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Hyperproliferation and Dysregulation of IL-4 Expression in NF-ATp-Deficient Mice

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Summary

NF-ATp is a member of a family of genes that encodes the cytoplasmic component of the nuclear factor of activated T cells (NF-AT). In this study, we show that mice with a null mutation in the NF-ATp gene have splenomegaly with hyperproliferation of both B and T cells. They also display early defects in the transcription of multiple genes encoding cytokines and cell surface receptors, including CD40L and FasL. A striking defect in early IL-4 production was observed after ligation of the TCR complex by treatment with anti-CD3 in vivo. The transcription of other cytokines including IL-13, GM–CSF, and TNFα was also affected, though to a lesser degree. Interestingly, the cytokines IL-2 and IFNγ were minimally affected. Despite this early defect in IL-4 transcription, Th2 development was actually enhanced at later timepoints as evidenced by increased IL-4 production and IgE levels in situations that favor the formation of Th2 cells both in vitro and in vivo. These data suggest that NF-ATp may be involved in cell growth, and that it is important for the balanced transcription of the IL-4 gene during the course of an immune response.

Introduction

Ligation of the T lymphocyte antigen receptor triggers the expression of multiple early activation genes encoding cytokines and surface receptor molecules. Nuclear factor of activated T cells (NF-AT) was first described as a transcriptional regulatory complex critical for the expression of the T cell cytokine, interleukin-2 (IL-2) (Shaw et al., 1988). Subsequently, the IL-4, granulocyte–macrophage colony-stimulating factor (GM–CSF), and tumor necrosis factor-α (TNFα) cytokine genes were shown to be regulated by NF-AT (Cockerill et al., 1995; Goldfeld et al., 1993; Rooney et al., 1994). NF-AT is composed of a cytoplasmic subunit, whose translocation to the nucleus is cyclosporin sensitive, and an inducible nuclear component composed of AP-1 family member proteins (Flanagan et al., 1991; Jain et al., 1992). The cytoplasmic subunit of NF-AT is encoded by a family of genes including NF-ATp, NF-ATc, NF-AT3, and NF-AT4/x, all of which can bind to and transactivate NF-AT target sequences in vitro (Hoey et al., 1995; Masuda et al., 1995; McCaffrey et al., 1993; Northrop et al., 1994). These family members share approximately 70% sequence identity within a region distantly related to the Rel homology domain of the NF-κB transcription factors. However, their tissue distibution differs (Hoey et al., 1995; Northrop et al., 1994), as does their inducibility after stimulation of T cells by T cell receptor (TCR) ligation or phorbol esters plus calcium ionophore (Northrop et al., 1994). NF-ATp and NF-AT3 are expressed in virtually all tissues, while NF-ATc and NF-AT4/x are preferentially expressed in lymphoid organs and thymus with particularly high expression of NF-AT4/x in thymus (Ho et al., 1995; Hoey et al., 1995; Masuda et al., 1995; Northrop et al., 1994). Further, while transcripts encoding NF-ATc are markedly increased after T cell stimulation, transcripts for NF-ATp are higher at baseline and do not change upon stimulation (Northrop et al., 1994). These observations raised the possibility that the functions of NF-AT family members may not be overlapping.

NF-ATp was the first member of this family to be purified and cloned (McCaffrey et al., 1993). To address whether NF-ATp may have unique functions, we generated mice with a targeted mutation in the NF-ATp gene. Mice that lack functional NF-ATp display hyperproliferation and dysregulation in the production of IL-4.

Results

Generation of NF-ATp-Deficient Mice

To generate mice deficient for NF-ATp, genomic DNA containing an exon encoding 47 aa of the highly conserved Rel homology domain was targeted for deletion and replaced by insertion of a neomycin resistance gene (*neo*$^{+}$) (Figure 1A). Germline chimeras generated from one gene-targeted embryonic stem (ES) cell clone (Figure 1B) produced heterozygous mice that were then bred to produce mice homozygous for the disrupted allele, hereafter referred to as (−/−) mice (Figure 1C). Western blot analysis of (−/−) extracts using a monoclonal antibody (MAb) specific for a region animo terminal to the NF-ATp Rel homology domain revealed an absence of full-length NF-ATp proteins (Figure 1D). Western blot analysis using an NF-ATc-specific MAb demonstrated that NF-ATc is expressed normally in NF-ATp (−/−) mice and that the extracts used to assess the status of NF-ATp were not degraded (Figure 1D). Two smaller polypeptides reactive with the NF-ATp antibody were observed in (−/−) cytoplasmic extracts, but a nearly undetectable amount of either form is found in the nucleus (Figure 1D). Extracts from heterozygous (+/−) cells contained polypeptides present in both the (+/+) and the (−/−) cell extracts (Figure 1D). Sequence analysis of RT–PCR products from (−/−) RNA revealed the presence of spliced in-frame transcripts, which precisely remove the targeted exon (amino acids 398–444; data not shown). Electrophoretic mobility shift assay (EMSA) of polypeptides corresponding to the mutant allele, generated from NF-ATp cDNA from (−/−) splenocytes (Figure 1E), indicated that this mutation leads to an

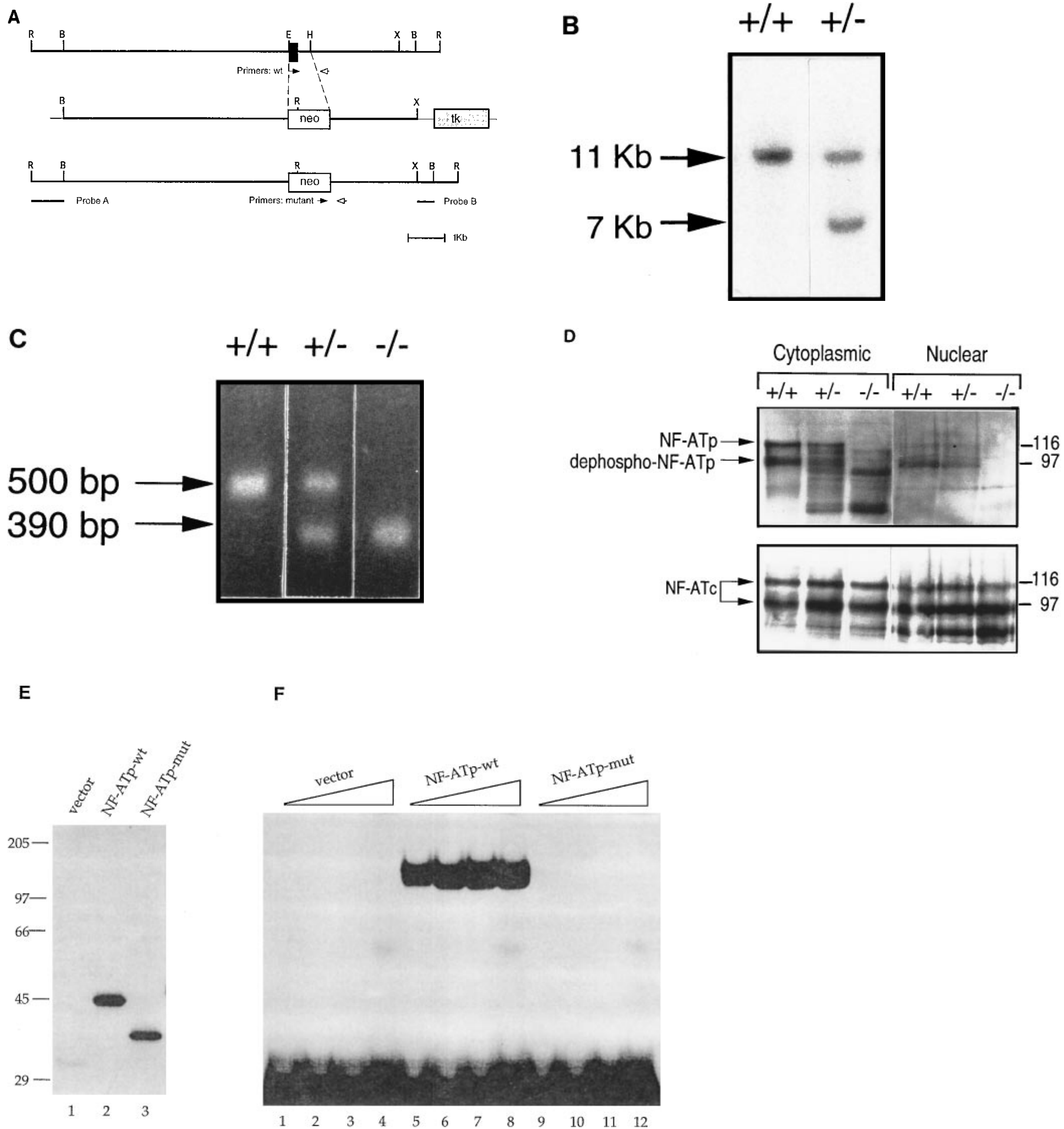

Figure 1. Disruption of the Murine NF-ATp Gene

(A) The structure of the genomic DNA encoding a portion of the NF-ATp Rel similarity domain isolated from a 129/sv genomic library (top). Replacement construct containing the *neo* cassette indicating the replacement of the segment containing the Rel homology domain exon with the *neo* cassette (middle). Gene structure of a portion of the locus bearing a deletion in the NF-ATp gene (bottom). R, B, E, H, and X indicate recognition sites for the restriction enzymes EcoRI, BamHI, Eco47III, HindIII, and XbaI, respectively.

(B) Southern blot analysis of EcoRI-digested genomic DNA from representative ES clones bearing disrupted (+/−) or intact (+/+) NF-ATp alleles.

(C) Genotype analysis of mice bearing mutant NF-ATp alleles.

(D) Western blot analysis of NF-AT polypeptides in extracts of NF-ATp mutant mice. Cytoplasmic or nuclear extracts from wild-type (+/+), heterozygous (+/−), or homozygous NF-ATp mutant (−/−) mice separated by SDS–PAGE were blotted and probed with anti-NF-ATp or NF-ATc antibodies. The positions of molecular mass markers (in kDa) are indicated to the right.

(E) Rel domain polypeptides from wild-type or mutant NF-ATp genes. Autoradiogram showing protein expression of the control (lane 1) or the wild-type (lane 2) and mutant (lane 3) forms of NF-ATp Rel domains. The proteins were expressed in an in vitro transcription/translation extract and labeled with ^{35}S. The positions of molecular mass size markers (in kDa) are indicated to the left of the gel.

(F) EMSA comparing the DNA binding activities of the wild-type and mutant NF-ATp proteins. Reactions in lanes 1–4 were carried out with 0.1, 0.3, 1, and 3 μl of the control extract; lanes 5–8 and lanes 9–12 contained the same amounts of extract containing wild-type or mutant NF-ATp Rel domain, respectively.

Table 1. Cell Recoveries from NF-ATp (−/−) Spleens

Age	Splenocytes (× 10^6 cells) (+/−)	(−/−)	Ratio
12 weeks	59	47	0.79
14 weeks	84	96	1.17
16 weeks	116	142	1.22
18 weeks	76	156	2.07
Average			1.31

Splenic cell suspensions from control (+/−) or NF-ATp (−/−) mice were depleted of erythrocytes and the cell number was determined. The number of NF-ATp (−/−) cells divided by the number of control cells for each timepoint or the average for all timepoints is expressed as Ratio.

abrogation of DNA binding activity (Figure 1F). Indeed, previous analyses of NF-ATp truncation mutants indicate that residues in the region from 375–410 are critical for high affinity DNA binding (Jain et al., 1995). Although the truncated protein cannot bind DNA, it could potentially interfere with accessory proteins that interact with other NF-AT family members. However, the analyses described below did not reveal a similar phenotype in the heterozygous and the deficient mouse, indicating that a dominant negative effect did not arise from this mutation. Taken together, these data indicate that this alteration of the NF-ATp gene is a loss of function mutation.

Splenomegaly and Hyperproliferation in NF-ATp (−/−) Mice

Homozygous mutant mice were born at the expected Mendelian ratio, were fertile, and appeared healthy until at least 6 months of age. Although NF-ATp is expressed in multiple organs, including the central nervous system (Ho et al., 1994), the only gross pathologic or histologic abnormality noted was moderate splenomegaly due to an increased number of both lymphoid and erythroid cells. The relative number of mononuclear cells in spleens of NF-ATp (−/−) mice compared with control mice increases with age with an approximately 2-fold increase by 18 weeks (Table 1). Although there were increased numbers of cells in spleens from (−/−) compared with (+/−) mice, the percentage of B cells, and $CD4^+$ and $CD8^+$ T cells, was not substantially different from control littermates (Figure 2A). Proliferative responses of B lymphocytes from NF-ATp (−/−) mice to saturating doses of anti-CD40 and anti-immunoglobulin M (IgM) were slightly elevated compared with those from control littermates (Figure 2B). In addition, the proliferation of B lymphocytes from (−/−) mice stimulated with anti-IgM appeared slightly less sensitive to cyclosporin A than those from (+/−) mice. These results suggest that other NF-AT family members can largely compensate for the lack of NF-ATp in mediating calcineurin-dependent events; alternatively, signaling pathways not involving NF-AT proteins may be involved.

Treatment of $CD4^+$T cells with anti-CD3 antibody in vitro also revealed an increased proliferative capacity of NF-ATp (−/−) T cells as detected by incorporation of [^{3}H]thymidine (Figure 2C) and by cell recoveries at 48 hr following stimulation (data not shown). Further, when the NF-ATp (−/−) mice were bred onto the DO11.10 ovalbumin TCR transgenic line, there was a 1.7-fold increase in numbers of lymphocytes recovered after a primary in vitro stimulation of transgenic spleen cells with ovalbumin (data not shown). We conclude that NF-ATp is important in regulating the proliferation of $CD4^+$ T cells in response to signals delivered through the TCR.

Since cytokine expression has been proposed to play a role in thymocyte development (Carding et al., 1991), and NF-AT activity can be detected in thymocytes (Sen et al., 1994), we examined the surface phenotype of thymocytes by flow cytometry (Figure 2D). Thymocytes from NF-ATp (−/−) mice resembled (+/−) or (+/+) control littermates with respect to CD4 and CD8 levels and distribution of subsets (Figure 2D). Examination of other markers of T cell development including CD44, CD69, HSA, IL-2Rα, and TCRα/β also did not reveal any defects in the maturation of NF-ATp (−/−) thymocytes (data not shown). Thymic development thus proceeds normally in the absence of wild-type NF-ATp.

Defective Induction of CD40 Ligand and Fas Ligand in NF-ATp (−/−) Mice

The T cell hyperproliferation observed above prompted us to examine the induction of several early activation genes in NF-ATp (−/−) mice. T cells were stimulated in vivo by ligation of the TCR by injection of antibodies to CD3, and early gene activation assessed by Northern blot analysis of spleen RNA harvested 1 hr postinjection. The mRNAs encoding CD40 ligand (CD40L) and Fas ligand (FasL) were substantially reduced following in vivo stimulation with anti-CD3 antibodies, while the expression of CD69 mRNA was only moderately affected (Figure 3A). These data are consistent with the presence of NF-AT target sites in the CD40L promoter (Tsitsikov et al., 1994).

Early Impairment of IL-4 Expression in NF-ATp (−/−) Mice

NF-ATp was initially described as a transcription factor important in the regulation of multiple cytokine genes. To test whether NF-ATp is indeed important in cytokine gene regulation upon signaling through the TCR, we chose the following system. In vivo ligation of the TCR complex by treatment with anti-CD3 antibodies results in a rapid (60–90 min) and transient increase in the levels of multiple cytokine transcripts, including IL-2, IL-4, and interferon-γ (IFNγ) (Scott et al., 1990; Yoshimoto and Paul, 1994). This system allowed us to test whether NF-ATp is indeed important in the initial regulation of multiple cytokine genes. Control and NF-ATp (−/−) mice were injected with antibodies to CD3. Anti-CD3-injected (−/−) mice exhibited a striking defect in the induction of IL-4, with little or no mRNA detectable 1 hr after treatment (Figure 3B). The transcription of the IL-13, GM-CSF, and TNFα genes was also reduced, although to a somewhat lesser degree. The defect in the induction of these cytokines was observed in at least five separate experiments. Surprisingly, IL-2, the cytokine whose regulation by NF-AT has been most extensively studied, did not appear to be affected significantly by the absence

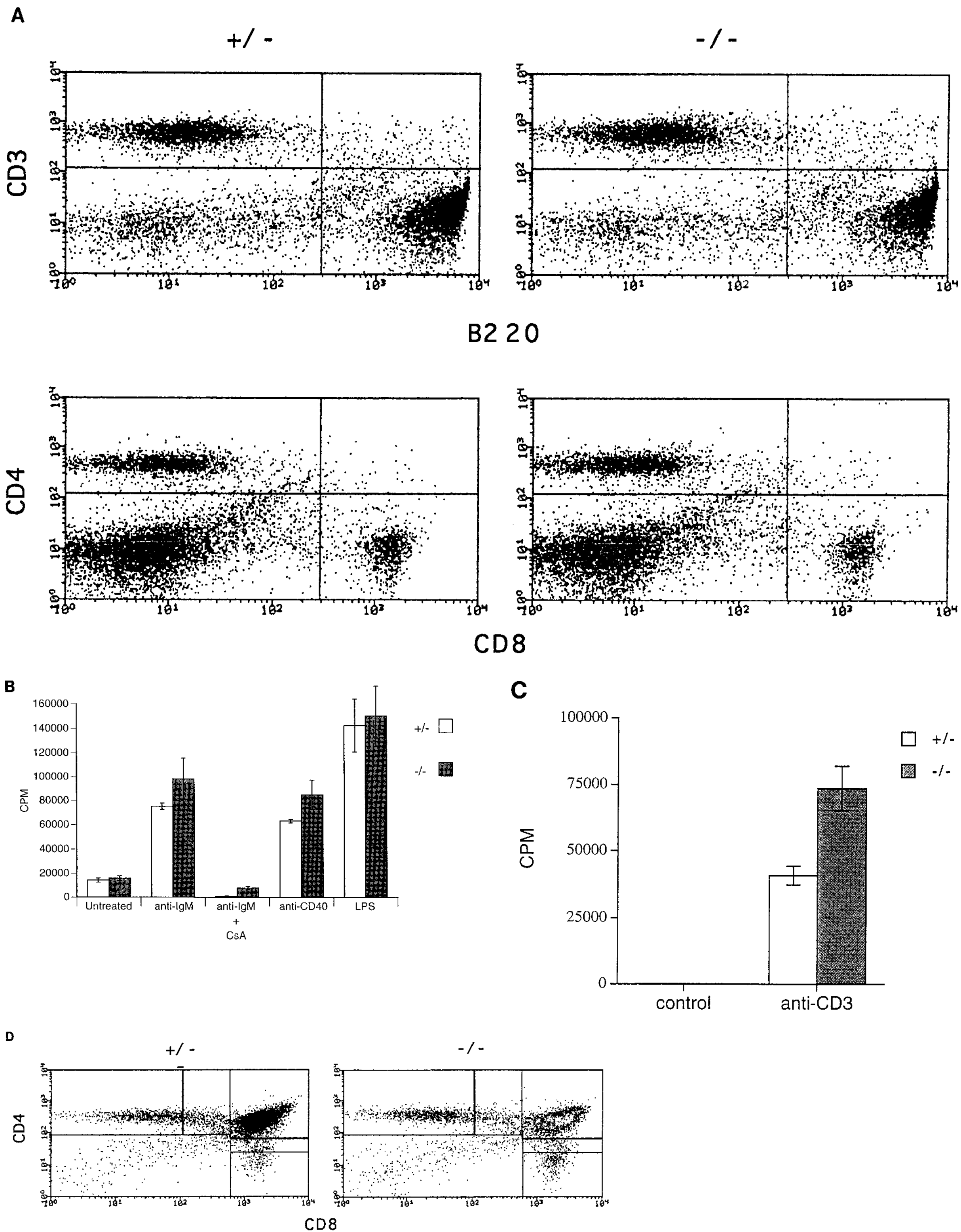

Figure 2. Flow Cytometric Analysis of Lymphocytes from NF-ATp (−/−) Mice

Splenocytes (A) or thymocytes (D) from 5-week-old control (+/−) or NF-ATp-deficient (−/−) mice were stained with fluorescein- (ordinate) or phycoerythrin- (abscissa) conjugated antibodies to murine surface markers as indicated. (B) Proliferation of splenocytes from NF-ATp-deficient mice. [^{3}H]thymidine incorporation (cpm) by control (+/−) (open bars) or (−/−) (closed bars) splenocytes in response to plate-bound anti-IgM (α-IgM), anti-CD40 (α-CD40) antibodies, or lipopolysaccharide (LPS). Anti-IgM treatment was also performed in the presence of 1 μg/ml cyclosporin A (CsA). (C) [^{3}H]thymidine incorporation (cpm) by control (+/−) (open bars) or (−/−) (closed bars) CD4$^+$ T cells in response to plate-bound anti-CD3 or control hamster immunoglobulin.

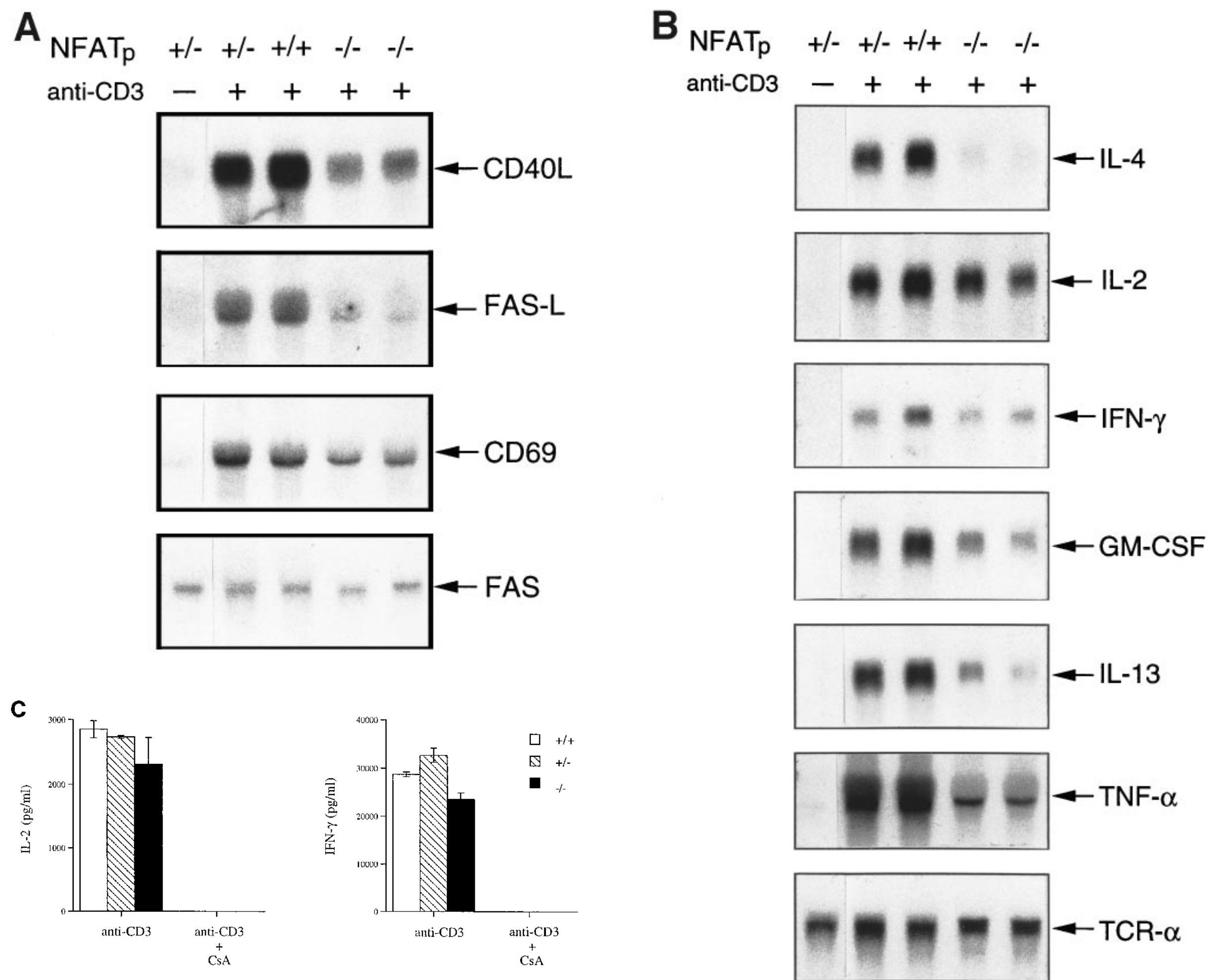

Figure 3. Northern Blot Analysis of Splenic RNA from Anti-CD3ε-Treated Mice

RNA prepared from spleens of control mice (+/+ and +/−) or NF-ATp mutant (−/−) mice injected with PBS (minus) or anti-CD3ε antibody (plus) was analyzed by Northern blot hybridization with probes specific for cytokine (A) or cell surface activation (B) genes as indicated. Hybridization with a TCRα probe was used to control for relative quantity of RNA in each sample. (C) ELISA of cytokine expression by NF-AT (−/−) lymphocytes. We incubated 5×10^5 splenocytes from control (+/−) or NF-AT mutant (−/−) mice for 48 hr in wells coated with 10 μg/ml of anti-CD3 antibody and supernatants assayed for levels of cytokines by ELISA (Pharmingen). Erythrocytes were removed as described in Figure 2.

of NF-ATp. Furthermore, transcripts for IFNγ were only modestly affected by the disruption of the NF-ATp gene.

Consistent with the in vivo data, a striking decrease in induction of IL-4 mRNA was also observed upon Northern blot analysis of RNA from spleen cells stimulated for 1 hr with anti-CD3 in vitro (data not shown). Consistent with the normal induction of IL-2 and IFNγ in vivo, primary in vitro stimulation of splenocytes from (−/−) mice with immobilized anti-CD3 antibody for 48 hr resulted in the secretion of normal levels of IL-2 and IFNγ both in the presence and absence of cyclosporin A (Figure 3C). We conclude that NF-ATp is particularly important in the early transcription of certain cytokine genes, most notably, IL-4.

Production of IL-4 at Later Timepoints as well as Th2 Development Is Increased in NF-ATp (−/−) Mice

The above experiments demonstrated a marked defect in early IL-4 production following administration of anti-CD3 in vivo. To test whether the production of IL-4 at later timepoints was impaired in NF-ATp (−/−) mice, the response of NF-ATp (−/−) or control littermates to two different stimuli that evoke the production of IL-4 and result in Th2-type responses in vitro and in vivo was examined. To dissociate the early defect in IL-4 production from an effect of NF-ATp in driving IL-4 production at later time periods, we utilized an in vitro model of T helper (Th) cell differentiation. Spleen cells from four NF-ATp (−/−) or four control mice were stimulated in vitro with anti-CD3 in the presence of recombinant IL-4, shown to be critical in priming for later IL-4 production, so that Th2 development was not dependent on endogeneous production. Cells were then restimulated with anti-CD3 and supernatants harvested after 48 hr. An increased level of IL-4 was clearly present in supernatants from NF-ATp (−/-) splenocytes as compared with control cells (Figure 4). An increase in IL-4 was also observed even in the absence of exogeneous IL-4 (data not shown). We also looked at an in vivo immune response. Immunization with trinitrophenol (TNP)–

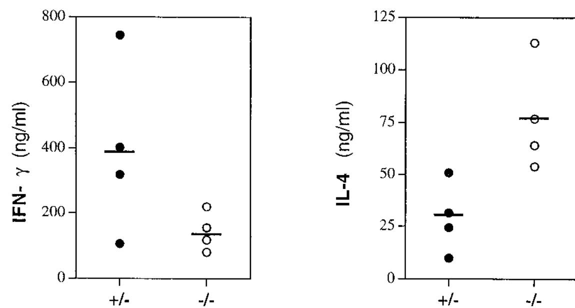

Figure 4. Cytokine Analysis of In Vitro-Differentiated T Lymphocytes from Control and NF-ATp (−/−) Mice

Spleen cells from four control and four NF-ATp (−/−) mice were activated with anti-CD3 and cultured in the presence of recombinant IL-4 as described in Experimental Procedures. After 7 days in culture, cells were restimulated and supernatants harvested 24 hr later and analyzed for the presence of cytokines by ELISA using specific antibodies according to the instructions of the manufacturer(Pharmingen). Horizontal bars represent the average cytokine expression for each group of mice.

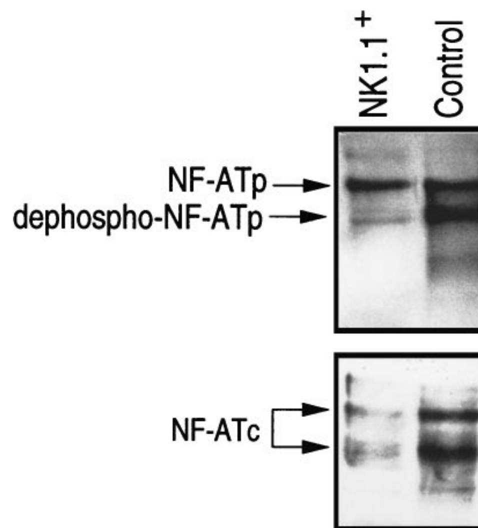

Figure 5. Western Blot Analysis of NF-AT Proteins in NK1.1$^+$ Cells

Extracts from purified NK1.1$^+$ cells and control lymph node cells were probed with NF-AT-specific antibodies as described in Figure 1D.

ovalbumin resulted in increased levels of TNP-specific serum IgE antibodies in three NF-ATp (−/−) mice as compared with three control mice (average 55.6 ng/ml versus 14.6 ng/ml). Taken together, these data demonstrate that, despite an initial defect in IL-4 production, the absence of NF-ATp during an ongoing immune response actually favors the formation of Th2 cells as evidenced both by increased IL-4 and IgE production.

NK1.1 Cells Preferentially Express NF-ATp

IL-4 is produced by multiple cell types including Th2 cells, mast cells, basophils, and a recently defined population of CD4$^+$ T cells that bear the NK1.1 surface marker (Aramburu et al., 1995; Yoshimoto and Paul, 1994). This small CD4$^+$, NK1.1$^+$ population of T cells has been shown to account for virtually all of the IL-4 produced in response to in vivo challenge with anti-CD3 (Carding et al., 1991; Sen et al., 1994). A possible explanation for the early in vivo defect in IL-4 transcription is that NF-ATp is critical in initiating IL-4 gene transcription in these cells, but not in Th2 cells where other NF-AT family members can substitute, thus providing for a Th2 response. To examine this issue, we therefore purified NK1.1$^+$ cells from splenocytes and performed Western blot analysis on lysates using anti–NF-AT-specific antibodies. While both NF-ATp and NF-ATc polypeptides were present, NF-ATp was enriched relative to NF-ATc in NK1.1$^+$ splenocytes compared with control unseparated spleen cells (Figure 5, lanes 1 and 2). Unfortunately, the prohibitively small number of these cells that can be obtained did not allow us to compare directly the binding activity of NF-ATp versus NF-ATc in nuclear extracts.

Discussion

Taken together, our analysis of NF-ATp (−/−) mice indicates a predominant and essential role for NF-ATp in balancing the transcription of the IL-4 gene during the course of an immune response, a function that cannot be compensated for by other NF-AT family members. Further, the splenomegaly and increased proliferation of lymphocytes in NF-ATp-deficient mice both at baseline and upon TCR ligation suggests a previously unknown role for this transcription factor in regulating cell growth.

The increased proliferation of lymphocytes in NF-ATp-deficient mice upon TCR ligation suggests a role for this transcription factor in regulating cell growth, potentially through the regulation of molecules such as CD40L and FasL. There are known NF-AT binding sites in the CD40L promoter, and our data raise the possibility that other genes involved in early T cell activation (such as FasL) will be found to contain NF-AT target sites. If so, then NF-ATp may play a global role in early T cell signaling. The increased lymphocyte numbers in these animals raise the possibility of a basal defect in apoptosis, dysregulation (or both) of the cell cycle, possibilities that we are currently exploring. The hyperproliferation observed in NF-ATp (−/−) lymphocytes after stimulation through the TCR may be related to the defective induction of the FasL receptor. Indeed, the genetic defect that accounts for the striking lymphoproliferation observed in the *gld* mouse strain is a mutation in the FasL gene resulting in a nonfunctional protein (Takahashi et al., 1994).

The cytokine phenotype of the NF-ATp-deficient mice revealed an important role for this factor in the regulation of the IL-4 gene over time. Interestingly, a similar defect in the early transcription of the IL-2 gene in vivo was not observed, suggesting a more prominent role for other NF-AT family members in the regulation of this cytokine. At early timepoints, NF-ATp is critical for initiating IL-4 gene transcription and other NF-AT family members cannot substitute for NF-ATp. However, as the immune response progresses, the absence of NF-ATp actually results in an increased generation of IL-4. We conclude that NF-ATp is critical for the balanced transcription of the IL-4 gene over time.

The mechanisms involved in the differential effects of NF-ATp on early cytokine transcription are not clear but

may be related to the types of lymphocytes that produce particular cytokines. There are two possible, and not mutually exclusive, explanations for these seemingly paradoxical effects of NF-ATp on IL-4 production. The first explanation relies on differences in the kinetics of binding of the different NF-AT family members to the IL-4 promoter. It is reasonable to hypothesize that NF-ATp, which is present in unstimulated T cells, might be the first NF-AT protein to translocate to the nucleus and bind DNA after T cell stimulation, while NF-ATc, which is induced after stimulation, could replace it at later timepoints. A deficiency in NF-ATp would then result in an early and transient defect in IL-4 as observed here. Indeed, in preliminary studies, levels of transcripts for IL-4 in CD4 T cells stimulated with anti-CD3 for varying lengths of time reveals an early defect followed by an increase at later timepoints (A. M. R., unpublished observations). The second explanation involves the preferential expression of NF-ATp in certain cell types responsible for the early, but not late, release of IL-4 and is supported by the experiments described above (Figure 5). Interestingly, it has recently been shown that human natural killer (NK) cells, upon ligation of the CD16 receptor, contain only NF-ATp and not NF-ATc binding activity (Aramburu et al., 1995). The presence of enhanced Th2 development in NF-ATp-deficient mice despite the impairment in the early release of IL-4 differs from the recently reported defect in Th2 development in SJL mice that have greatly diminished numbers of NK1.1 cells (Yoshimoto et al., 1995). Strain background or the compensatory increase in IL-4 production that occurs at later time periods in NF-ATp-deficient mice may account for these differences. Appropriate backcrossing of NF-ATp (−/−) mice to strains (e.g., C57BL/6) that express the NK1.1 marker should allow us to establish definitively the role of NF-ATp in cytokine gene transcription in this interesting subpopulation of T cells.

The molecular basis for the effect of NF-ATp in controlling IL-4 production at later timepoints is unknown at present. The increase in IL-4 and IgE levels observed in in vitro-differentiated T cells and in the response to TNP-ovalbumin demonstrated a clear preference for the expansion of Th2 cells. Indeed, we have also found increased amounts of IL-4 in spleen cells harvested from NF-ATp (−/−) mice 5 days after in vivo challenge with anti-IgD antibodies when compared with control animals (M. R. H. and A. M. R., unpublished data).

Taken together, our analysis of NF-ATp (−/−) mice indicates a critical role for NF-ATp in the regulation of IL-4 expression and in Th2 development. The extent of overlap in the function of the other NF-AT family members in regulating cell proliferation and transcription of cytokine and surface receptor genes will be best assessed by the generation of mice deficient for these proteins.

Experimental Procedures

Disruption of the NF-ATp Gene

A cDNA fragment corresponding to the Rel homology domain of the NF-ATp gene was used as a probe to isolate clones from a 129/sv genomic library. An Eco47III–HindIII fragment from the NF-ATp genomic clone was replaced with a thymidine kinase promoter–*neo*–poly(A) cassette from pMC1*neo*poly(A) (Stratagene). The replacement construct was prepared by transferring a BamHI to XhoI fragment containing 6 Kb of 5′ NF-ATp sequence, the *neo* cassette, and 3.5 kb of 3′ NF-ATp sequence, to a plasmid encoding the thymidine kinase drug resistance gene. ES cell clones transfected with the targeting construct were selected for drug-resistance in 180 μg/ml G418 and 2 μM gancyclovir. To screen for homologous recombination, genomic DNA from drug-resistant clones was digested with EcoRI and analyzed by Southern blot hybridization to a 0.9 Kb BamHI–EcoRI probe (Figure 1, probe A). This probe detects an 11 kb EcoRI fragment in the wild-type allele and a 7 kb fragment in the *neo* disrupted allele. NF-ATp disruption in selected clones was also confirmed by analysis with a probe specific for the 3′ end of the deletion (Figure 1, probe B) (data not shown). Three different primers indicated in Figure 1 were used to amplify a 500 bp fragment from the wild-type allele or a 390 bp band from the disrupted NF-ATp allele using genomic DNA from individual mice. The common primer NpIn1 (5′-GCAAGCCTCAGTGACAAAGTATCCACTTCA-3′) was used at 1 μM and the two allele-specific primers NpEx1 (5′-CCACGA GCTGCCCATGGTGGAGAGACAAGA-3′) and *neo*PCR (5′-AGCGTT GGCTACCCGTGATATTGCTGAAGA-3′) were used at a concentration of 0.5 μM.

Western Blot Analysis of NF-AT Polypeptides

Splenic extracts were prepared by treating 1×10^6 splenocytes with lysis buffer containing 1% NP-40 and separating nuclei by centrifugation at $500 \times g$. NK1.1$^+$ cells were prepared from six MHC class II–deficient C57BL/6 mice by depletion of CD8$^+$ and HSA$^+$ cells by antibody-dependent complement lysis and adsorption to anti-murine IgG-coated plates. Remaining live cells were stained with biotinylated anti-NK1.1 plus streptavidin–phycoerythrin and anti-CD4–flourescein isothiocyanate (Pharmingen), sorted into NK1.1$^+$ and NK1.1$^-$ populations, and used to prepare whole cell extracts. Extracted proteins were separated by 6% PAGE followed by electrotransfer to nitrocellulose membranes. Proteins were detected by probing with MAbs specific for NF-ATp (4G6-G5 specific for amino acids 145–189 of NF-ATp, gift of L. Timmerman) or NF-ATc (7A6) (Ho et al., 1995) plus horseradish peroxidase–conjugated sheep F(ab′)$_2$ anti-mouse immunoglobulin and enhanced chemiluminescence according to the instructions of the manufacturer (Amersham).

DNA Binding Assay of Mutant NF-AT Polypeptides

The mutant form of NF-ATp cDNA was isolated by RT–PCR from mRNA obtained from spleens of (−/−) mice. DNA sequence analysis indicated that in the mutant form of NF-ATp, the residues 398–444 in the C-terminal half of the Rel domain were deleted. A portion of the wild-type murine NF-ATp cDNA (McCaffrey et al., 1993) encoding amino acids 187–519, which spans the Rel homology domain, and the equivalent region from the mutant NF-ATp cDNA, were subcloned into the T7 expression vector TP7 (Hoey et al., 1995). Proteins were expressed in an in vitro transcription/translation reticulocyte extract (TnT, Stratagene) and labeled with [^{35}S]methionine. DNA binding activity was tested by gel mobility shift assay as previously described (Hoey et al., 1995), using a labeled sequence corresponding to the distal NF-AT site from the murine IL-2 promoter (5′-CAAAG AGGAAAATTTGTTTCATA-3′).

Flow Cytometric Analysis

Single cell suspensions of 1×10^6 cells were incubated at 4°C with fluorescein- or phycoerythrin-conjugated antibodies (Pharmingen) and analyzed using a FACScan (Becton Dickenson) flow cytometer using Lysis software. Dead cells were gated out by propidium iodide (0.5 μg/ml) exclusion. Erythrocytes were depleted from splenic cell suspensions by treatment with 8.3 g/ml NH_4Cl in 10 mM Tris–HCl (pH 7.5). Staining and analysis was performed on at least five animals of each genotype with similar results.

Analysis of Cell Proliferation

Splenocytes (5×10^5) from control (+/−) or NF-ATp (−/−) mice were incubated per well of a 96-well plate previously coated with antibody. Cultures were pulsed at 48 hr with 1 μCi of [^{3}H]thymidine (New England Nuclear) and harvested 14 hr later. For analysis of B

cell proliferation, plates were coated with 10 μg/ml anti-IgM, or 20 μg/ml anti-CD40. IL-4 was added to a concentration of 100 U/ml in samples treated with fixed anti-IgM antibodies. Lipopolysaccharide and cyclosporin A were added at a concentration of 20 μg/ml and 1 μg/ml, respectively. $CD4^+$ T cells were purified from splenocytes and lymph node cells by depletion of MHC class II^+, $CD8^+$, and HSA+ cells using antibody-dependent complement lysis and adsorption to anti-IgG-coated plates. We incubated 1×10^5 purified $CD4^+$ cells per well previously coated with anti-CD3ε (1 μg/ml) antibody and assayed for proliferation as described above.

In Vivo Anti-CD3 Analysis

Mice (5 weeks old) were injected with 100 μl PBS or PBS containing 4 μg of purified anti-CD3ε (145–2C11) antibody into the orbital sinus. At 1 hr postinjection, mice were sacrificed and spleens snap frozen in liquid N_2. Splenic RNA was prepared by the guanidinium/CsCl method (Chirgwin et al., 1979) and 10 μg fractionated by electrophoresis on 1.2% agarose, 6% formaldehyde gels. Identical gels were blotted to Hybond N (Amersham) and hybridized with at least 1×10^6 cpm of random primer-labeled cDNA fragments per milliliter of QuickHyb solution according to the instructions of the manufacturer (Stratagene). cDNA fragments were purified from plasmids: IL-2, IL-4, and IFNγ (Yoshimoto and Paul, 1994), GM–CSF (Miyatake et al., 1985), IL-10 (Hsu et al., 1990), TNFα (Caput et al., 1986), TCRα (Saito et al., 1984), CD40L (Armitage et al., 1992), Fas-L (Takahashi et al., 1994), CD69 (Ziegler et al., 1994), Fas (Rouvier et al., 1993), or amplified using PCR with specific primers for IL-13 (McKenzie et al., 1993) (IL-13.5, 5′-CTTGCTTGCCTTGGTGGTCTCGC-3′, and IL-13.3, 5′-GCAGTTTTGTTATAAAGTGGGCT-3′) and cDNA prepared from anti-CD3-stimulated D10.G4 T cell RNA (Kaye et al., 1983).

Measurement of Cytokine and Antibody Levels by ELISA

We incubated 5×10^5 splenocytes from control or NF-AT-deficient mice for 48 hr in wells coated with 10 μg/ml of anti-CD3ε antibody and supernatants assayed for levels of IL-2 or IFNγ by ELISA (Pharmingen). Erythrocytes were depleted from splenic cell suspensions by treatment with 8.3 g/ml NH_4Cl in 10 mM Tris–HCl (pH 7). Mice injected with TNP–ovalbumin were bled by the tail vein to obtain preimmunization serum. At 7 days postinjection, mice were sacrificed and bled by cardiac puncture. IgG1 and IgE were analyzed by ELISA as previously described (Markowitz et al., 1993).

In Vitro T Cell Differentiation

For in vitro T cell differentiation assays, 2×10^6 spleen cells/ml were cultured in medium and stimulated with 1 μg/ml plate-bound anti-CD3 in the presence of 1000 U/ml IL-4. Cultures were fed with fresh medium after 4 days, washed, and restimulated after 7 days with plate-bound anti-CD3 in the absence of any additional rIL-4. Supernatants were collected 24 hr later and cytokines measured by ELISA as above.

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