10-4-2006

Transcranial Magnetic Stimulation: A Stimulating New Method for Treating Depression, but Saddled with the Same Old Problems

Mark S. George
Medical University of South Carolina

Published July 2, 2001

Tumor Necrosis Factor Receptor–associated Factor (TRAF)2 Represses the T Helper Cell Type 2 Response through Interaction with NFAT-interacting Protein (NIP45)

Rebecca Lieberson,[1] Kerri A. Mowen,[1] Kathryn D. McBride,[1] Veronica Leautaud,[1] Xiankui Zhang,[5] Woong-Kyung Suh,[4] Lin Wu,[3] and Laurie H. Glimcher[1,2]

[1]*Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115*
[2]*Department of Medicine, Harvard Medical School, Boston, MA 02115*
[3]*Arthur D. Little, Cambridge, MA 02140*
[4]*Ontario Cancer Institute, Department of Medical Biophysiology and Immunology, Toronto, Ontario M5G 2M9, Canada*
[5]*Center for Molecular and Structural Biology, Hollings Oncology Center, Medical University of South Carolina, Charleston, SC 29425*

Abstract

Recently we have identified a novel protein NIP45 (nuclear factor of activated T cells [NFAT]-interacting protein) which substantially augments interleukin (IL)-4 gene transcription. The provision of NIP45 together with NFAT and the T helper cell type 2 (Th2)-specific transcription factor c-Maf to cells normally refractory to IL-4 production, such as B cells or Th1 clones, results in substantial IL-4 secretion to levels that approximate those produced by primary Th2 cells. In studies designed to further our understanding of NIP45 activity, we have uncovered a novel facet of IL-4 gene regulation. We present evidence that members of the tumor necrosis factor receptor–associated factor (TRAF) family of proteins, generally known to function as adapter proteins that transduce signals from the tumor necrosis factor receptor superfamily, contribute to the repression of IL-4 gene transcription and that this effect is mediated through their interaction with NIP45.

Key words: NIP45 • interleukin-4 • cytokines • TRAF2 • transcription

Introduction

IL-4, originally identified as a B cell growth factor, has proven to be a critical and multifunctional modulator of immune processes (1). As virtually all hematopoietic cells possess surface receptors for IL-4, this cytokine affects the function of T cells, B cells, macrophages, dendritic cells, and mast cells among others. Interestingly, IL-4 itself plays a key role in the generation and maintenance of IL-4–producing cells. $CD4^+$ T cells which classically deliver helper signals via secreted cytokines can be divided into two groups based on their different patterns of cytokine production: Th1 (IFN-γ, TNF-β, and IL-2) and Th2 (IL-4, IL-5, IL-6, IL-10, and IL-13) (2, 3). Polarization of $CD4^+$ T helper precursor cells (which are functionally naive and referred to as Thp) to either a Th1 or Th2 dominant population is an important determinant of the type of immune response that is initiated and whether this immune response is ultimately protective or pathogenic. Th1 cells function to promote host resistance to many intracellular microbes by activating macrophages whereas Th2 cells are known to be important in certain humoral responses such as eradicating extracellular helminthic parasites and also are critical in allergic responses. The influence from IL-4 on uncommitted Thp cells is twofold. IL-4 signaling during primary activation of the Th cell can initiate a program of Th2 development and also suppress Th1 development by inhibiting IFN-γ production (3–5).

The importance of IL-4 is underscored by the physiological consequences of its dysregulation. Mice harboring targeted mutations in the IL-4 locus, or the IL-4 signaling pathway — that is the IL-4 receptor through which IL-4 signaling occurs or a downstream signaling molecule Stat6

Address correspondence to L.H. Glimcher, Department of Immunology and Infectious Diseases, Harvard School of Public Health, 651 Huntington Ave., Boston, MA 02115. Phone: 617-432-0622; Fax: 617-432-0084; E-mail: lglimche@hsph.harvard.edu

Published July 2, 2001

— show marked impairment in Th2 development and diminished IgE/IgG1 production (5–11). And reciprocally, mice overexpressing an IL-4 transgene in T cells developed allergic-like disorders of the eye, skin, and/or lung (12). Additionally, in humans, overproduction of IL-4 is associated with allergic diseases. Another important IL-4–mediated effect is to downregulate inflammatory responses which, left unchecked, may lead to tissue damage and chronic inflammatory disorders (for a review, see reference 13).

Expression of the IL-4 gene is tightly regulated. IL-4 production is limited to a subset of lymphoid cells: Th2 cells, NK T cells, Tc2 $CD8^+$ cells, mast cells and basophils, eosinophils, and possibly γ/δ T cells (for a review, see reference 14). Moreover, IL-4 gene transcription occurs in these cell types only in response to antigen-T cell receptor stimulation. Recent progress has been made in identifying the transcription factors that control the expression of the IL-4 gene in Th2 cells (for a review, see reference 15). In vitro analyses of the proximal IL-4 promoter demonstrate that both the c-maf proto-oncogene (a member of the activating protein 1 [AP-1] family of basic region/leucine zipper proteins) and nuclear factor of activated T cells (NFAT)* family members (NFATc1, c2, and c3 also called NFATc, NFATp [NFAT1], and NFAT4 [NFATx], respectively) transactivate the proximal IL-4 promoter. The expression and activity of both c-maf and NFAT are regulated through T cell receptor–mediated signals (16, 17). In vivo studies substantiate an essential role for c-maf in tissue-specific regulation of the IL-4 gene, as mice deficient for c-maf have severely impaired production of IL-4 and serum IgG1, whereas IL-4 levels are elevated in transgenic mice whose T cells overproduce c-maf (18, 19). Other findings highlight the complexity involved in regulatory control of the IL-4 gene locus. For instance, the capability of c-maf–deficient T cells to produce low levels of IL-4 when provided with exogenous IL-4 suggests the existence of other factors which have the capacity to drive IL-4 production in a tissue-specific manner (18). It may be that GATA3, another Th2-specific factor, can serve this function, although it is thought that its primary action is to control the coordinate regulation of Th2 cytokines by remodeling chromatin (20–22). Additionally, while mice lacking NFATc1 in T cells have a decrease in IL-4 production and a selective loss of IgE and IgG1 isotypes, supporting the notion that NFAT is critical in potentiating IL-4 gene transcription, mice lacking NFATc2 or doubly deficient for both NFATc2 and NFATc3 show elevated levels of IL-4 (strikingly so for the double mutant mice) (23–26). Clearly, NFAT family members play vital roles in both initiation and termination of IL-4 gene production.

Recently we have identified a novel protein NIP45 (NFAT-interacting protein) which substantially augments IL-4 gene transcription (27). NIP45 was initially identified as a factor which interacts with the REL homology domain (which is required for sequence-specific DNA binding) of NFAT family members. We have shown that it is possible to force endogenous IL-4 production in non-producer cells. For example, the ectopic expression of c-maf and NFAT in cells normally refractory to IL-4 production such as B cells or Th1 clones allows the production of measurable, albeit low, amounts of IL-4. However, the addition of NIP45, in conjunction with c-maf and NFAT, to the same non-producing cell types now results in appreciable levels of endogenous IL-4, levels that approximate those produced by primary Th2 cells. Indeed, this synergy was so pronounced that cells transfected with NIP45 produced 50- to 200-fold more IL-4 than cells that did not receive NIP45 (27). The mechanism by which NIP45 augments IL-4 production remains unresolved. For example, although it is clear that while NIP45 does cooperate with all NFAT family members to enhance NFAT-driven transcription, it does not transactivate the IL-4 promoter on its own.

In studies designed to further our understanding of NIP45 activity we have uncovered a novel facet of IL-4 gene regulation. We present evidence that members of the TRAF family of proteins, generally known to function as adapter proteins that transduce signals from the TNFR superfamily, may also contribute to the repression of IL-4 gene transcription and that this effect is mediated through their interaction with NIP45.

Materials and Methods

Mice and Cell Lines. TRAF2.DN transgenic mice (which overexpress a lymphocyte-specific mutant form of TRAF2 transgene such that the N-terminal RING finger domain and zinc fingers are deleted) were provided by Dr. Y. Choi (The Rockefeller University, New York, NY) (28). Age-matched wild-type C57BL/6 mice (The Jackson Laboratory) were used as controls. Mice were used from 12 to 16 wk of age. M12C3 (M12) is a murine B lymphoma cell line and Jurkat is a human T lymphoma cell line. Cell lines and primary cells were maintained in complete media (CM) containing RPMI 1640 supplemented with heat-inactivated 10% fetal calf serum (Hyclone Laboratories), glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), Hepes (100 mM), and β-ME (50 μM).

Yeast Two-Hybrid Screen. The full-length NIP45 cDNA was engineered by PCR to be flanked by EcoR1 and Xho1 and cloned inframe, N-terminal to the Lex A DNA-binding domain, into the bait vector pEG202 to produce pEG202-NIP45. This bait was used to screen two expression libraries: one constructed from cDNA derived from the Th1 clone OF6 (29) and the other from a Th2 clone D10 (16) as described (27).

Coimmunoprecipitation. Immunoprecipitation experiments were performed using anti-Flag and anti-MYC antibodies essentially as described previously (30).

Th Cell Purification and In Vitro Differentiation. Single cell suspensions were prepared from spleen and lymph node and depleted of red blood cells using RBC lysis buffer (Sigma-Aldrich) and B cells by panning against IgG and IgM positive. CD4-positive T cells were then isolated by incubating with anti-CD4 MACS micromagnetic beads (Miltenyi Biotec) and positively selecting by high-gradient magnetic cell separation using VS+ MACS columns (Miltenyi Biotec) according to the manufacturer's instructions. Naive cells were obtained by sorting (MO

*Abbreviations used in this paper: Jnk, c-Jun NH_2-terminal kinase; NFAT, nuclear factor of activated T cells; NIP45, NFAT-interacting protein.

Published July 2, 2001

FLO; Cytomation) cells that had been harvested from lymph node and spleen, depleted of B cells, stained with FITC-conjugated anti-CD4 antibody and PE-conjugated Mel-14 (CD62L) antibody, resuspended in RPMI supplemented with 1% fetal calf serum at 10^7 cells/ml. The CD4-, Mel-14 (CD62L) high population were collected at >98% purity. For in vitro differentiation assays, purified naive or total CD4 cells (10^6/ml) were stimulated with 2 μg/ml plate-bound anti-CD3ε antibody and 2 μg/ml plate-bound anti-CD28 antibody. IL-12 and anti–IL-4 were added for Th1 differentiation and IL-4 and anti–IFN-γ for Th2 differentiation. On day 3, 200 U/ml of IL-2 was added to all cultures. On day 8, cells were harvested, washed, and restimulated (at 10^6 cells/ml) with 2 μg/ml plate-bound anti-CD3ε and 2 μg/ml plate-bound anti-CD28 antibody for 24 h. Culture supernatants were collected and levels of cytokine production were measured by ELISA.

Intracellular Staining. Purified, naive CD4 cells were differentiated in culture for 7 d and restimulated with 50 ng/ml PMA (Sigma-Aldrich) and 1 μM ionomycin (Calbiochem) for 5 h with the addition of monensin for the last 3 h of stimulation. Cells were then fixed in 4% paraformaldehyde for 20 min, permeabilized by washing in PBS supplemented with 0.1% saponin/1% FCS, and incubated with either PE-conjugated and FITC-conjugated anticytokine antibodies. PE-conjugated and FITC-conjugated, isotype-matched monoclonal antibody controls were used to evaluate background staining. After a final wash, cells were analyzed on a FACScan™ flow cytometer (Becton Dickinson).

Expression Constructs, Transfections, and Luciferase Assays. The following mammalian expression vectors and reporter constructs have been described previously: NFATC2 (referred to as NFATp), c-maf, NIP45, TRAF1, TRAF 2, TRAF3, TRAF4, TRAF 5, TRAF6, IL-4-luc (which contains 807 bp upstream of the murine IL-4 promoter), Egr3-luc (which contains bases −2,952 to +86 of the Egr3 promoter) (18, 27, 31–34). M12 or Jurkat cells (at 5×10^6 cells in 0.4 ml of RPMI were transiently transfected by electroporation using a Bio-Rad Laboratories electroporator. In brief, DNA (quantities stated in figure legends) was combined with cells in a 0.4 μM cuvette, electroporated (280 V, 975 μF for M12 and 270 V, 975 μF for Jurkat), and plated in 6 ml of complete medium. At 18 to 36 h after transfection, cells were harvested and whole cell lysates were prepared with reporter lysis buffer (Promega) and used for luciferase assays. Values were calculated as fold increase over the reporter luciferase construct alone. Each transfection experiment was repeated between four and seven times.

ELISA. Cytokine protein levels in tissue culture supernants were measured by ELISA essentially as described previously (23). All anticytokine capture antibodies and anticytokine biotinylated detection antibodies were purchased from BD PharMingen. Calculated values are expressed as means ± SEM.

Western Blot Analyses. Whole cell extracts or nuclear extracts were prepared as described (4). 25 μg of protein was run on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose. The filters were probed with a combination of two separate anti-NIP45 monoclonal antibodies, NIP451-1 and NIP454-7 of the IgG1 isotypes, respectively. Blots were developed with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemoluminescence (ECl).

Results and Discussion

NIP45 Interacts with TRAF Family Members. Previously we have shown that NIP45 can synergize with the transcription factors NFAT and c-maf to potently upregulate IL-4 production. However, the mechanism by which NIP45 augments IL-4 gene activity remains unclear. For example, we cannot detect direct binding of NIP45 to IL-4 promoter DNA as measured by electrophoretic mobility shift assays (unpublished data). It may be that other proteins, in addition to NFAT and c-maf, are required for the stable association of NIP45 within the context of the IL-4 transcription complex. Therefore, to explore the possibility that NIP45 may associate with other proteins in addition to NFAT, we used the yeast two-hybrid system to search for potential interacting partners using full-length NIP45 cDNA as "bait". Two T cell cDNA expression libraries were screened. One of the interacting proteins isolated was TRAF1, a member of the TRAF family of proteins known to function as signal transduction molecules (for a review, see reference 34). A coimmunoprecipitation assay was used to determine whether NIP45 and any of the six known TRAF family members (identified in human and mouse) could associate in vivo. cMyc-tagged NIP45 was coexpressed with each Flag-tagged TRAF (1 through 6) in transiently transfected human embryonic kidney 293T cells. NIP45-cMyc immunoprecipitated with anti-Flag antibody only in the presence of flag-tagged TRAFs demonstrating that NIP45 can form a physical association with all six TRAF proteins (Fig. 1).

By definition, all members of the TRAF family of proteins have in common a "TRAF domain" which is a unique, highly homologous stretch of ~200 amino acids positioned near the COOH terminus and is the only predicted structural domain shared by all six TRAF proteins (35, 36). As NIP45 is able to coimmunoprecipitate with all TRAF proteins, it was likely that this interaction was mediated via the TRAF domain. A series of progressive 5′ to 3′ truncation mutants of TRAF5 fused to the activation

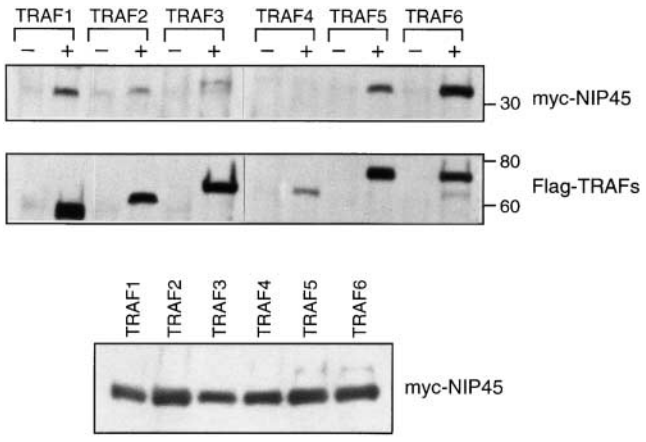

Figure 1. Interaction of NIP45 and TRAF family members in 293 cells. Tagged expression vectors (myc-NIP45 and Flag-TRAF1 through 6) were transfected into 293 cells. Whole cell lysates were prepared for immunoprecipitation with an antibody to Flag (top and middle panels). Western blot analysis was done with an antibody to myc (top panel) or Flag (middle panel). To evaluate the amount of myc-NIP45 protein produced, lysates were analyzed by Western blotting with an antibody to myc (bottom panel).

Published July 2, 2001

domain of GAL4 were tested for their ability to interact with NIP45 in yeast. In fact, expression of just the TRAF domain was sufficient to promote interaction with NIP45 (data not shown). However, because all of the mutants evaluated retained the TRAF domain, we cannot rule out the possibility that NIP45 can also interact with other regions of the TRAF proteins.

TRAF2 Represses Both IL-4 Promoter Transactivation and Production of Endogenous IL-4. To directly assess whether TRAFs can exert an effect on IL-4 promoter transactivation, M12 or Jurkat cells were transiently cotransfected with combinations of plasmids expressing c-maf, NFATc2, NIP45, and TRAF family members (TRAF1 through 6) in conjunction with an IL-4 promoter reporter construct (Fig. 2 A). NIP45 typically functions to enhance c-maf/NFATc2-driven transactivation of the IL-4 promoter. The provision of TRAF2 in addition to NIP45 resulted in marked repression of c-maf/NFATc2-driven transactivation of the IL-4 promoter. TRAF1 and TRAF4 were also found to significantly inhibit IL-4 promoter transactivation. TRAF1, 2, and 4 differ in their tissue distribution (34). TRAF1 has been identified in spleen, lung, and testis,

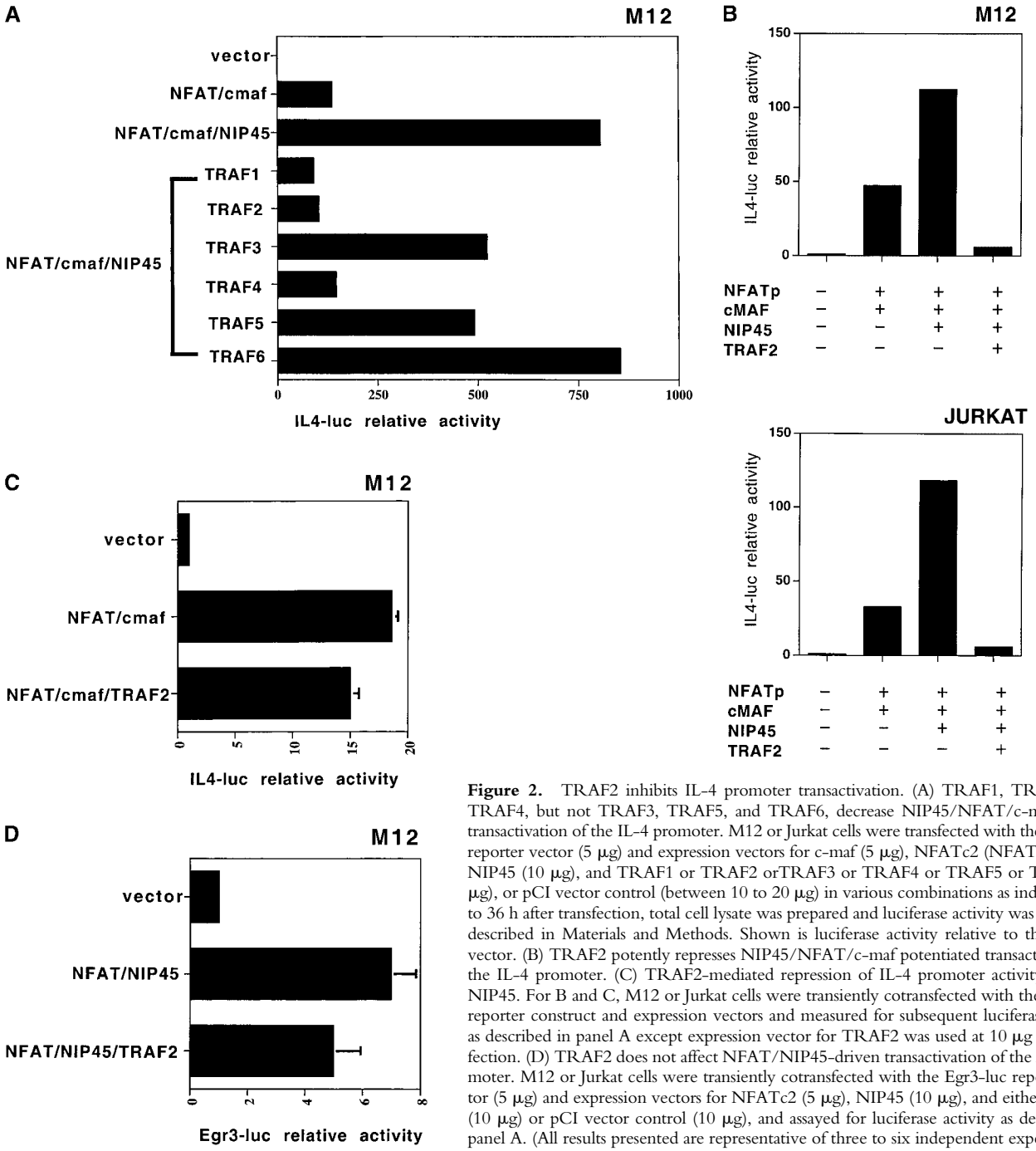

Figure 2. TRAF2 inhibits IL-4 promoter transactivation. (A) TRAF1, TRAF2, and TRAF4, but not TRAF3, TRAF5, and TRAF6, decrease NIP45/NFAT/c-maf-driven transactivation of the IL-4 promoter. M12 or Jurkat cells were transfected with the IL-4-luc reporter vector (5 μg) and expression vectors for c-maf (5 μg), NFATc2 (NFATp) (5 μg), NIP45 (10 μg), and TRAF1 or TRAF2 orTRAF3 or TRAF4 or TRAF5 or TRAF6 (5 μg), or pCI vector control (between 10 to 20 μg) in various combinations as indicated. 18 to 36 h after transfection, total cell lysate was prepared and luciferase activity was assayed as described in Materials and Methods. Shown is luciferase activity relative to the control vector. (B) TRAF2 potently represses NIP45/NFAT/c-maf potentiated transactivation of the IL-4 promoter. (C) TRAF2-mediated repression of IL-4 promoter activity requires NIP45. For B and C, M12 or Jurkat cells were transiently cotransfected with the IL-4-luc reporter construct and expression vectors and measured for subsequent luciferase activity as described in panel A except expression vector for TRAF2 was used at 10 μg per transfection. (D) TRAF2 does not affect NFAT/NIP45-driven transactivation of the Egr3 promoter. M12 or Jurkat cells were transiently cotransfected with the Egr3-luc reporter vector (5 μg) and expression vectors for NFATc2 (5 μg), NIP45 (10 μg), and either TRAF2 (10 μg) or pCI vector control (10 μg), and assayed for luciferase activity as described in panel A. (All results presented are representative of three to six independent experiments.)

Published July 2, 2001

whereas TRAF2 appears to be ubiquitously expressed. TRAF4 is highly expressed during embryogenesis and in regions of the adult brain. Thus, the tissue distribution of TRAF1 and TRAF2 is compatible with a potential role in IL-4 gene regulation. To interpret these findings we needed to address the following points: (a) is this TRAF mediated repression of IL-4 promoter activity dependent on NIP45? (b) Is this TRAF-mediated repression specific to the IL-4 promoter?

To address these issues we performed the following experiments using TRAF2 which repeatedly led to the greatest inhibition of c-maf/NFATc2-driven transactivation of the IL-4 promoter (Fig. 2 B). First, the promoter transactivation assays were repeated in the absence of NIP45. In these conditions, TRAF2 no longer significantly repressed c-maf/NFATc2-driven IL-4 promoter activity, demonstrating that TRAF2-mediated repression does require NIP45 (Fig. 2C). The slight decrease in c-maf/NFAT-driven transactivation that is observed upon addition of TRAF2 may be because TRAF2 is interacting with endogenous NIP45 present in both M12 and Jurkat cells. In support of this interpretation, Fig. 3 shows low levels of endogenous NIP45 are present in untreated M12 cells. Second, we asked whether the effect of the TRAF2/NIP45 interaction was selective for IL-4 transcription or was a generalized phenomenon. NIP45 has been shown to augment NFAT-driven transactivation of other promoters such as the Egr3 promoter (31). As shown in Fig. 2 D, overexpression of TRAF2 had no influence on NIP45/NFATc2-driven transactivation of the Egr3 promoter. These additional transfection results suggest that TRAF2-mediated repression of IL-4 is not due to a competition between NFAT and TRAF2 for partnering with NIP45. If that were the case, one might have predicted that all TRAFs would have repressive activity since they all can physically associate with NIP45 (as demonstrated by the above described coimmunoprecipitation experiments) and that TRAF2 would have blocked the NIP45/NFAT association in the context of Egr3 promoter transactivation. Thus, overexpression of TRAF2 significantly and specifically inhibits the very potent NIP45/NFATc2/c-maf transactivation of the IL-4 promoter.

We then sought to test possible TRAF2 effects on endogenous IL-4 production using a model system in which we were able to induce IL-4 in non-producer cells such as the B lymphoma line, M12, by transiently overexpressing the three factors: c-maf, NFAT, NIP45. Consistent with the promoter assays, we found that providing the cells with TRAF2 rendered them unable to produce endogenous IL-4 (Fig. 4).

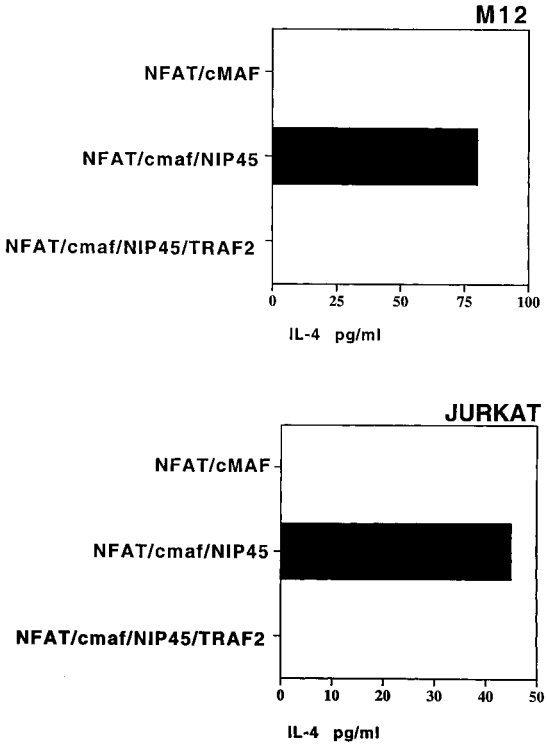

Figure 4. Overexpression of TRAF2 blocks the ability of c-maf, NFAT, and NIP45 to initiate endogenous IL-4 production. M12 murine B lymphoma cells or Jurkat human T lymphoma cells were transiently cotransfected with expression plasmids for c-maf (5 μg), and NFATc2 (5 μg), together with either NIP45 (10 μg), and TRAF2 (10 μg), or pCI vector control (between 10 to 20 μg) as indicated. The concentration of IL-4 in supernatants harvested 72 h after transfection was measured by ELISA as described in reference 27 (Similar results were obtained in three additional independent experiments).

CD4 Cells from TRAF2.DN Mice Overproduce IL-4. The TRAF proteins have been extensively characterized as a family of cytoplasmic adapter proteins which are recruited to numerous ligand-bound receptors within the TNF receptor family such as CD27, CD30, CD40, LMP-1, IL-1, TNFR1, and TNFR2 (34). As signaling via these receptors has not been shown to influence IL-4 gene transcription, we thought it important to determine whether there is indeed a biological role for TRAF molecules in IL-4 gene activity. To address this issue we sought to look for perturbations in IL-4 production in mice harboring a mutation in TRAF2. We initially chose to examine mice expressing a

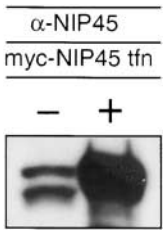

Figure 3. Relative comparison of levels of endogenous and overexpressed NIP45 protein. Lysates prepared from M12 cells transiently transfected with myc-tagged NIP45 or vector only expression plasmids were immunoblotted and probed with a combination of two anti-NIP45 monoclonal antibodies.

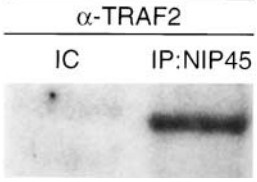

Figure 5. NIP45 associates with TRAF2. Lysates prepared from Bjab cells (human B cells) were immunoprecipitated with a combination of two anti NIP45 monoclonal antibodies (according to the methods used in reference 47). Western blot analysis was performed using an anti-TRAF2 antibody (Santa Cruz Biotechnology, Inc.). IC, isotype control; IP, immunoprecipitation.

Published July 2, 2001

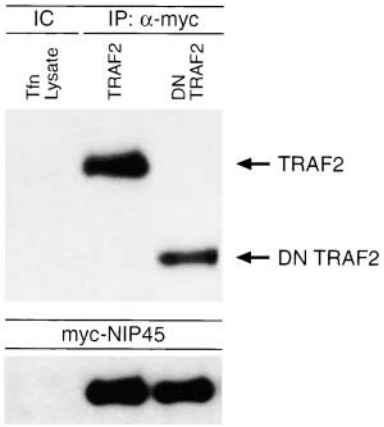

Figure 6. NIP45 associates with TRAF2 dominant negative mutant protein in 293 cells. Myc-tagged NIP45 and either TRAF2 or TRAF2.DN expression plasmids were transiently transfected into 293 cells. Whole cell lysates were prepared for immunoprecipitation with an antibody to myc. Western blot analysis was done using antibodies to TRAF2 (Santa Cruz Biotechnology, Inc.) (top panel) or myc (bottom panel). IC, isotype control; IP, immunoprecipitation.

TRAF2 dominant negative transgene (TRAF2.DN) (28). Unlike TRAF2-deficient mice which are postnatal lethal, T lymphocyte development appears to occur normally in TRAF2.DN mice which have typical ratios of $CD4^+$ to $CD8^+$ T cells in the thymus and lymph nodes (37, 38). Additional rational to perform these experiments came from our observations of in vivo associations of (a) endogenous TRAF2 and endogenous NIP45 protein (Fig. 5) and (b) overexpressed myc-tagged NIP45 and overexpressed TRAF2 dominant negative mutant protein (Fig. 6).

An important physiological consequence of IL-4 production is to direct developing naive $CD4^+$ T cells toward a Th2 phenotype—that is a subset of $CD4^+$ T cells which secrete IL-4, IL-5, IL-10, IL-13, but not IFN-γ or TNFβ. An in vitro differentiation assay was therefore used to examine Th2 development in TRAF2.DN mice. $CD4^+$ Th cells were primed with immobilized anti-CD3/CD28 mAbs in the presence of either Th1, or Th2-polarizing conditions, or nonskewing conditions. After 8 d of culture, cells were rested and then restimulated with anti-CD3/CD28 mAb. 24 h after secondary stimulation, culture supernatants were collected and the levels of various cytokines were determined by ELISA (Fig. 7). The most dramatic results were observed in the nonskewing conditions. Between 300 and 2,500 pg/ml of IL-4 was detected in supernatants from TRAF2.DN cells contrasted with supernatants from wild-type cells in which virtually no IL-4 was detected. Similar increases in IL-5 and IL-13 production was observed in the nonskewing conditions (data not shown). Under Th2-polarizing conditions, IL-4, IL-5, and IL-13 levels were also each elevated relative to wild-type levels (Fig. 7). In contrast, levels of the Th1-type cytokine, IFN-γ, were nearly equivalent between TRAF2.DN and wild-type control mice. It is interesting to note that in the original characterization of the TRAF2.DN mice, TCR-induced T cell proliferation was substantially reduced compared with wild-type controls. This suggests that the marked increase in Th2-like cytokines is not secondary to a lymphoproliferative disorder as has been noted in several mouse mutants (i.e., NFATc2/c3-deficient mice, CTLA4-deficient mice) (25, 39, 40). TRAF2.DN mice do display lymphadenopathy but this has been attributed to a massive increase in the number of $CD3^-B220^+$ B cells (28).

As CD4 cells are a mixed population of memory, naive, and activated T helper cells we could not rule out the formal possibility that overproduction of IL-4 was a secondary consequence of a proliferative or activation defect. Therefore, parallel experiments were performed using highly pu-

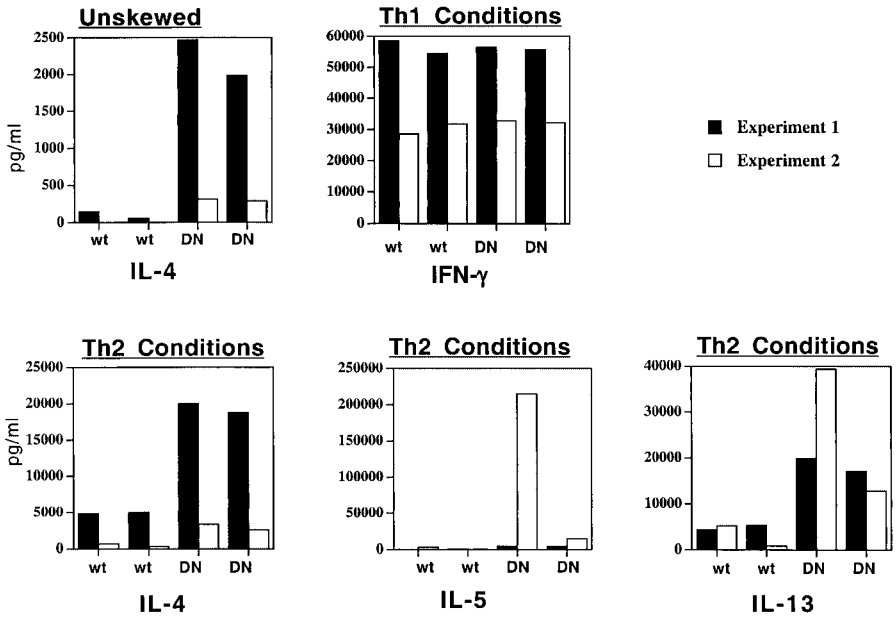

Figure 7. TRAF2.DN T cells have enhanced production of Th2 type cytokines. CD^+ T cells were magnetically-purified from spleen and lymph nodes differentiated in vitro under nonskewing, or Th1-, or Th2-skewing conditions as described in Materials and Methods. Supernants from cultured cells were harvested 24 h after secondary stimulation with plate bound anti-CD3ε and levels of IL-4, IL-5, IL-13, and IFN-γ were quantitated by ELISA. Results from two independent experiments are represented. Two transgenic (DN) and two wild-type (wt) mice were analyzed per experiment.

Published July 2, 2001

rified naive CD4+ Thp cells isolated from spleen and lymph nodes. Again we observed overproduction of IL-4, IL-5, and IL-13 in the culture supernatants from TRAF2.DN T cells which had initially been primed in nonskewing or Th2-polarizing conditions and then reactivated (Fig. 7). In addition, we found that the proliferative responses of naive CD4+ Th cells from TRAF2.DN to plate-bound anti-CD3 were normal (data not shown).

To evaluate cytokine production on a per cell basis, intracellular staining was also performed (Fig. 8). Purified,

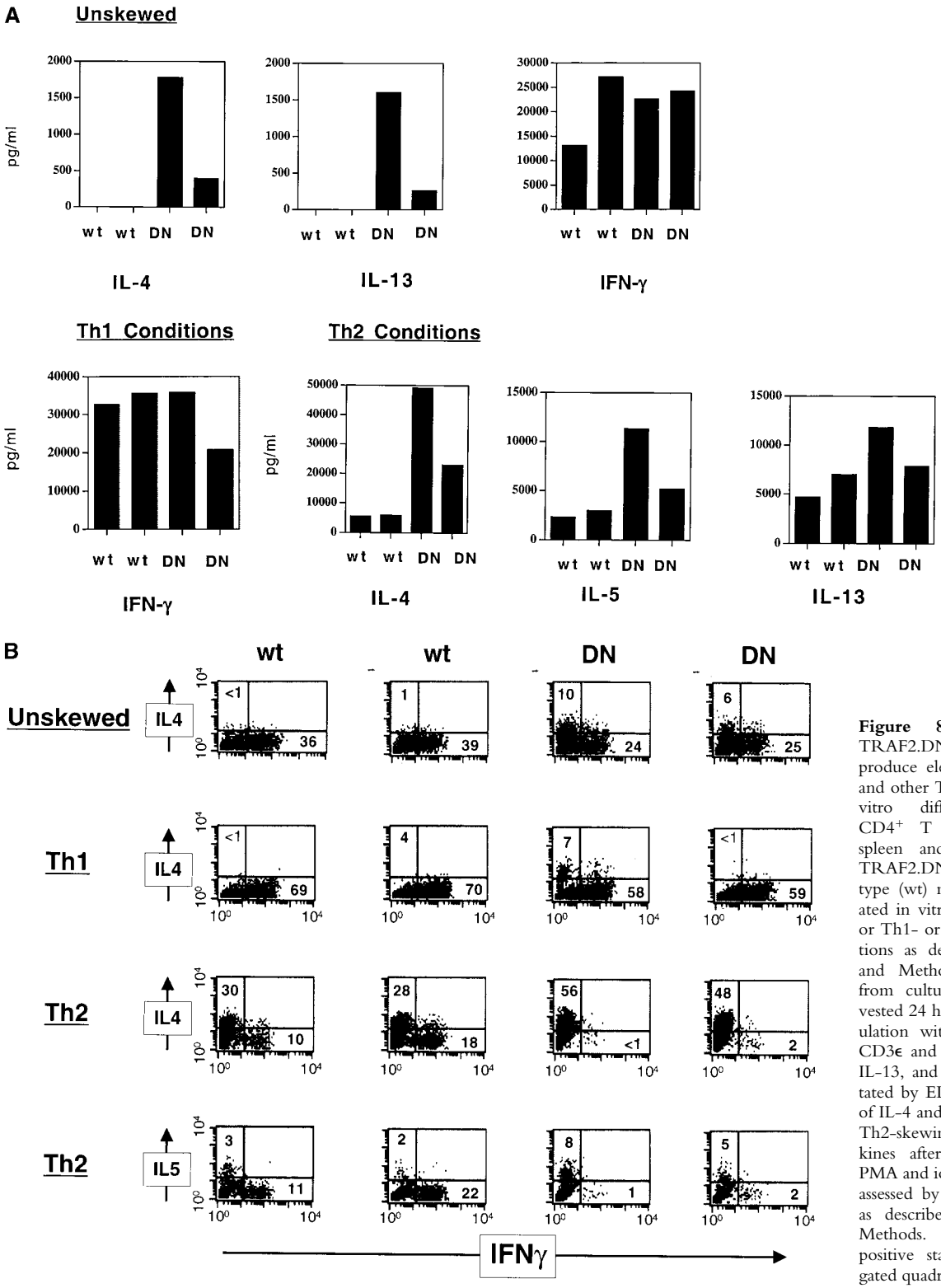

Figure 8. Th cells from TRAF2.DN transgenic mice produce elevated levels of IL-4 and other Th2 cytokines after in vitro differentiation. Naive CD4+ T cells isolated from spleen and lymph nodes of TRAF2.DN (DN) and wild-type (wt) mice were differentiated in vitro under nonskewing or Th1- or Th2-skewing conditions as described in Materials and Methods. (A) Supernants from cultured cells were harvested 24 h after secondary stimulation with plate-bound anti-CD3ε and levels of IL-4, IL-5, IL-13, and IFN-γ were quantitated by ELISA. (B) Production of IL-4 and IFN-γ (and IL-5 for Th2-skewing conditions) cytokines after restimulation with PMA and ionomycin for 5 h was assessed by intracellular staining as described in Materials and Methods. The percentage of positive staining cells in each gated quadrant is indicated.

Published July 2, 2001

naive CD4+ T cells isolated from TRAF2.DN or wild-type control mice underwent primary differentiation in Th1, Th2 or nonskewing culture conditions for 7 d as described above. Cells were then rested overnight and stimulated with PMA/ionomycin and cytokine production was measured by intracellular staining. Results from two separate mice show that in all three of our priming culture conditions (Th1, Th2, and nonskewing) there was an increase in the percentage of TFAF2.DN-derived cells producing IL-4 and a concomitant decrease in the percentage of IFN-γ–producing cells as compared with wild-type cells. Again, the largest differential between TRAF2.DN and wild-type IL-4 production was observed in the nonskewing conditions. Under Th2 culture conditions a higher percentage of TRAF2.DN cells were positive for intracellular IL-5. No IL-5–producing cells were detected among wild-type and TRAF2.DN cells from the Th1 or nonskewing conditions. It is interesting to speculate that the potential increase in IL-4 production by T cells may contribute to the overabundance of B cells observed in the TRAF2.DN mice.

Presently, it is an open question as to the conditions under which TRAF-mediated repression of IL-4 production might occur in vivo. An obvious possibility would be the differential expression of TRAFs or NIP45 in Th1 but not Th2 cell types; however, assessment of TRAF2 and NIP45 in naive and Th1 or Th2 skewed CD4+ T cells gave no indication of this type of tissue specificity (data not shown). Furthermore, we did not observe differential upregulation of NIP45 protein during in vitro differentiation of naive CD4 cells into either Th1 or Th2 cells (Fig. 9), although it is conceivable that the NIP45/TRAF2 interaction may qualitatively differ in Th1 versus Th2 cells. TRAF1 and TRAF2 have been reported to bind the cytoplasmic domain of CD30 which has been found to be highly expressed on human Th2 cells, although no function has yet been attributed to this molecule in the development of Th2 cells (41, 42). An intriguing scenario is that CD30 signaling might trigger upregulation of IL-4 gene transcription by disrupting NIP45/TRAF partnering possibly by recruiting TRAF to the cell membrane and thus presumably away from its association with NIP45. Additionally,

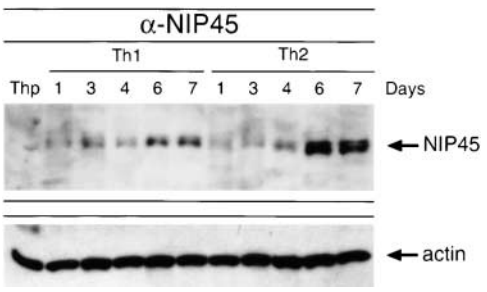

Figure 9. Upregulation of NIP45 protein during in vitro differentiation of naive CD4 cells into either Th1 or Th2 cells. In vitro T cell differentiation was performed as described in the Materials and Methods. Western blot analysis was performed on whole-cell lysates prepared from cells harvested at the indicated time points using a combination of two anti-NIP45 monoclonal antibodies.

IL-4 has been implicated in the downregulation of inflammatory cytokines. Given the clear evidence of cross-regulation among different cell types within the immune system (for example during Th1/Th2 development, cytokines produced by each subset promote same cell type development and expansion and inhibit maturation and proliferation of the opposing cell type), one might predict that the proinflammatory cytokines (such as TNFα, and TNFβ which are ligands for TNFR1 and TNFR2) would in turn inhibit the production of IL-4. Conceivably TRAF2-mediated repression of IL-4 gene transcription might be part of this intracellular pathway. Both of these possibilities warrant future investigation.

Several genetically altered mouse mutants exhibit elevated levels of Th2-type cytokines. Among these are mice lacking Jnk1/2 (c-Jun NH_2-terminal kinase) which phosphorylates the AP-1 transcription factors that participate in the regulation of growth signals and cytokine gene transactivation. Thus, Jnk can contribute to the development of Th2 cytokine–producing T cells (43, 44). It should be noted that lymphocytes from TRAF2.DN mice and TRAF2-deficient fibroblasts were defective in the TNF-induced activation of the Jnk signal transduction pathway. It will be important to determine whether the TRAF/NIP45-mediated effect on IL-4 production is occurring via a direct modification of the transcriptional machinery at the IL-4 promoter or rather through an indirect pathway such as perturbing Jnk signaling. At present we cannot distinguish between these two possibilities. The former is possible, since in the TRAF overexpression studies we observed inhibition of IL-4 promoter transactivation and IL-4 endogenous gene activity presumably in the absence of Jnk activation. On the other hand, overexpression of the dominant negative did not have a direct effect on IL-4 expression.

What is the likelihood that endogenous NIP45 and TRAF proteins colocalize within the cell, clearly requisite if their physical association is to occur? Numerous studies have classified the TRAFs as cytoplasmic adapter proteins that interact with intracellular domains of cell surface receptors and therefore must localize to the soluble fraction of the cytoplasm of cells (for a review, see reference 34). The exception is TRAF4 which possesses a putative nuclear localization signal and appears to be a predominantly nuclear protein. Interestingly, it was recently reported that endogenous TRAF2 is present in the nuclei of vascular endothelial cells and that the N-terminal "ring finger" domain targets TRAF2 to the nucleus (45). Additional evidence that TRAFs may be recruited to the nucleus comes from a study of the tissue distribution of TRAF3 which detected TRAF3 in both the nuclei of splenic B lymphocytes and a subset of neuronal cells from the cerebellum (46). And, although originally identified as a nuclear factor, further investigation shows that NIP45 is also present in the cytoplasmic fraction of lymphoid cells (unpublished data). Therefore, it is possible that NIP45 and TRAFs might interact within either the cytoplasmic and/or nuclear compartment of the cell. Furthermore, there is precedent for molecules with dual function as cytoplasmic signal transducers and as nuclear regula-

tors of gene expression such as the Stat family of proteins and β-catenin. In addition, it was recently reported that TRAF2 may directly participate in the transcriptional activation of the TNF target gene, E-selectin (45).

In summary, we demonstrate that TRAF2 is an interacting partner of NIP45 and that a functional consequence of the TRAF2/NIP45 association is the repression of IL-4 production due to downregulation of promoter activity. Furthermore, we have implicated TRAF2 in IL-4 regulation in vivo. These data taken together with our previous finding that NIP45 potently augments NFAT-driven IL-4 gene transcription allows us to envision NIP45 as a modulator of IL-4 gene transcription, functioning to enhance or repress depending on whom it partners with.

We wish to thank Dr. Yongwon Choi for very generously providing TRAF2.DN mice and TRAF plasmids and Jyothi Rengarajan, Mohammed Oukka, and Adrian Erlebacher for critical reading of the manuscript. We also wish to thank David Goeddel for his helpful comments.

This work was supported by a grant from the National Institutes of Health 1 RO1 AI43953-01 (L.H. Glimcher) and a gift from The G. Harold and Leila Y. Mathers Charitable Foundation (L.H. Glimcher).

Submitted: 4 January 2001
Revised: 30 April 2001
Accepted: 7 May 2001

References

1. Brown, M.A., and J. Hural. 1997. Functions of IL-4 and control of its expression. *Crit. Rev. Immunol.* 17:1–32.
2. Mosmann, T.R., and R.L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145–173.
3. Seder, R.A., and W.E. Paul. 1994. Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. *In* Annual Review of Immunology, Vol. 12. W.E. Paul, C. Garrison Fathman, and L.H. Glimcher, editors. Annual Reviews, Inc., Palo Alto, CA. 635–673.
4. Szabo, S.J., A.S. Dighe, U. Gubler, and K. Murphy. 1997. Regulation of the interleukin (IL)-12R β2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J. Exp. Med.* 185:817–824.
5. Ouyang, W., S.H. Ranganath, K. Weindel, D. Bhattacharya, T.L. Murphy, W.C. Sha, and K.M. Murphy. 1998. Inhibition of Th1 developmental mediated by GATA-3 through an IL-4 independent mechanism. *Immunity.* 9:745–755.
6. Kuhn, R., K. Rajewsky, and W. Muller. 1991. Generation and analysis of interleukin-4 deficient mice. *Science.* 254:707–710.
7. Noben-Trauth, N., L.D. Shultz, F. Brombacher, J.F.J. Urban, H. Gu, and W.E. Paul. 1997. An interleukin 4 (IL-4) independent pathway for $CD4^+$ T cell IL-4 production is revealed in IL-4 receptor-deficient mice. *Proc. Natl. Acad. Sci. USA.* 94:10838–10843.
8. Kopf, M., G. Le Gros, M. Bachmann, M.C. Lamers, H. Bluethmann, and G. Kohler. 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature.* 362:245–248.
9. Kopf, M., and G. LeGros. 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature.* 362:245–248.
10. Takeda, K., T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto, and S. Akira. 1996. Essential role of Stat6 in IL-4 signalling. *Nature.* 380:627–630.
11. Kaplan, M.H., Y.L. Sun, T. Hoey, and M.J. Grusby. 1996a. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature.* 382:174–177.
12. Tepper, R.I., D.A. Levinson, B.Z. Stanger, J. Campos-Torres, A.K. Abbas, and P. Leder. 1990. IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice. *Cell.* 62:457–467.
13. Paul, W.E., and R.A. Seder. 1994. Lymphocyte responses and cytokines. *Cell.* 76:241–251.
14. Nelms, K., A.D. Keegan, J. Zamorano, J.J. Ryan, and W.E. Paul. 1999. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* 17:701–738.
15. Szabo, S.J., L.H. Glimcher, and I.C. Ho. 1997. Genes that regulate interleukin-4 expression in T cells. *Curr. Opin. Immunol.* 9:776–781.
16. Ho, I.-C., M.R. Hodge, J.W. Rooney, and L.H. Glimcher. 1996. The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. *Cell.* 85:973–983.
17. Rao, A., C. Luo, and P.G. Hogan. 1997. Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* 15:707–747.
18. Kim, J., I.C. Ho, M. Grusby, and L.H. Glimcher. 1999. The transcription factor c-Maf controls the production of IL-4 but not other Th2 cytokines. *Immunity.* 10:745–751.
19. Ho, I.-C., D. Lo, and L.H. Glimcher. 1998. C-maf promotes Th2 and attenuates Th1 differentiation by both IL-4 dependent and independent mechanisms. *J. Exp. Med.* 188:1859–1866.
20. Zhang, D.H., L. Cohn, P. Ray, K. Bottomly, and A. Ray. 1997. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J. Biol. Chem.* 272:21597–21603.
21. Zheng, W.-P., and R.A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell.* 89:587–596.
22. Ouyang, W., M. Lohning, Z. Gao, M. Assenmacher, S. Ranganath, A. Radbruch, and K.M. Murphy. 2000. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity.* 12:27–37.
23. Hodge, M.R., A.M. Ranger, F. Charles de la Brousse, T. Hoey, M.J. Grusby, and L.H. Glimcher. 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity.* 4:1–20.
24. Ranger, A.M., M.R. Hodge, E.M. Gravallese, M. Oukka, L. Davidson, F.W. Alt, F.C. de la Brousse, T. Hoey, M. Grusby, and L.H. Glimcher. 1998. Delayed lymphoid repopulation with defects in IL-4-driven responses produced by inactivation of NFATc. *Immunity.* 8:125–134.
25. Ranger, A.M., M. Oukka, J. Rengarajan, and L.H. Glimcher. 1998. Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. *Immunity.* 9:627–635.
26. Yoshida, H., H. Nishina, H. Takimoto, L.E.M. Marengère, A.C. Wakeham, D. Bouchard, Y.-Y. Kong, T. Ohteki, A. Shahinian, M. Bachmann, et al. 1998. The transcription fac-

tor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. *Immunity*. 8:115–124.
27. Hodge, M.R., H.J. Chun, J. Rengarajan, A. Alt, R. Lieberson, and L.H. Glimcher. 1996. NFAT-driven interleukin-4 transcription potentiated by NIP45. *Science*. 274:1903–1905.
28. Lee, S.Y., A. Reichlin, A. Santana, K.A. Sokol, M.C. Nussenzweig, and Y. Choi. 1997. TRAF2 is essential for JNK but not NF-kB activation and regulates lymphocyte proliferation and survival. *Immunity*. 7:703–713.
29. Szabo, S.J., S.T. Kim, G.L. Costa, X. Zhang, G.C. Fathman, and L.H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 100:655–669.
30. Woronicz, J.D., X. Gao, Z. Cao, M. Rothe, and D.V. Goeddel. 1997. IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. *Science*. 278:866–869.
31. Rengarajan, J., P.R. Mittelstadt, H.W. Mages, A.J. Gerth, R.A. Kroczek, J.D. Ashwell, and L.H. Glimcher. 2000. Sequential involvement of NFAT and Egr transcription factors in FasL regulation. *Immunity*. 12:293–300.
32. Song, H.Y., M. Rothe, and D.V. Goeddel. 1996. The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-kappaB activation. *Proc. Natl. Acad. Sci. USA*. 93:6721–6725.
33. Rothe, M., V. Sarma, V.M. Dixit, and D.V. Goeddel. 1995. TRAF2-mediated activation of NF-kB by TNF receptor 2 and CD40. *Science*. 269:1424–1427.
34. Arch, R.H., R.W. Gedrich, and C.B. Thompson. 1998. Tumor necrosis factor receptor-associated factors (TRAFs) - a family of adapter proteins that regulates life and death. *Genes Dev*. 12:2821–2830.
35. Rothe, M., S.C. Wong, W.J. Henzel, and D.V. Goeddel. 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell*. 78:681–692.
36. Cao, Z., J. Xiong, M. Takeuchi, T. Kurama, and D.V. Goeddel. 1996. TRAF6 is a signal transducer for interleukin-1. *Nature*. 383:443–446.
37. Yeh, W.C., A. Shahinian, D. Speiser, J. Kraunus, F. Billia, A. Wakeham, J.L. Delapompa, D. Ferrick, B. Hum, N. Iscove, et al. 1997. Early lethality, functional NF-κB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity*. 7:715–725.
38. Xu, Y., G. Cheng, and D. Baltimore. 1996. Targeted disruption of TRAF3 leads to postnatal lethality and defective T-dependent immune responses. *Immunity*. 5:407–415.
39. Tivol, E.A., F. Borriello, A.N. Schweitzer, W.P. Lynch, J.A. Bluestone, and A.H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity*. 3:541–547.
40. Waterhouse, P., J.M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K.P. Lee, C.B. Thompson, H. Griesser, and T.W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in *Ctla-4*. *Science*. 270:985–988.
41. Lee, S.Y., C.G. Park, and Y. Choi. 1996. T cell receptor-dependent cell death of T cell hybridomas mediated by the CD30 cytoplasmic domain in association with tumor necrosis factor receptor-associated factors. *J. Exp. Med*. 183:669–674.
42. Romagnani, S., and E. Maggi. 1995. T_H2 downregulation of macrophage HIV-1 replication. *Science*. 267:538–539.
43. Dong, C., D.D. Yang, C. Tournier, A.J. Whitmarsh, J. Xu, R.J. Davis, and R.A. Flavell. 2000. JNK is required for effector T-cell function but not for T-cell activation. *Nature*. 405: 91–94.
44. Ip, Y.T., and R.J. Davis. 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)- from inflammation to development. *Curr. Opin. Cell Biol*. 10:205–219.
45. Min, W., J.R. Bradley, J.J. Galbraith, S.J. Jones, E.C. Ledgerwood, and J.S. Pober. 1998. The N-terminal domains target TNF receptor-associated factor-2 to the nucleus and display transcriptional regulatory activity. *J. Immunol*. 161: 319–324.
46. Krajewski, S., J.M. Zapata, M. Krajewska, T. VanArsdale, A. Shabaik, R.D. Gascoyne, and J.C. Reed. 1997. Immunohistochemical analysis of in vivo patterns of TRAF-3 expression, a member of the TNF receptor-associated factor family. *J. Immunol*. 159:5841–5852.
47. Mowen, K.A., J. Tang, W. Zhu, B.T. Schurter, K. Shuai, H.R. Herschman, and M. David. 2001. Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription. *Cell*. 104:731–741.