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Inhibitory Function of Two NFAT Family Members in Lymphoid Homeostasis and Th2 Development

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Summary

Nuclear factor of activated T cells (NFAT) is a critical regulator of early gene transcription in response to TCR-mediated signals. Here, we show that mice lacking both NFATp and NFAT4 develop a profound lymphoproliferative disorder likely due to a lowered threshold for TCR signaling coupled with increased resistance to apoptosis secondary to defective FasL expression. NFAT mutant mice also have allergic blepharitis, interstitial pneumonitis, and a 10^3 to 10^4 fold increase in serum IgG1 and IgE levels, secondary to a dramatic and selective increase in Th2 cytokines. This phenotype may be ascribed to unopposed occupancy of the IL-4 promoter by NFATc. Our data demonstrate that lymphoid homeostasis and Th2 activation require a critical balance among NFAT family members.

Introduction

Nuclear factor of activated T cells (NFAT) is a critical regulator of early gene transcription in response to TCR-mediated signals. First identified as a transcriptional regulatory complex important for the expression of the T cell cytokine, IL-2 (Shaw et al., 1988; Crabtree, 1989; Rao et al., 1997), NFAT target sequences have since been identified in the promoters of multiple cytokine genes, including IL-4, GM-CSF, IL-3, and TNFα (Del Prete et al., 1991; Miyatake et al., 1991; Chuvpilo et al., 1993; Goldfeld et al., 1993; Masuda et al., 1993; Rooney et al., 1994, 1995; Cockerill et al., 1995), as well as in the promoters of the FasL and CD40L cell surface receptors (Tsitsikov et al., 1994; Latinis et al., 1997). NFAT expression has also been observed in B lymphocytes (Choi et al., 1994; Venkataraman et al., 1994) as well as in multiple cell types (Timmerman et al., 1997) within the innate immune system (NK, macrophage, and mast cells), although the endogenous target genes regulated by NFAT in these cells have not yet been identified. More recently, NFATc has been shown to regulate HIV-1 replication in T cells (Kinoshita et al., 1997) and to be critical in cardiac valve formation (Luis de la Pompa et al., 1998; Ranger et al., 1998a).

The NFAT complex contains a cytoplasmic subunit and a *ras*/protein kinase C–responsive inducible nuclear component (Flanagan et al., 1991) composed in part of AP-1 family member proteins (Jain et al., 1992, 1993; Boise et al., 1993; Rooney et al., 1995). Following activation through the T cell receptor (TCR), BCR, or CD40 accessory molecules, the cytoplasmic subunit translocates into the nucleus. NFAT nuclear translocation is controlled by the calcium-regulated phosphatase calcineurin that is a target for the immunosuppressive drugs cyclosporin A (CsA) and FK506 (Flanagan et al., 1991; Clipstone and Crabtree, 1992; Beals et al., 1997). Treatment of T cells with CsA or FK506 prevents NFAT nuclear translocation and subsequent activation of cytokine gene transcription (Emmel et al., 1989).

There are currently four NFAT genes encoding the cytoplasmic subunit , NFATp (NFATc2, NFAT1), NFATc (NFATc1, NFAT2), NFAT3 (NFATc4), and NFAT4 (NFATc3, NFATx) (McCaffrey et al., 1993; Northrop et al., 1994; Ho et al., 1995; Hoey et al., 1995; Masuda et al., 1995). In vitro, all these factors can bind to and transactivate the promoters of multiple cytokine genes, although in T cell extracts, only NFATc and NFATp bind to these sites (Timmerman et al., 1997). The sequence variability among NFAT family members in N- and C-terminal regions that contain transactivation domains (Luo et al., 1996), together with their differing tissue distribution (Masuda et al., 1995), suggested functional differences among NFAT family members.

Indeed, the phenotype of mice harboring single mutations at the NFATc, NFATp, and NFAT4 loci demonstrated unique functions for each of these family members. Mice lacking NFATc in the lymphoid system (as evaluated by RAG-$2^{-/-}$ blastocyst complementation) have mildly impaired proliferation and a selective decrease in IL-4 production (Ranger et al., 1998b; Yoshida et al., 1998) consistent with a function of NFATc as a positive regulator of the immune system. Conversely, some evidence that NFATp and NFAT4 might negatively control proliferative responses, Th2 cell formation, and lymphocyte activation was obtained from the characterization of NFATp- and NFAT4-deficient animals (Hodge et al., 1996; Xanthoudakis et al., 1996; Kiani et al., 1997; Oukka et al., 1998). We and others (Hodge et al., 1996; Xanthoudakis et al., 1996) demonstrated that mice lacking NFATp display modest splenomegaly, T and B cell hyperproliferation, and cytokine dysregulation during the course of an immune response with a moderate increase in Th2-type cytokines. Mice lacking NFAT4 have normal cytokine production although an increased percentage (50% versus 20% in control) of T and B cells display a phenotype characteristic of memory/activated cells (Oukka et al., 1998). The modest inhibitory effects of the single NFATp and NFAT4 gene deletions, however, suggested either that the repressive effect of each was independent but not profound or that these proteins were functionally redundant.

To test these hypotheses, we intercrossed NFATp and NFAT4 null mice to generate mice doubly deficient in these two NFAT proteins (DKO). DKO mice display massive lymphadenopathy and selective activation of the Th2 compartment, demonstrating that NFAT family members must act together to generate a normal, balanced immune response.

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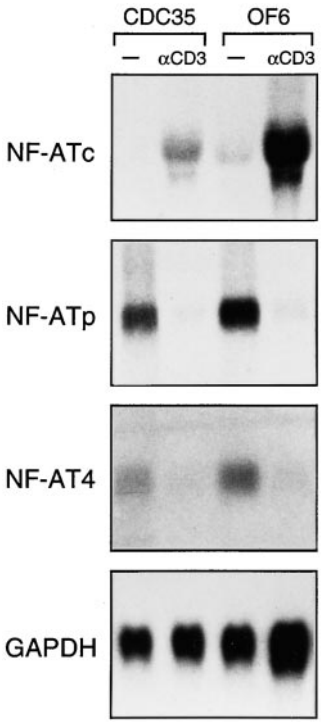

Figure 1. Induction of NFATc and Repression of NFATp and NFAT4 Expression upon T Cell Activation

The T helper clones CDC35 (Th2) and OF6 (Th1) were stimulated with plate-bound anti-CD3 (1 μg/ml) and RNA prepared at 6 hr. Blots were hybridized with cDNA probes encoding the NFATc, NFAT4, and NFATp genes (Hoey et al., 1995; Hodge et al., 1996)

Results and Discussion

Opposing Patterns of Expression of NFATs upon T Cell Activation

The phenotype of mice singly deficient in each of the three NFAT proteins as described above raised the possibility that NFATc acts as an activator of, and NFAT4 and NFATp as repressors of, the immune response. Interestingly, while examination of NFAT family member expression in T helper clones upon activation revealed a rapid induction of transcripts encoding NFATc, a downregulation of both NFATp and NFAT4 expression was observed (Figure 1). These data are consistent with a function for NFATp and NFAT4 as repressors of immune activation.

Allergic Blepharitis, Pneumonitis, and Lymphadenopathy in Mice Lacking Both NFATp and NFAT4

To test whether the repressive effects of NFATp and NFAT4 were functionally redundant, or independent, mice bearing a targeted disruption of each of these alleles were intercrossed. DKO mice were born at a less than Mendelian frequency similar to what we had observed for mice lacking only NFAT4 (Oukka et al., 1998). DKO mice demonstrated modest growth retardation and developed severe bilateral blepharitis by approximately 4 weeks after birth. In all DKO animals examined ($n = 5$), the eyelids displayed edema and ulceration with underlying granulation tissue and a marked inflammatory infiltrate (data not shown). Examination of the lungs revealed an acute and chronic interstitial pneumonitis characterized by an intense inflammatory infiltrate consisting of lymphocytes, macrophages, plasma cells, neutrophils, and mast cells (Figure 2A). There was no evidence of inflammatory disease in the heart, kidney, or liver, and no evidence of renal or pancreatic dysfunction as judged by the absence of urinary glucose and protein. DKO mice exhibited massive splenomegaly and lymphadenopathy by 7 weeks of age (Figure 2B), far more severe than the modest splenomegaly observed for the single NFATp deletion. Histological analysis of the spleen (Figures 2C–2H) and lymph node (data not shown) revealed disruption of the normal architecture by numerous granulomas (Figure 2F) and a marked increase in mast cells (Figure 2G) and eosinophils (Figure 2H) as compared to the wt control (Figures 2C–2E). Examination of the bone marrow revealed a dramatic increase in eosinophils (approximately 60% of BM cells) (Figure 2J) compared to wt (Figure 2I). The absence of multiorgan lymphoid infiltration and immune complex–mediated pathology distinguishes the NFAT DKO from other mouse strains that display massive lymphadenopathy such as CTLA-4 and IL-2 receptor α–deficient and TRAF2 dominant negative mutant mice (Sadlack et al., 1995; Tivol et al., 1995; Waterhouse et al., 1995; Willerford et al., 1995). This is consistent with the normal protein or RNA levels of these genes in NFAT DKO lymphocytes (A.R. and M.O., unpublished data).

Hyperactivation of DKO T and B Lymphocytes

In contrast to the increased size of the peripheral lymphoid organs, the thymus was normal or modestly smaller than wild-type with a somewhat increased percentage of CD8 SP thymocytes. Flow cytometric analysis of peripheral lymphoid organs revealed a modest increase in the percentage of $B220^+$ cells in LN and a corresponding decrease in $CD3^+$ T cells in both the spleen (Figure 3B) and LN (Figure 3C). The ratio of $CD4^+$/$CD8^+$ T cells was decreased in LN and increased in spleen. This may be secondary to increased apoptosis of CD8 cells in spleen versus LN secondary to hyperactivation (see below). An increased number of non-T, non-B cells of unclear identity, possibly mast cells, were present. In the absence of NFATp and NFAT4, there was a dramatic increase in the percentage of peripheral T cells with a memory/activated phenotype as indicated by low levels of Mel-14 and CD45RB and elevated levels of CD44 (data not shown) and CD69 on spleen (Figure 3D) and LN cells (data not shown). The activated/memory cells did not represent a clonal expansion of T cells as evaluated by their Vβ and Vα usage (A.R. and M.O., unpublished data). DKO B cells were also hyperactivated as demonstrated by upregulation of MHC class II and increased numbers of IgM-negative $B220^+$ cells (Figure 3E).

DKO T Cells Display Increased Spontaneous Proliferation and Hyperresponsiveness to TCR-Mediated Signals

The massive lymphadenopathy in the DKO mice could potentially be explained by increased proliferation and/or

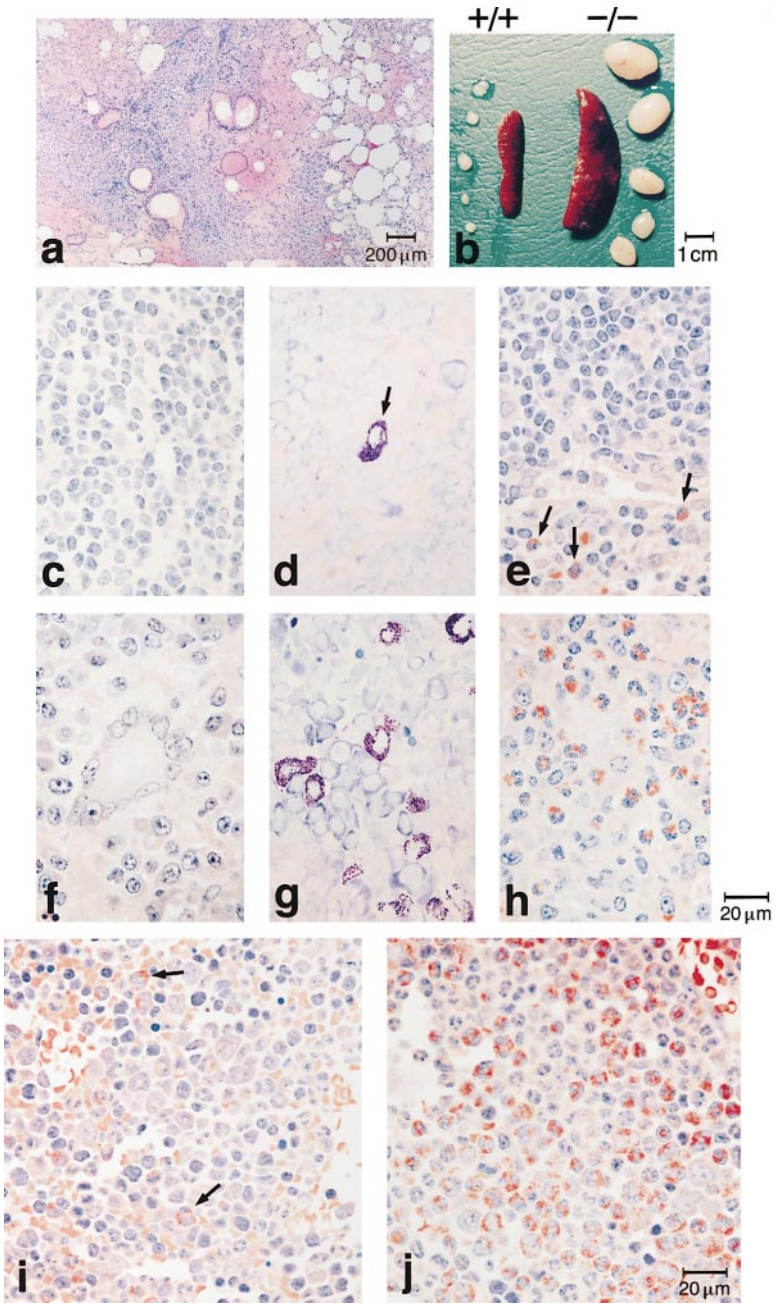

Figure 2. Interstitial Pneumonitis and Lymphadenopathy with Mast Cell and Eosinophil Infiltrates and Granulomas in NFAT DKO Mice

(A) Interstitial pneumonitis in a DKO mouse characterized by infiltration of mast cells, macrophages, plasma cells, neutrophils, and lymphocytes (40×). (B) Lymphadenopathy and splenomegaly in DKO mice. Shown are the spleen and lymph nodes (L to R: inguinal, popliteal, axillary, and cervical) removed from wild-type BALB/c control and DKO mice aged 14 weeks. Representative of five animals. (C-E) Spleen of wt control mouse with arrows showing the occasional mast cell (D) and eosinophil (E). Stains are Congo Red (C and E) and toluidine blue (D). Magnification, 200×. (F) Granulomas in spleen of DKO mice. Congo Red–stained section of spleen from wild-type (C) and DKO (F) mice (200×). The architecture of the spleen and lymph node (data not shown) of the DKO is disrupted by granulomatous lesions containing multinucleated giant cells. Congo Red stain, 200×. (G) Increased mast cell numbers in DKO spleen. Toluidine blue–stained spleen sections from DKO mice (200×) show numerous mast cells identified by intense staining of intracellular granules. (H) Increased eosinophils in DKO spleen. Congo Red–stained spleen section (200×). (I) Wt bone marrow stained with Congo Red with arrows pointing to eosinophils (200×). (J) Increased numbers of eosinophils in DKO bone marrow. Congo Red stain, 200×.

decreased apoptosis. A 2- to 3-fold increase in spontaneous proliferation of freshly isolated DKO splenocytes and LN cells (Figure 4A) and in the percentage of CD4 and CD8 T cells and B cells in S phase as evaluated by propidium iodide staining (Figure 4B) was observed. Since NFAT responds to signals delivered by the TCR, this increased proliferation might reflect a lower threshhold for activation via TCR signals. Because so few naive T cells were present in the NFAT DKO, we could not obtain sufficient numbers to test this in naive resting cells. Therefore, we used wild-type and DKO LN T cells that had been stimulated in culture, rested for an 8 day period, and then restimulated with varying doses of anti-CD3 antibody. DKO T cells displayed markedly increased proliferation in response to ligation of CD3 compared to wild-type T cells at all doses tested with a marked shift to the left of the dose response curve (Figure 4C) and this proliferation was blocked by addition of CsA (data not shown). These data suggest that NFATp and NFAT4 act, like CTLA-4, to raise the threshold at which a T cell can be activated by occupancy of the TCR. In the absence of both NFATp and NFAT4, this threshhold is lowered, resulting in hyperproliferation and hyperactivation. Since the NFATp and NFAT4 single gene deletions do not show this dramatic phenotype (Hodge et al., 1996; Xanthoudakis et al., 1996; unpublished data), this function can clearly be performed by both NFATp and NFAT4, but not by NFATc, proteins. Indeed, NFATc-deficient T cells displayed hypoproliferation, suggesting that NFATc serves the opposite function—to raise the threshold at which a T cell can respond to signals (Ranger et al., 1998b; Yoshida et al., 1998).

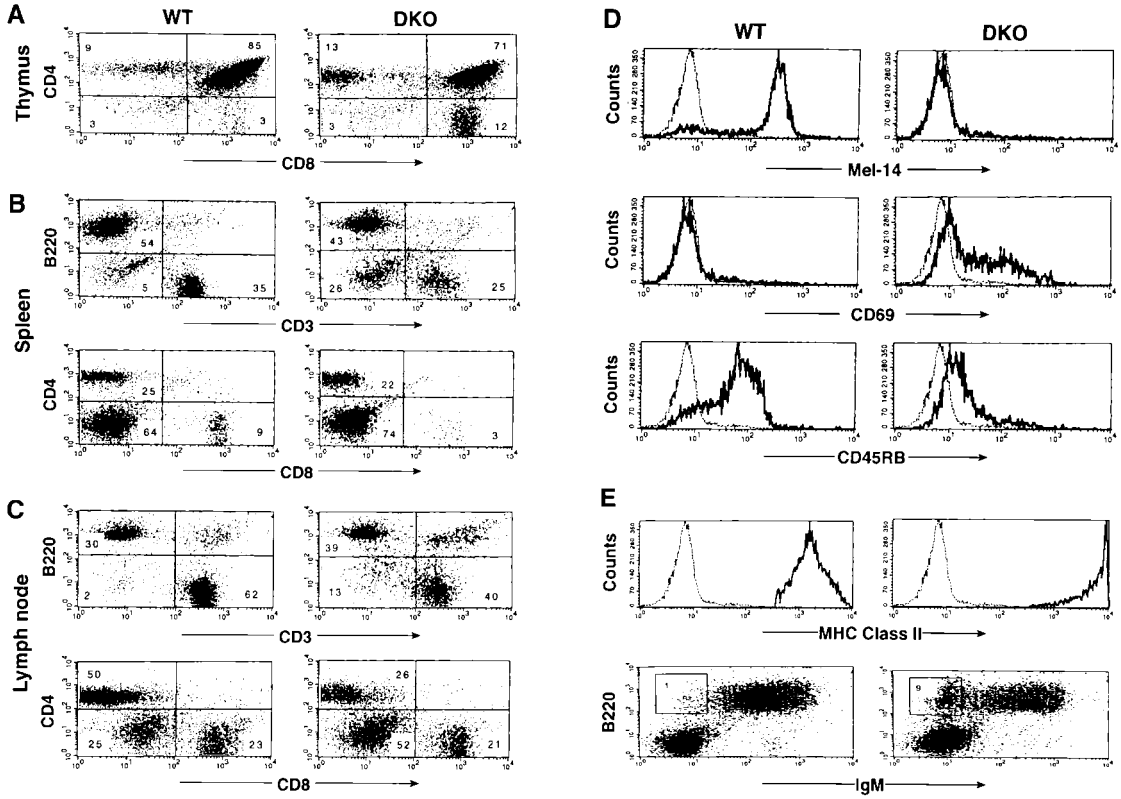

Figure 3. Flow Cytometric Analysis of Lymphocytes from Wild-Type and DKO Mice from Thymus, Spleen and Lymph Node

Single cell suspensions were stained with antibodies as indicated: anti-CD4-TC, anti-CD8-PE, anti-B220-PE, and anti-CD3-FITC. (D and E) Increased memory/activated T and B cells in DKO spleen. (D) Data were electronically gated on $CD3^+$ cells and levels of CD69, and CD45RB were evaluated with specific FITC-conjugated antibodies (solid line). A FITC-conjugated isotype control was included (dotted line). (E) Data were electronically gated on $B220^+$ cells and MHC class II $I\text{-}A^d$ and IgM expression evaluated as above. All data are representative of three mice aged 7–14 weeks.

Resistance to Apoptosis and Defective Induction of Fas Ligand in DKO T Cells

In addition to an increased sensitivity to positive signals delivered via the TCR, DKO T cells also might have an altered sensitivity to signals that result in cell death (apoptosis). Given our earlier observation that the induction of FasL 1 hr after stimulation with anti-CD3 was impaired in mice lacking NFATp, we examined the induction of FasL in DKO T cells at later time points after TCR stimulation. Northern blot analysis revealed a nearly complete absence of FasL transcripts in DKO T cells after 6 hr of stimulation with anti-CD3 (Figure 4D). Consistent with the impaired expression of FasL, DKO T cells displayed a marked resistance to antigen-induced cell death upon anti-CD3 stimulation (Figure 4E).

These data and that of Koretsky and colleagues (Latinis et al., 1997) demonstrate that NFAT proteins regulate the FasL gene in vivo. However, the NFAT DKO phenotype cannot be solely explained on the basis of impaired FasL expression, as evidenced by comparison with the phenotype of *gld* mice. *gld* animals have a slower onset of lymphadenopathy (3–5 months) secondary to expansion of a DN $B220^+$ T cell subpopulation not present in NFAT DKO mice and manifest autoimmunity with immune complex glomerulonephritis (Cohen and Eisenberg, 1991; Watanabe-Fukunaga et al., 1992; Takahashi et al., 1994). NFAT DKO mice have rapid onset (by 7 weeks) of lymphadenopathy comprised of SP T cells and B cells and no evidence of autoimmune disease although they do have anti-nuclear antibodies (A.R., unpublished data). We conclude that the massive splenomegaly and lymphadenopathy observed in DKO mice is due both to increased proliferation secondary to a lowered threshold for TCR-mediated positive signals and to compromised FasL expression resulting in defective apoptosis over time.

Extreme Overproduction of Th2 Cytokines and Th2-Dependent Immunoglobulins in DKO Mice

The presence of blepharitis, interstitial pneumonitis, increased mast cell numbers, and granulomas in the spleen and LN of NFAT DKO mice suggested overproduction of Th2-type cytokines in these animals. Indeed, a dramatic increase in Th2 cytokine production in response to anti-CD3 stimulation of DKO spleen and LN cells was observed (Figure 5A). The amount of IL-4 produced by unfractionated DKO spleen cells in a primary response was approximately 75-fold greater than wild-type, and this increased to 600-fold in a secondary response (Figure 5C). The levels of other Th2-type cytokines, IL-5, IL-6, and IL-10, were also very high (Figure

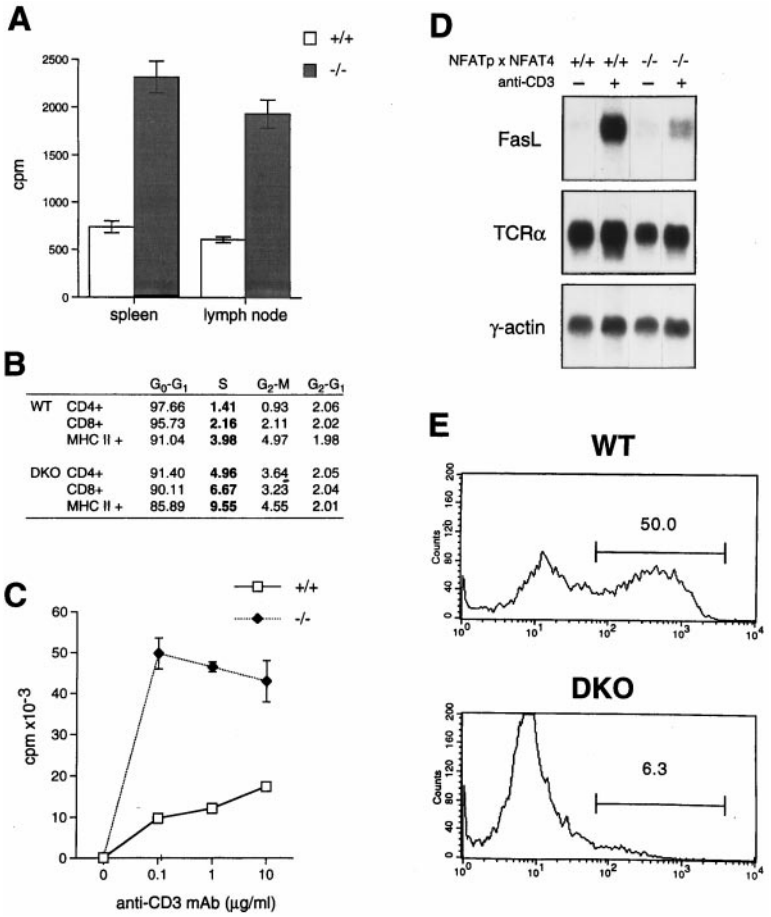

Figure 4. Hyperproliferation and Decreased Apoptosis in DKO T Cells Correlates with Lowered TCR-Signaling Threshhold and Impaired FasL Expression

(A) Increased spontaneous proliferation of DKO LN cells (2.5×10^6 cells/ml, 96-well plate). 1 μCi/well of [^{3}H]-thymidine was added at 6 hr and cells harvested 12 hr later. (B) Modest increase in the percentage of freshly isolated CD4, CD8, and MHC class II$^+$ cells in S phase as evaluated by propidium iodide staining. (C) Increased sensitivity to signaling via the TCR as measured by proliferation of DKO T cells to varying doses of anti-CD3. Data are representative of three experiments. (D) Impaired induction of FasL in response to anti-CD3 antibody treatment in DKO T cells. Unfractionated LNC were stimulated with 1 μg/ml of anti-CD3 antibody for 6 hr. RNA blots were hybridized with a FasL-specific probe (Takahashi et al., 1994). An actin probe was used to verify equal RNA loading and a TCRα probe used to control for differences in T cell numbers. (E) Increased resistance to AICD in DKO T cells. LN T cells from a tertiary stimulation were restimulated with 1 μg/ml of plate-bound anti-CD3 antibody for 20 hr and TUNEL assay performed. The percentage of cells undergoing apoptosis is shown above the bar.

5A). In contrast, levels of the Th1-type cytokines, IFN-γ, IL-2, and TNFα, were modestly to significantly decreased (Figure 5B). Levels of GM-CSF, a cytokine produced by both Th1 and Th2 cells, were elevated (Figure 5A) and together with IL-4 likely account for the formation of granulomas and infiltration of mast cells observed (Wynn and Cheever, 1995). This overproduction of IL-4 resulted in a massive increase in the levels of the IL-4-dependent isotypes IgG1 (2 to 3 logs) and IgE (3 to 4 logs) in the sera of unimmunized mice (Table 1). The extraordinarily large amounts of IgE and IgG1 produced far exceed those present in single NFATp-deficient mice (Hodge et al., 1996; Xanthoudakis et al., 1996) or in mice that overexpress the IL-4 gene itself (Tepper et al., 1990). In contrast to other mouse strains with lymphoproliferative disorders (CTLA4-deficient, *lpr* and *gld* strains), the hypergammaglobulinemia was very isotype-specific, as levels of IgG2a and 2b were only minimally increased (Table 1). This is consistent with the modest reduction of IFNγ observed in a primary stimulation of NFAT DKO lymphocytes.

Constitutive Nuclear Localization of NFATc in DKO T Cells

NFAT family members have traditionally been thought to activate cytokine gene expression, and the phenotype of lymphocytes lacking NFATc is consistent with that idea (Ranger et al., 1998b; Yoshida et al., 1998). Here, we have revealed that two other NFAT family members can act as repressors of lymphocyte proliferation and Th2 development. Disruption of the balance between NFAT family members such that NFATc, the family member necessary for optimal IL-4 gene transcription (Ranger et al., 1998b; Yoshida et al., 1998), is unopposed then results in the extraordinary increase in IL-4 production. To investigate the status of NFATc in these mice, we performed Western blot analysis on nuclear extracts prepared from cultured wild-type and DKO T cells that had been rested for 8 days and then activated for 6 hr with PMA/ionomycin. In nuclear extracts prepared from wild-type resting T cells, little NFATc protein was detected consistent with the cytoplasmic location of NFATc in unactivated cells. Upon activation by PMA/ionomycin, the three dephosphorylated isoforms of NFATc can clearly be detected. In DKO T cells, in contrast, large amounts of dephosphorylated NFATc peptides are present in the nucleus in the resting state and actually decrease upon activation suggesting that these cells are already activated (Figure 6A). A particular predominance of the smallest MW isoform is observed for reasons we do not understand. To determine whether NFATc proteins bound to the IL-4 promoter in DKO T cells, EMSA was performed with nuclear extracts prepared from freshly isolated LN cells using an oligonucleotide from the IL-4 promoter containing the proximal NFAT site as probe (Figure 6B). A complex that was

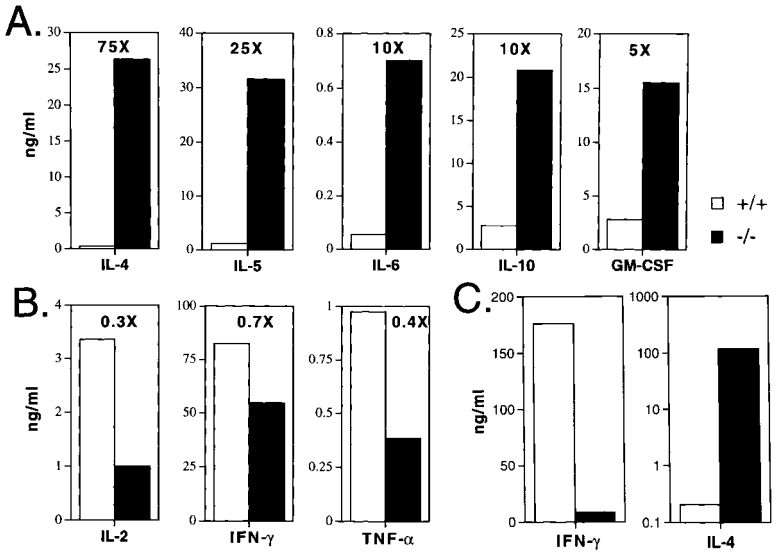

Figure 5. Extremely Elevated Levels of Th2 Cytokines and IgE and IgG1 Antibodies in DKO Mice

(A) Increased Th2 cytokine production and (B) decreased Th1 cytokine production upon primary stimulation of DKO spleen cells. Freshly isolated splenocytes from wild-type or DKO mice were cultured at 2×10^6 cells/ml with 1 μg/ml of plate-bound anti-CD3 for 48 hr. Cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, GM-CSF, IFNγ, and TNFα) were measured by ELISA in supernatants taken at 24 hr for IL-2 and 48 hr for all others. Numbers above the bars represent the approximate DKO/WT ratio for each cytokine. (C). Secondary stimulation of spleen cells from DKO mice. Cytokines were measured as above at 48 hr. Note that IL-4 is expressed on a log scale. Data from one representative pair of mice of three pairs are shown.

specifically competed with excess unlabeled probe and that was supershifted with MAbs to both NFATc and NFATp was present in extracts prepared from PMA/ionomycin-stimulated wild-type LN. This complex was present only at low levels in unstimulated wild-type LN cells (data not shown) and was induced upon treatment with PMA/ionomycin. In contrast, nuclear extracts from DKO LN cells contained only NFATc and not NFATp binding activity, and this binding activity was present at high levels in both unstimulated, freshly isolated (data not shown), and stimulated LN cells. These data suggest that the overproduction of IL-4 in the NFAT DKO mice is secondary to the unopposed activity of NFATc, likely acting in concert with the Th2-specific transcription factors c-maf and GATA-3 (Ho et al., 1996; Zheng and Flavell, 1997) in driving IL-4 gene transcription.

Taken together, the phenotype of the DKO mice leads us to conclude that in the absence of NFATp and NFAT4, the threshold for signaling via the TCR is lowered, resulting in constitutive T cell activation and the continual import of NFATc into the nucleus where it transactivates the IL-4 gene. It is also possible that the phenotype of the NFAT DKO reflects the dysregulation of other as yet unknown NFAT regulated target genes, critical in maintaining homeostasis of the lymphoid system. For example, such target genes may have a unique role in the maintenance of the resting state, in control of the cell cycle, or in the regulation of a Th2-specific death pathway. In either case, our data demonstrate that NFAT family member proteins must cooperate together to achieve normal generation and activation of the immune system.

Experimental Procedures

Mice

NFATp- and NFAT4-deficient mice (Hodge et al., 1996; Oukka et al., 1998) were backcrossed seven and five generations, respectively, onto the BALB/c background and then intercrossed to generate doubly deficient mice. Wild-type controls were either littermates or age-matched BALB/c mice. Mice were housed in sterilized microisolator cages, fed autoclaved food and water, and handled in laminar airflow hoods.

Histologic Analysis

Tissues were fixed in OmniFix II, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin, toluidine blue, Giemsa, or Congo Red, as indicated.

Flow Cytometric Analysis

Single cell suspensions were incubated at 4°C with fluorescein-, TC- or phycoerythrin-conjugated antibodies (PharMingen) and analyzed using a FACScan (Becton Dickenson) with Cell Quest software. The following antibodies were used: anti-CD3 (145-2C11), anti-CD4 (RM4-4), anti-CD8α (53-6.7), anti-CD45R/B220 (RA3-6B2), anti-IgMα (DS-1), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), and anti-I-$A^{b,d}$ (25-9-17). The rat anti-mouse FcRγII/III MAb (2.4G2) was used to block nonspecific binding.

Table 1. Serum Immunoglobulin Levels from Wild-Type and NFATpxNFAT4-Deficient (DKO) Mice

	Serum Immunoglobulin (μg/ml)							
	Age (Weeks)	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA	IgE
Wild-type 1	12	293	192	1193	109	340	125	0.2
Wild-type 2	13	223	45	274	19	195	26	0.2
Wild-type 3	14	230	22	201	10	180	26	0.1
DKO 1	12	1110	29,856	1233	139	386	118	755
DKO 2	13	1238	44,769	2441	243	1060	161	1,063
DKO 3	14	927	26,472	2920	799	184	182	828

Serum immunoglobulin levels were determined by isotype-specific ELISA in 12- to 14-week-old wild-type or DKO mice.

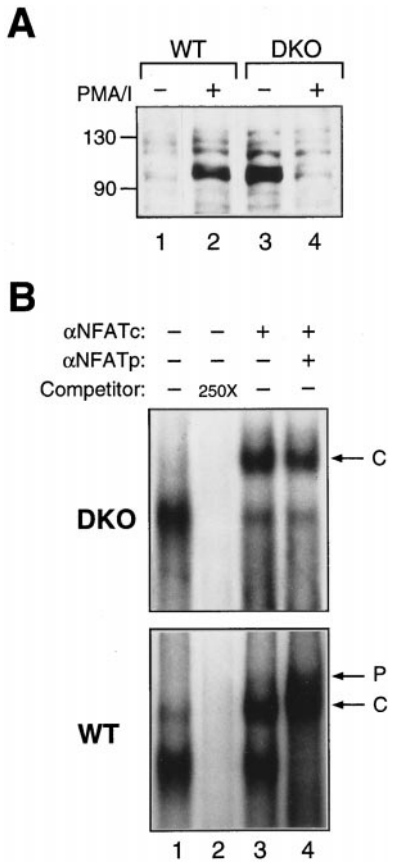

Figure 6. Constitutive Nuclear Localization of NFATc in DKO T Cells

(A) Western blot analysis of unstimulated (lanes 1 and 3) and PMA/ionomycin-stimulated (lanes 2 and 4) cultured LN T cells from wild-type (lanes 1 and 2) and DKO (lanes 3 and 4) mice. (B) EMSA and supershift analyses of nuclear extracts from PMA/iono-stimulated freshly harvested wild-type (bottom panel) and DKO (top panel) LN cells. An oligonucleotide from the proximal IL-4 promoter containing the NFAT target sequence was used as the probe. Lane 2, excess unlabeled homologous probe; lane 3, anti-NFATc antibody; lane 4, anti-NFATp plus anti-NFATc antibody. C, anti-NFATc supershifted complex; P, anti-NFATp supershifted complex.

Analysis of Cell Activation

For primary stimulation of unseparated splenocyte or LN cell populations, 2×10^6 cells were stimulated in 1 ml cultures in 24-well plates with 1 μg/ml plate-bound anti-CD3ε antibody. Unless otherwise indicated, at 48 hr supernatants were harvested for cytokine analysis and the cells were resuspended in IL-2 containing media for 7–10 days. Restimulation was performed with 2×10^5 cells per 96-well plate and 0.1–10 μg/ml of plate-bound anti-CD3ε. For in vitro proliferation, 1 μCi of [^{3}H] thymidine was added for the final 12 hr of a 60 hr incubation. Results are the mean of triplicate wells. The TUNEL assay was performed as per manufacturer's instructions (Boehringer Mannheim) on wild-type and DKO LN cell populations that had undergone multiple rounds of anti-CD3ε stimulation as described above.

Measurement of Cytokine and Antibody Levels by ELISA

Supernatants were assayed for levels of IL-2, IL-4, IFN-α, TNF-α, GM-CSF, IL-6, IL-10, and IL-5 by ELISA (PharMingen) as previously described (Hodge et al., 1996). Chimeric mice were bled by cardiac puncture and sera analyzed for Ig isotypes as previously described (Markowitz et al., 1993).

Western Blotting and Electrophoretic Mobility Shift Assays

T cells from lymph nodes in a resting state or after stimulation for 6 hr with PMA (50 μg/ml) and ionomycin (1 μM) were used and nuclear extracts prepared as described previously (Rooney et al., 1995). 2×10^6 cell equivalents/lane of nuclear extracts were resolved by 7.5% SDS-PAGE and immunoblotted with anti-NFATc antibody 7A6 (kind gift of G. Crabtree) as described (Timmerman et al., 1997). Mobility shift assays were performed (Timmerman et al., 1997) using 5–8 μg nuclear extract and 2×10^5 cpm of ^{32}P end-labeled oligo nucleotide containing a proximal NFAT site from the murine IL-4 promoter −61 to −88 (AATGTAACCTTTTAAAATAATGTGGTCT). Supershifts using MAb specific for NFATc (7A6), NFATp (G1-D10), and control ascites were performed using 2 μl of 1:10 dil of 7A6 (anti NFATc), 2 μl of 1:5 dil of G1-D10 (anti NFATp), or 2 μl of control ascites.

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