resulting posttranslational modification might inhibit the ability of NFAT1 to interact effectively with the general transcriptional machinery while allowing it to remain bound to DNA. Alternatively, NFAT1 might uniquely activate genes whose products directly or indirectly switch off IL-4 gene transcription late during the response. It is not unprecedented that a single protein can have both positive and negative effects on a signaling pathway leading to gene transcription: for instance, ligation of a cell surface receptor typically leads to activation of positively acting kinases as well as negatively acting phosphatases, and a single kinase may phosphorylate multiple target proteins in a signaling pathway, thus leading to opposing effects on cellular function (Rao, 1991; Hill and Treisman, 1995; Hunter, 1995).

Examples of such inhibitory, NFAT1-regulated gene products might include members of the recently described family of STAT-induced inhibitors of STAT signaling (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997); direct repressors or corepressors acting at the level of the nuclear transcription complex; or proteins inducing activation-induced cell death in all T cells or selectively in IL-4 producing cells. The latter possibility would be consistent with the higher proliferation rate of NFAT1$^{-/-}$ cells (Hodge et al., 1996b; Xanthoudakis et al., 1996) and with the observation that spleen cells of an independent strain of NFAT1$^{-/-}$ mice showed little or no expression of mRNA encoding Fas ligand (FasL) when evaluated 1 hr after in vivo injection with anti-CD3 (Hodge et al., 1996b). However, several studies have indicated that Th1 cells are more susceptible than Th2 cells to Fas/FasL-mediated apoptosis (Varadhachary et al., 1997; Zhang et al., 1997); thus, if NFAT1$^{-/-}$ cells differed from wild-type cells by the absence of a Fas/FasL-mediated apoptotic mechanism, skewing toward a Th1-dominated immune response would be expected, rather than the Th2 skewing actually observed. Furthermore, NFAT1$^{-/-}$ cells, differentiated and restimulated in vitro, were equally capable of inducing cell death in a Fas-expressing target cell line, indicating equivalent expression of FasL (A. Marshak-Rothstein, personal communication). Certainly, other pathways of activation-induced cell-death (Wong and Choi, 1997) could be defective in NFAT1$^{-/-}$ mice, which might selectively lead to the survival of IL-4–producing cells.

The maintained transcription of the IL-4 gene in NFAT1$^{-/-}$ cells clearly has pathophysiologically relevant consequences in vivo. Resistance to infection with L. major had been shown to depend on the ability to generate a Th1-dominated immune response, whereas susceptibility to the disease was associated with differentiation into Th2 cytokine-producing cells (Reiner and Locksley, 1995). As expected, NFAT1$^{-/-}$ mice were less able to control local infection with L. major than wild-type mice, consistent with increased levels of IL-4 mRNA in cells of the draining lymph nodes (Figure 6). However, the course of the disease in NFAT1$^{-/-}$ mice was different from that seen in mice of the highly susceptible BALB/c strain (Hsieh et al., 1995; Reiner and Locksley, 1995), in that four of five NFAT1$^{-/-}$ mice were eventually able to control the infection while the BALB/c mice were not (Figure 6A). Control of L. major infection depends primarily on the effective activation of macrophages by IFNγ and other factors (Reiner and Locksley, 1995), and susceptibility or resistance to infection with L. major in BALB/c is linked to several independent chromosomal loci, indicating a multigenic control of the infection (Beebe et al., 1997). Notably, transcript levels of IFNγ were comparable in cells of the draining lymph nodes of NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice (Figure 6B). We suggest that the early susceptibility of NFAT1-deficient mice to L. major reflects their inability, relative to wild-type mice,

to down-regulate IL-4 transcription effectively. In contrast, additional mechanisms, such as the maintenance of responsiveness to IL-12 (Güler et al., 1996), may limit the further expansion of the infection in the resistant background strains C57BL/6 and 129/SvJ, but not in BALB/c mice.

In summary, we have shown that the transcriptional activator NFAT1 controls, either directly or indirectly, the late down-regulation of IL-4 gene transcription. These data constitute evidence that NFAT proteins regulate not only the early inductive phase but also the termination of cytokine gene transcription. Our increasing knowledge of the structure and regulation of NFAT proteins, and thus the prospect of individually targeting NFAT1, might allow us to bias immune responses specifically, with crucial consequences for disease and infection.

Experimental Procedures

Mice

The generation of NFAT1$^{-/-}$ mice, on a mixed C57BL/6 and 129Sv/J background, has been described (Xanthoudakis et al., 1996). All mice were maintained in pathogen-free conditions in barrier facilities at the Center For Animal Resources and Comparative Medicine, Harvard Medical School, and were used at an age of 8–12 weeks for experiments.

Cell Culture Conditions and Reagents

All cell cultures were done in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, L-glutamine, streptomycin–penicillin, nonessential amino acids, sodium pyruvate, vitamins, HEPES (all from GIBCO, Gaithersburg, MD), and 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). Single-cell suspensions were prepared from lymph node (inguinal, axillary, brachial, and cervical) and spleen cells. Where indicated, CD4$^+$ T cells were purified using magnetic beads (Dynabeads L3T4, Dynal, Lake Success, NY). After each separation, the efficiency of CD4$^+$ cell purification was analyzed by flow cytometry and was routinely greater than 95%. In selected experiments, CD4-purified T cells were further separated by cell sorting in Mel-14^{high} and Mel-14^{low} populations (see below). All experiments were done in parallel using equal numbers of cells from NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice.

Purified T helper cells ($1–10 \times 10^6$) or unseparated lymph node and spleen cells ($10–20 \times 10^6$) were stimulated in vitro at $1–2 \times 10^6$ cells/ml with 1 μg/ml of plate-bound anti-CD3 (2C11; Pharmingen, San Diego, CA) for the indicated time points. In some experiments, CsA (2 μM; courtesy of Sandoz Pharma, Basel, Switzerland) was added to the cultures.

For in vitro differentiation assays (Gorham et al., 1996; Kaplan et al., 1996a; Lederer et al., 1996), cells were stimulated with plate-bound anti-CD3 alone (default conditions); with anti-CD3 plus 10 μg/ml anti-IL-4 (11B11; a gift from the Biological Response Modifiers Program, National Cancer Institute, Frederick, MD) and 5 ng/ml recombinant IL-12 (courtesy of Hoffmann-La Roche, Nutley, NJ) to promote Th1 cell differentiation; or with anti-CD3 plus 1000 U/ml IL-4 to promote Th2 differentiation (IL-4 was added as supernatant from the cell line I3L6 transfected with a constitutively expressed murine IL-4 cDNA [Tepper et al., 1989]) to direct Th2 cell differentiation. In some experiments, cells were differentiated with anti-CD3 plus anti-IL-4 alone.

All cultures were supplemented with 20 U/ml IL-2 (Collaborative Biomedical Products, Bedford, MA) after 24 hr, and fresh medium (30% of the initial volume) was added after 48 hr of stimulation. After 4 days, cells were extensively washed, rested for 24–48 hr in IL-2 (20 U/ml), counted, and restimulated at $1–2 \times 10^6$ cells/ml with 1 μg/ml plate-bound anti-CD3. For the quantitation of cytokine mRNA or protein, as described below, total cellular RNA was isolated and supernatants were collected at the indicated time points.

Flow Cytometry

The fluorescein isothiocyanate (FITC)–labeled monoclonal antibodies (MAbs) anti-CD3, anti-CD8, and anti-Mel-14 (CD62L), as well as the phycoerythrin (PE)–labeled MAb anti-CD4, were purchased from Pharmingen. The MAbs anti-CD25-PE, anti-CD44-PE, anti-CD45RB-PE, anti-Mel-14-PE, and anti-CD69-PE (Pharmingen) were kindly provided by A. Abbas (Harvard Medical School); the biotinylated MAb against the marker NK1.1 (Pharmingen) was a gift from F. Alt (Harvard Medical School). Unseparated spleen and lymph node cells or purified CD4$^+$ T cells were stained for 30 min at 4°C, washed, and either fixed with 1% paraformaldehyde or analyzed directly on a Becton Dickinson FACScan Flow Cytometer. For cell sorting experiments, CD4-purified T cells were stained with the MAb anti-Mel-14-FITC and sorted on a Flow Cytometer (Epics Elite ESP). The sorted cell population was greater than 98% Mel-14^{high} CD4$^+$.

RNase Protection Assay

After primary or secondary in vitro stimulation, cells were harvested at the indicated time points and the total cellular RNA immediately extracted (Ultraspec, Houston, TX). Cytokine RNA levels were analyzed by RNase protection assay using the RiboQuant multiprobe kit (Pharmingen, San Diego, CA) following the instructions of the manufacturer. In brief, equal amounts of target RNA (3–15 μg) were hybridized overnight to a ^{32}P-labeled RNA probe, which had been synthesized in vitro from a multicytokine template set, after which free probe and other single-stranded RNA were digested with RNases. The protected mRNAs were purified and resolved on a 6% denaturing polyacrylamide gel. Transcript levels were quantified by autoradiography and densitometric scanning (IS-1000 Digital Imaging System, Alpha Innotech, San Leandro, CA) of autoradiograms with exposures within the linear range. The cytokine transcripts were identified by the length of the respective fragments. RNA loading was estimated by measuring the intensities of protected fragments representing two housekeeping genes, those encoding L32 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

ELISA

Cell culture supernatants of cells activated in vitro in a primary or secondary stimulation as described above were collected at 24 and 48 hr, and cytokine levels for IL-4 and IFNγ were analyzed using commercial ELISA kits (Endogen, Woburn, MA) according to the manufacturer's instructions. Calculated values are expressed as means ± SEM.

L. major infection

Infections were performed with promastigotes of L. major (strain Friedlin V1; courtesy of Dr. Steven Beverley, Harvard Medical School) grown in M199 medium supplemented with 10% fetal calf serum and antibiotics. Promastigotes were harvested from early stationary-phase cultures by centrifugation and washed in phosphate-buffered saline before use. Female NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice (five animals per group) as well as BALB/c control mice were inoculated with 2×10^6 parasites in the left hind footpad. Designated mice were injected intraperitoneally with 1 mg of the MAb 11B11 (anti-IL-4) 24 hr prior to infection and weekly thereafter with 0.5 mg of the antibody. The course of infection was monitored every fifth day by measuring both footpads with a metric caliper and subtracting the size of the noninjected from the injected footpad. For in vitro experiments, three mice per group were injected with L. major in both footpads. Four weeks after infection, the mice were sacrificed, the cells of the popliteal lymph nodes draining the lesions isolated and pooled within groups, and the total cellular RNA extracted immediately. The cytokine mRNA was analyzed by RNase protection assay, as described above.

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