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IFN- γ Production by CD8⁺ T Cells Depends on NFAT1 Transcription Factor and Regulates Th Differentiation¹

Leonardo K. Teixeira,* Bruna P. F. Fonseca,* Adriana Vieira-de-Abreu,[†] Bianca A. Barboza,* Bruno K. Robbs,* Patrícia T. Bozza,[†] and João P. B. Viola^{2*}

CD8⁺ T lymphocytes are excellent sources of IFN- γ ; however, the molecular mechanisms that dictate IFN- γ expression upon TCR stimulation in these cells are not completely understood. In this study, we evaluated the involvement of NFAT1 in the regulation of IFN- γ gene expression in murine CD8⁺ T cells and its relevance during Th differentiation. We show that CD8⁺, but not CD4⁺, T cells, represent the very first source of IFN- γ upon primary T cell activation, and also that the IFN- γ produced by naive CD8⁺ T cells may enhance CD4⁺ Th1 differentiation *in vitro*. TCR stimulation rapidly induced IFN- γ expression in CD8⁺ T lymphocytes in a cyclosporin A-sensitive manner. Evaluation of CD8⁺ T cells showed that calcium influx alone was sufficient to activate NFAT1 protein, transactivate IFN- γ gene promoter, and induce IFN- γ production. In fact, NFAT1-deficient mice demonstrated highly impaired IFN- γ production by naive CD8⁺ T lymphocytes, which were totally rescued after retroviral transduction with NFAT1-encoding vectors. Moreover, NFAT1-dependent IFN- γ production by the CD8⁺ T cell compartment was crucial to control a Th2-related response *in vivo*, such as allergic inflammation. Consistently, CD8 α - as well as IFN- γ -deficient mice did not mount a Th1 immune response and also developed *in vivo* allergic inflammation. Our results clearly indicate that IFN- γ production by CD8⁺ T cells is dependent of NFAT1 transcription factor and may be an essential regulator of Th immune responses *in vivo*. *The Journal of Immunology*, 2005, 175: 5931–5939.

Upon T cell stimulation, CD4⁺ T lymphocytes may undergo a Th1/Th2 differentiation that is mostly characterized by the distinct pattern of cytokines they secrete. Th1 cells produce IFN- γ , which is essential for the eradication of intracellular pathogens, whereas Th2 cells secrete IL-4, IL-5, and IL-13, which are crucial to the elimination of extracellular organisms and to sustain allergic reactions. Several factors can influence the differentiation pathway of CD4⁺ Th cells, especially the cytokines prevailing within the microenvironment where these cells encounter Ags (1, 2). IL-12 and IFN- γ are known to be the major Th1-inducing cytokines (3). IFN- γ is a pleiotropic cytokine that is essential for both innate and adaptive immunities (4), and its role in CD4⁺ Th1 differentiation has been intensely addressed. *In vitro* studies have shown that IFN- γ exerts both indirect and direct effects during Th1 development (5, 6). It induces APCs to produce IL-12, which is of great importance during Th1 cell commitment (7, 8). In addition, IFN- γ is responsible for inducing/maintaining the expression of the β -chain of the IL-12R (IL-12R β 2) on CD4⁺ T cells, indicating an important role for IFN- γ in the Th1 effects mediated by IL-12 (9, 10). Furthermore, IFN- γ and Th1 responses are considered to be protective against Th2-related disorders such

as asthma and allergy (11–13). In animal models, the adoptive transfer of IFN- γ -producing cells into allergen-sensitized recipients has protected from airway eosinophilia after Ag challenge (14, 15). Defective IFN- γ production also predisposes toward the development of allergic diseases, and patients with severe asthma present significantly reduced IFN- γ production in response to allergen compared with control individuals (16, 17).

Different transcription factors have been shown to regulate IFN- γ gene expression in T lymphocytes (3, 18). In CD4⁺ T cells, T-bet, the master switch of the Th1 response, is a key regulator of IFN- γ production in developing Th1 lymphocytes (19). However, T-bet expression is induced by IFN- γ signaling pathway through STAT1, and thus is dependent on an initial source of IFN- γ (10, 20). Several cellular compartments of the immune system have been characterized as potential sources of IFN- γ *in vivo*, including NK cells and CD8⁺ T cells (21, 22). It has been suggested that CD8⁺ T cells may represent an early source of IFN- γ , which acts directly on CD4⁺ Th1 differentiation (22). Nevertheless, there are no available data concerning the involvement of TCR-induced transcription factors in the regulation of IFN- γ expression in CD8⁺ T cells.

NFAT proteins are pre-existing cytoplasmic transcription factors that are rapidly activated in T lymphocytes upon TCR stimulation (23). The activation of NFAT proteins requires sustained intracellular calcium levels that are induced shortly after TCR triggering (23, 24). Calcium influx then activates the Ca²⁺-dependent phosphatase calcineurin, which dephosphorylates NFAT (25, 26). Once activated, NFAT translocates to the nucleus, where it binds to regulatory sequences and regulates the expression of several cytokine genes, including IFN- γ (27, 28). The process of NFAT activation is blocked by immunosuppressive drugs, such as cyclosporin A (CsA)³ and FK506, which inhibit the phosphatase activity of calcineurin (29, 30). In fact, NFAT1-deficient mice

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³ Abbreviations used in this paper: CsA, cyclosporin A; EGFP, enhanced GFP; Eomes, eomesodermin; IRES, internal ribosomal entry sequence.

(NFAT1^{-/-}) present a preferential differentiation toward a Th2 phenotype, including low levels of IFN- γ and high levels of IL-4 (31, 32). Consistently, CD4⁺ T cells from NFAT1^{-/-} mice present an impaired IFN- γ production (33), but no reports regarding the participation of NFAT1 in the regulation of IFN- γ in CD8⁺ T lymphocytes have been described.

Thus, we addressed the involvement of NFAT1 transcription factor in the regulation of IFN- γ production in CD8⁺ T cells and its influence on Th1/Th2 immune responses using *in vitro* and *in vivo* models of Th differentiation. In this study we show that naive CD8⁺ T cells do produce high levels of IFN- γ upon TCR triggering during the primary response, which is dependent on NFAT1 transcription factor. Also, we demonstrate that IFN- γ production by the CD8⁺ T cell compartment enhances CD4⁺ Th1 differentiation *in vitro* and is crucial to control allergic inflammation, which has been related to a Th1/Th2 immune response deregulation. Our results thus suggest that NFAT1 protein plays a positive regulatory role in IFN- γ production in CD8⁺ T cells, which may modulate Th immune response *in vivo*.

Materials and Methods

Animals, cells, and reagents

C57BL/6, NFAT1^{-/-}, CD8 α ^{-/-}, and IFN- γ ^{-/-} 8- to 12-wk-old female mice were used in all experiments. Animals were bred and maintained in the Brazilian National Cancer Institute animal facility. Animals were treated according to the animal care guidelines of the Council for International Organizations of Medical Sciences. All primary cells (lymph nodes, CD3⁺, CD4⁺, and CD8⁺ T lymphocytes) and the mouse CD8⁺ CTLL-R8⁺ cell line were cultured in DMEM supplemented with 10% FCS, L-glutamine, streptomycin-penicillin, essential and nonessential amino acids, sodium pyruvate, vitamins, and 2-ME (all from Invitrogen Life Technologies). Hybridomas 2C11 (anti-CD3) and 53-6.7 (anti-CD8) were cultured in RPMI 1640 supplemented with 10% FCS. All Abs were purified from hybridoma supernatants by chromatography over protein G (Amersham Biosciences), and their activities were functionally tested by cellular proliferation, complement-dependent depletion, and ELISA. The cytokines IFN- γ , IL-2, IL-12, and IL-18 were purchased from PepruTech. The polyclonal Ab 67.1 (Dr. A. Rao, Harvard University, Boston, MA) was used to detect the NFAT1 protein. PMA and ionomycin were obtained from Calbiochem, and the immunosuppressive drug CsA was obtained from LC Laboratories. CFA and OVA were purchased from Sigma-Aldrich. The solutions of May-Grünwald and Giemsa were obtained from Merck. Polybrene (hexadimethrin bromide) was obtained from Fluka Chemie, and chondroitin 6-sulfate sodium salt was purchased from Sigma-Aldrich.

Cell isolation and flow cytometry

In all experiments, different cell populations (CD3⁺, CD4⁺, and CD8⁺ T cells) were obtained from lymph nodes (inguinal, brachial, axillary, and superficial cervical). Purified single-cell suspensions were isolated by negative selection with magnetic beads (Micro Beads, MACS technology) according to the manufacturer's instructions (Miltenyi Biotec). Streptavidin magnetic beads were conjugated to specific-biotinylated Abs (anti-CD4, anti-CD8, and anti-B220/CD45R) to sort out undesired cell populations. For cytometric analysis, cells were stained with specific fluorochrome-labeled Abs as previously described (32). Labeled mAbs were all obtained from BD Pharmingen, and cells were analyzed by flow cytometry on a FACScan (BD Biosciences). Cell populations were isolated to >95% purity.

CD4⁺ Th differentiation

CD4⁺ and CD8⁺ T cells were purified from C57BL/6 (IFN- γ ^{+/+}) or IFN- γ ^{-/-} as described. For cocultivation assays, CD4⁺ T cells from IFN- γ ^{+/+} mice were cultured together with CD8⁺ T cells from either IFN- γ ^{+/+} or IFN- γ ^{-/-} mice (CD4:CD8 ratio, 2:1) and primarily stimulated *in vitro* for 72 h with plate-bound anti-CD3 (1 μ g/ml) in the presence of IL-12 (10 ng/ml). On the third day, cells were harvested, washed, and rested for 48 h. After resting, CD4⁺ T cells were isolated again as described and were left unstimulated or were *in vitro* stimulated for 48 h with plate-bound anti-CD3 (1 μ g/ml). After stimulation, cell-free supernatants were assessed for IFN- γ or IL-4 by ELISA.

ELISA and intracellular cytokine staining

Cells were left unstimulated or were *in vitro* stimulated for 24, 48, or 72 h at 37°C with different stimuli as indicated. Cell-free supernatant was as-

essed for IFN- γ and IL-4 protein levels by ELISA according to the manufacturer's instructions (BD Pharmingen). For intracellular cytokine staining, indicated cell populations (1 \times 10⁶ cells) from naive C57BL/6, NFAT1^{+/+}, and NFAT1^{-/-} mice were stimulated *in vitro* for 72 h with plate-bound anti-CD3 (1 μ g/ml). Brefeldin A (1/1000; Cytofix/Cytoperm; BD Pharmingen) was added to the culture 5 h before the staining procedure. Briefly, cells were harvested, and surface markers were stained with anti-CD8-FITC or anti-CD4-FITC Ab. Cells were next fixed and permeabilized for intracellular cytokine staining with anti-IFN- γ -PE Ab, then analyzed by flow cytometry.

RNase protection assays, Western blot, and immunofluorescence staining

For RNase protection assay analysis, purified CD8⁺ T cells (2 \times 10⁶ cells) from C57BL/6 mice were left unstimulated or were *in vitro* stimulated for 6 h at 37°C with plate-bound anti-CD3 (1 μ g/ml) as indicated. CsA (5 μ M) was added to cells 15 min before anti-CD3 stimulation. Total RNA was immediately extracted with TRIzol reagent according to the manufacturer's protocol (Invitrogen Life Technologies). mRNA expression was analyzed with a multiprobe RNase protection assay kit (Ribo-Quant; BD Pharmingen). For IFN- γ expression analysis, mCK-1 and mCK-2 multiprobe sets were used, and RNA loading was estimated by measuring GAPDH and L32 housekeeping genes.

To detect the presence of the NFAT1 protein, purified CD8⁺ T cells from C57BL/6 mice or the CTLL-R8⁺ cell line (2 \times 10⁶ cells) were left unstimulated or were *in vitro* stimulated for 15 min at 37°C with ionomycin (5 μ M). CsA (5 μ M) was added to cells 15 min before stimulation. Total protein lysates were obtained as previously described (26). Briefly, cells were lysed in buffer containing 40 mM Tris (pH 7.5), 60 mM sodium pyrophosphate, 10 mM EDTA, and 5% SDS, followed by incubation at 100°C for 20 min. Small-scale nuclear extracts were made as previously described (34). Briefly, cells were resuspended in buffer containing 0.5% Nonidet P-40, 10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM MgCl₂, 0.5 mM DTT, 0.1 mM EGTA, 2 mM leupeptin, 1 mg/ml aprotinin, and 1 mM PMSF. The supernatant was removed to a separate tube, and the nuclear pellet was lysed in the same buffer as described above. Cell extracts were analyzed by electrophoresis on 6% SDS-PAGE. The separated proteins were transferred to nitrocellulose membrane, and NFAT1 protein was detected by the polyclonal Ab 67.1 as previously described (26).

Intracellular localization of NFAT1 protein was addressed in purified CD8⁺ T cells (2 \times 10⁵ cells) from C57BL/6 mice by immunofluorescence staining, also as previously described (26). Briefly, cells were attached to coverslips previously coated for 1 h with 2% gelatin and were left unstimulated or were stimulated for 16 h at 37°C with ionomycin (5 μ M). CsA (5 μ M) was added to cells 15 min before ionomycin. Then cells were fixed in 3% paraformaldehyde, permeabilized with 0.1% Nonidet P-40, and stained with anti-NFAT1 67.1 Ab. The cells were photographed under \times 100 magnification with a Zeiss Axiovert S100 microscope.

Retroviral construction and lymphocyte transduction

The pLIREs-EGFP bicistronic vector was constructed by inserting a 1.4-kb *BgIII-NoI* fragment from the pIRES2-EGFP vector (BD Clontech), comprising the encephalomyocarditis virus internal ribosomal entry sequence (IRES) and the enhanced GFP (EGFP) coding region, into the pLEGFP-N1 retroviral vector (BD Clontech; pLIREs-EGFP-empty). To generate the retroviral vector encoding for NFAT1, the full-length cDNA from NFAT1 isoform C was cloned into the pLIREs-EGFP (pLIREs-EGFP-NFAT1). Then, the BD Eco-Pack2 ecotropic packing cell line (BD Biosciences) was transiently transfected with retroviral vectors by calcium phosphate precipitation for 16 h. Cell-free virus-containing supernatant was collected 48 h after transfection, and concentrated as previously described (35). The supernatant was supplemented with IL-2 (20 U/ml), and immediately used for spin infection (twice, 45 min each time, 1800 rpm, room temperature) of purified CD8⁺ T cells from naive NFAT1^{+/+} and NFAT1^{-/-} mice previously stimulated with anti-CD3 (1 μ g/ml) for 24 h. Infected cells were incubated at 37°C for an additional 48 h, supplemented with fresh medium, and stimulated with anti-CD3 (1 μ g/ml) for another 48 h. Cells were then stained for intracellular cytokine as described above, and EGFP⁺ CD8⁺ lymphocytes were analyzed by flow cytometry for IFN- γ production as described.

Transactivation assays

CTLL-R8⁺ cell line (2 \times 10⁶ cells) was electroporated (950 μ F, 250 V) in a 0.4-cm GenePulser Cuvette (Bio-Rad) with 5 μ g of the indicated IFN- γ -promoter constructs fused to a luciferase reporter gene (Luc; donated by Dr. C. Wilson, University of Washington, Seattle, WA) (28) in serum-free

medium. After 24 h, cells were washed and left unstimulated or were stimulated in vitro for 16 h at 37°C with PMA (10 nM) or ionomycin (5 μ M) as indicated. CsA (5 μ M) was added to cells 15 min before any other treatment when indicated. The next day, cells were harvested, and lysis was performed for 20 min at room temperature with 50 μ l of 1 \times cell culture lysis reagent (Promega). Crude extract (10 μ l) was added to 100 μ l of luciferase assay substrate (Promega). Luciferase activity was promptly measured in a Monolight 3010 Luminometer (Analytical Luminescence Laboratory) and was expressed as relative light units.

Eomesodermin (Eomes) RT-PCR

Purified CD8⁺ T cells (2×10^6 cells) from naive NFAT1^{+/+} and NFAT1^{-/-} mice were left unstimulated (0 h) or were in vitro stimulated for the indicated time periods at 37°C with plate-bound anti-CD3 (1 μ g/ml). Total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies), and semiquantitative RT-PCR for murine Eomes expression was performed using the Ready-To-Go You-Prime First-Strand Beads, according to the manufacturer's instructions (Amersham Biosciences). The primers used were as follows: Eomes, 5'-GCC CAC GTC TAC CTG TGC AAC CG-3' and 5'-TGT TAT TGG TGA GTT TTA ACT TCC C-3' (334-bp product); and GAPDH, 5'-TGA AGG TCG GTG TGA ACG GAT TTG-3' and 5'-ACG ACA TAC TCA GCA CCA GCA TCA-3' (276-bp product). PCR conditions were as follows: 95°C for 3 min; 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s; and final elongation at 72°C for 10 min. PCR products were resolved on 1.5% agarose gels and visualized with ethidium bromide.

Pleurisy model

Naive animals were s.c. sensitized with 200 μ g (0.1 ml) of OVA emulsified in CFA in a hind footpad, as previously described (36). Fifteen days later, animals were intrathoracically challenged with PBS or OVA (12 μ g) as indicated. After 24 h, thoracic cavity was rinsed with 1 ml of PBS/0.1% BSA. Cells were then cytocentrifuged and stained with May-Grünwald/Giemsa for differential leukocyte analysis. Draining lymph nodes (popliteal and inguinal) were harvested, and cells (2×10^6 cells) were stimulated in vitro for 48 h with plate-bound anti-CD3 (1 μ g/ml). Cell-free supernatants were then assessed for IFN- γ and IL-4 by ELISA. For in vivo CD8⁺ T cell depletion, NFAT1^{+/+} mice were i.v. treated with anti-CD8 Ab (100 μ g/animal) every 2 days in a total of five injections before sensitization (NFAT1^{+/+} and anti-CD8). Thereafter, mice continued to be treated with anti-CD8 Ab (100 μ g/animal) every 2 days until challenge. CD8⁺ T cell depletion was evaluated by flow cytometry of lymph nodes, which always showed <3% CD8⁺ T cells.

Statistical analysis

Statistical analysis of values from wild type (+/+) and knockout (-/-) mice and between control and treated groups was performed using unpaired Student's *t* test for single comparison. A value of *p* < 0.05 was considered statistically significant.

Results

Influence of IFN- γ produced by CD8⁺ T cells on CD4⁺ Th1 differentiation

The cytokine IL-12 was initially characterized as the dominant cytokine influencing the Th1 phenotype (3, 7, 8). However, additional studies have shown that other cytokines, such as IFN- γ , may also play a role during Th1 development (5, 6). In an in vitro model of Th differentiation, disruption of IFN- γ R signaling pathway drastically reduced IFN- γ production by CD4⁺ T cells even when stimulated in the presence of IL-12, indicating that IFN- γ directly enhances CD4⁺ Th1 differentiation (data not shown). Several cellular sources of IFN- γ may enhance CD4⁺ Th1 differentiation, such as NK, dendritic cells and CD8⁺ T cells (3, 21, 22). As shown in Fig. 1A, upon TCR stimulation of primary lymph node cells, CD8⁺ T lymphocytes represent the very first source of IFN- γ production. To specifically address the influence of the IFN- γ produced by CD8⁺ T cells on CD4⁺ Th1 differentiation, naive CD4⁺ T lymphocytes cultivated together with CD8⁺ T cells from IFN- γ ^{+/+} or IFN- γ ^{-/-} mice were stimulated in vitro for 72 h with anti-CD3. After this primary stimulation, CD4⁺ T lymphocytes were purified, stimulated in vitro, and assessed for their ability to produce IFN- γ and IL-4. We observed in this study that

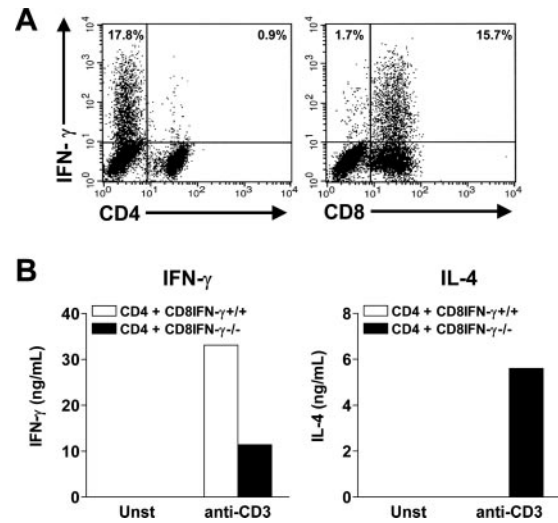


FIGURE 1. IFN- γ produced by CD8⁺ T lymphocytes enhances CD4⁺ Th1 differentiation. *A*, Total lymph node cells from naive C57BL/6 mice were stimulated in vitro with anti-CD3 (1 μ g/ml). After stimulation, cells were analyzed for intracellular IFN- γ production at 72 h as described. Data are representative of three independent animals. *B*, Purified CD4⁺ T lymphocytes from IFN- γ ^{+/+} or IFN- γ ^{-/-} mice were cultivated together with CD8⁺ T cells from IFN- γ ^{+/+} or IFN- γ ^{-/-} mice, and then stimulated in vitro for 72 h with anti-CD3 (1 μ g/ml) in the presence of IL-12 (10 ng/ml). On the third day, cells were rested for 48 h. After resting, CD4⁺ T cells were purified again and were left unstimulated (Unst) or were stimulated in vitro for 48 h with anti-CD3 (1 μ g/ml). Cell-free supernatants were assessed for IFN- γ and IL-4 by ELISA. All results are from a pool of three mice and are representative of two independent experiments.

CD4⁺ T cells, which were cocultivated with IFN- γ -competent CD8⁺ T cells, clearly produce more IFN- γ and less IL-4 than those cocultivated with IFN- γ -deficient CD8⁺ T lymphocytes (Fig. 1B). Thus, our results demonstrate that the IFN- γ produced by CD8⁺ T cells enhances Th1 phenotype development of CD4⁺ T lymphocytes during primary stimulation in vitro.

IFN- γ production by CD8⁺ T lymphocytes

The molecular mechanisms that control IFN- γ gene expression in CD4⁺ Th cells are widely known; however, the mechanisms that dictate its expression in CD8⁺ T lymphocytes are not completely elucidated. Thus, we decided to address the molecular pathways by which CD8⁺ T cells produce IFN- γ . We show in this study that purified naive CD8⁺ T cells produce IFN- γ upon TCR stimulation, but not in response to IL-12/IL-18 treatment alone (Fig. 2A). We also observed that the IFN- γ production level was not altered when anti-CD28 costimulation was administered together with anti-CD3 (data not shown). However, IL-12/IL-18 administration highly increased IFN- γ levels when associated with TCR triggering, suggesting that both cytokines may play a secondary role on TCR-induced IFN- γ production by CD8⁺ T lymphocytes (Fig. 2B). To evaluate the influence of other pathways in IFN- γ production, we stimulated naive CD8⁺ T cells with different stimuli. Surprisingly, ionomycin-driven Ca²⁺ influx was sufficient to induce IFN- γ production in CD8⁺ T cells, which was slightly potentiated by PMA administration (Fig. 2C). However, PMA stimulation alone was not sufficient to induce IFN- γ production in these cells (Fig. 2C). In addition, we found that IFN- γ expression is totally dependent on TCR stimulation, because IFN- γ transcript levels could be detected as early as 6 h after TCR triggering, but not in unstimulated CD8⁺ T cells (Fig. 2D). In this study, we also show that IFN- γ expression/production by CD8⁺ T lymphocytes is totally inhibited when CsA is administered together

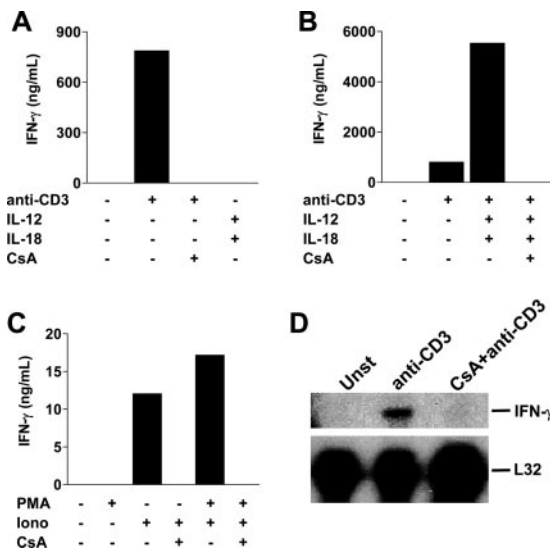


FIGURE 2. TCR triggering induces IFN- γ production in CD8⁺ T lymphocytes in a CsA-sensitive manner. CD8⁺ T lymphocytes were purified from naive C57BL/6 mice as described, and then left unstimulated or stimulated in vitro as indicated: anti-CD3 (1 μ g/ml), IL-12 (10 ng/ml), IL-18 (50 ng/ml), PMA (10 nM), ionomycin (Iono; 5 μ M), or CsA (5 μ M). CsA was added 15 min before the other treatments. *A–C*, Analysis of IFN- γ production by CD8⁺ T cells after 72 h of stimulation as indicated. Cell-free supernatant was assessed for IFN- γ by ELISA. *D*, Analysis of IFN- γ expression in CD8⁺ T cells by RNase protection assay after 6 h of stimulation. RNA loading was estimated by measuring the intensity of L32 housekeeping ribosomal protein gene. All results are from a pool of three mice and are representative of three independent experiments.

with any other stimulus (Fig. 2). These results clearly demonstrate that CD8⁺ T lymphocytes are competent for IFN- γ production upon TCR engagement, and IFN- γ expression was totally inhibited by CsA at the transcriptional level.

CD8⁺ T lymphocytes present functional NFAT1 protein

The activation of NFAT transcription factors requires sustained intracellular Ca²⁺ levels induced by TCR engagement (23). Calcium influx then activates the Ca²⁺-dependent phosphatase calcineurin, which is blocked by the immunosuppressive drug CsA (23). Taking into account that IFN- γ expression is Ca²⁺ dependent and blocked in a CsA-sensitive manner, we decided to investigate whether CD8⁺ T lymphocytes presented NFAT transcription factor. We demonstrate for the first time, to our knowledge, that naive CD8⁺ T cells do present inactive endogenous NFAT1 transcription factor (phosphorylated form), which is activated by ionomycin-induced Ca²⁺ influx (dephosphorylated form), and blocked by CsA (Fig. 3). In fact, ionomycin treatment not only led to NFAT1 dephosphorylation, but also resulted in NFAT1 nuclear translocation, which was again blocked by CsA as observed by Western blot of nuclear lysate and immunofluorescence staining of CD8⁺ T lymphocytes (Fig. 3).

Effect of NFAT transcription factor on IFN- γ promoter

We then examined the ability of NFAT transcription factor to regulate the IFN- γ promoter in a CD8⁺ T cell line. The CTLL-R8⁺ cell line presents endogenous NFAT1 whose activation is induced by ionomycin and blocked by CsA (Fig. 4A). Moreover, the CTLL-R8⁺ cell line did not produce IFN- γ when maintained in culture without stimulation, but ionomycin treatment was sufficient to induce its production, as assessed by ELISA (data not shown). We then transiently transfected these cells with different IFN- γ promoter constructs containing NFAT-binding sites and driving

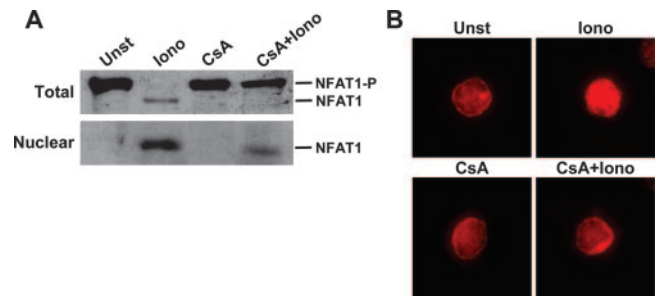


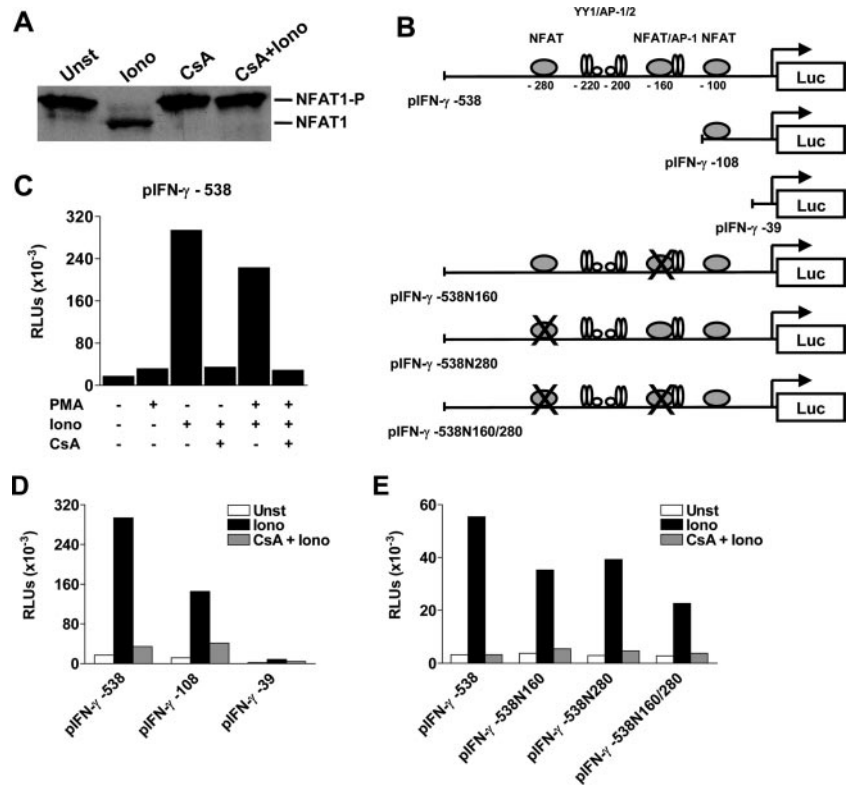
FIGURE 3. NFAT1 is dephosphorylated and translocated to the nucleus in CD8⁺ T lymphocytes after Ca²⁺ influx. CD8⁺ T lymphocytes were purified from naive C57BL/6 mice as described, and then left unstimulated (Unst) or stimulated in vitro as indicated: ionomycin (Iono; 5 μ M) or CsA (5 μ M). CsA was added 15 min before ionomycin. *A*, Detection of NFAT1 transcription factor in CD8⁺ T cells in total lysates (Total; upper panel) or nuclear lysates (Nuclear; lower panel) by Western blot analysis after 15 min of stimulation. NFAT1, dephosphorylated NFAT1; NFAT1-P, phosphorylated NFAT1. *B*, Cellular localization of NFAT1 protein in CD8⁺ T cells by immunofluorescence staining after 15 min of stimulation. All results are representative of at least two independent experiments.

the luciferase reporter gene (Fig. 4B). Three NFAT-binding sites were approximately identified at positions -100, -160, and -280 bp through the proximal 538 bp of the IFN- γ promoter (pIFN- γ -538; Fig. 4B) (28). Interestingly, ionomycin stimulation was sufficient to highly transactivate the IFN- γ proximal promoter construct pIFN- γ -538 (Fig. 4C). In accordance with primary CD8⁺ T cells results, PMA administration alone did not induce luciferase activity or enhance reporter expression when associated with ionomycin, indicating that ionomycin-driven Ca²⁺ influx alone was sufficient to induce optimal response (Fig. 4C). The partial promoter construct pIFN- γ -108, which contained a single NFAT-binding site, still showed 50% luciferase activity compared with the full-length promoter construct (Fig. 4D). Again, IFN- γ promoter activity remained sensitive to CsA (Fig. 4, C and D). However, promoter construct pIFN- γ -39 showed no luciferase expression at all (Fig. 4D). To evaluate the direct role of NFAT on IFN- γ promoter transactivation, we analyzed the effects of point mutations in different NFAT binding sites (Fig. 4B) (28). As shown in Fig. 4E, point mutations at the NFAT -160 site (N160), -280 site (N280), or both sites (N160/N280) decreased luciferase activity compared with the wild-type pIFN-538 construct. Point mutation at both sites (N160/N280) decreased luciferase expression by an average of 50% compared with the pIFN-538 construct, which is similar to the reduction observed in the pIFN- γ -108 construct (Fig. 4, D and E). It is still important to note in this study the presence of a third intact NFAT binding site at position -100, which could explain at least some of the luciferase activity observed in the double mutant (N160/N280) and pIFN-108 constructs (Fig. 4, D and E). This site might play an important unrecognized role in IFN- γ promoter regulation in CD8⁺ T cells. Moreover, luciferase expression always remained sensitive to CsA in all constructs (Fig. 4, D and E). Based on these results, we conclude that NFAT transcription factor plays a positive role in regulation of the IFN- γ promoter in the CD8⁺ T cell compartment.

Involvement of NFAT1 in IFN- γ production by CD8⁺ T cells

To unequivocally test the hypothesis that NFAT1 is required for IFN- γ production in CD8⁺ T lymphocytes, we evaluated intracellular IFN- γ production by these cells in NFAT1-deficient mice (NFAT1^{-/-}). Strikingly, upon stimulation of purified naive CD3⁺ T lymphocytes from NFAT1^{+/+} mice, CD8⁺, but not CD4⁺, T

FIGURE 4. NFAT is able to transactivate IFN- γ promoter in the CD8⁺ T cell line. CTLL-R8⁺ cells were left unstimulated (Unst) or were stimulated in vitro as indicated: ionomycin (Iono; 5 μ M), PMA (10 nM), or CsA (5 μ M). CsA was added 15 min before the other treatments. **A**, Detection of NFAT1 transcription factor in CTLL-R8⁺ cells by Western blot analysis 15 min after stimulation. NFAT1, dephosphorylated NFAT1; NFAT1-P, phosphorylated NFAT1. **B**, Schematic view of the plasmid constructs used in the transactivation assay. Different IFN- γ -promoter constructs containing NFAT-binding sites were used: pIFN- γ -538 (proximal 538 bp of IFN- γ promoter), pIFN- γ -108, pIFN- γ -39, pIFN- γ -538N160 (point mutation in the NFAT -160 site), pIFN- γ -538N280 (point mutation in the NFAT1 -280 site), and pIFN- γ -538N160/N280 (double mutation). Element binding sites for the transcription factors NFAT, AP-1, AP-2, and YY-1 are indicated. **C–E**, CTLL-R8⁺ cells were transfected with the indicated IFN- γ -promoter constructs and left unstimulated (Unst) or stimulated in vitro for 16 h as indicated. Then luciferase activity (Luc) was measured, and gene reporter activity was expressed as relative light units (RLUs). All results are representative of at least two independent experiments.



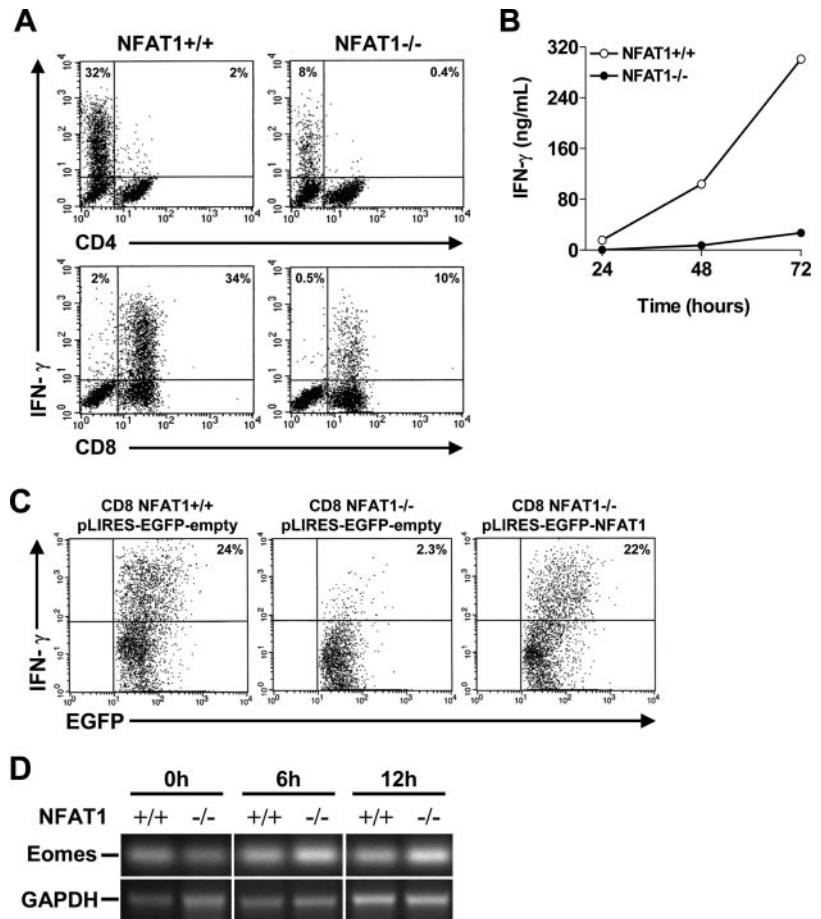
cells represented the main source of IFN- γ during the primary response (Fig. 5A). Furthermore, NFAT1^{-/-} mice showed a 3-fold decrease in the frequency of IFN- γ -producing CD8⁺ T cells compared with NFAT1^{+/+} mice after 72 h of in vitro stimulation (Fig. 5A). Also, NFAT1^{-/-} CD8⁺ T cells showed a drastic impairment of IFN- γ production compared with NFAT1^{+/+} CD8⁺ T cells, as assessed by ELISA (Fig. 5B). To investigate the essential role of NFAT1 transcription factor during IFN- γ expression in CD8⁺ T cells, NFAT1^{-/-} CD8⁺ T lymphocytes were retrovirally transduced with an NFAT1-encoding vector. As shown in Fig. 5C, CD8⁺ T cells from NFAT1^{-/-} mice infected with pLIREs-EGFP-empty construct showed drastically decreased IFN- γ -producing cells compared with NFAT1^{+/+} CD8⁺ T cells infected with the same construct. However, the frequency of IFN- γ -producing cells was rescued in NFAT1^{-/-} CD8⁺ T cells retrovirally transduced with NFAT1-encoding vector (pLIREs-EGFP-NFAT1) to the same levels as the NFAT1^{+/+} CD8⁺ T cells (Fig. 5C). Although these results clearly demonstrate that IFN- γ expression in CD8⁺ T lymphocytes is extremely dependent on NFAT1, we also decided to investigate the expression levels of the transcription factor Eomes, which has been shown to be responsible for IFN- γ production in CD8⁺ T lymphocytes (37). As shown in Fig. 5D, no differences were observed on Eomes gene expression levels in CD8⁺ T lymphocytes from NFAT1^{+/+} compared with NFAT1^{-/-} mice. Thus, our results clearly demonstrate that the IFN- γ produced by T lymphocytes upon TCR stimulation primarily originates from CD8⁺ T cells and depends mostly on NFAT1 transcription factor.

In vivo consequences of NFAT1-dependent IFN- γ production

To further characterize the relevance of the IFN- γ produced by CD8⁺ T cells during in vivo Th immune responses, we took advantage of a well-defined pleurisy model. Allergic diseases are usually characterized by eosinophil tissue infiltration, increase in the level of serum IgE, and a Th2 pattern of cytokine production,

including IL-4, IL-5, and IL-13, which is totally dependent on CD4⁺ T lymphocytes (38). It has been clearly shown that Th1 cytokines, such as IFN- γ and IL-12, may suppress and counteract the Th2 response of some allergic diseases (11, 12). Thus, we asked whether NFAT1-dependent IFN- γ production by CD8⁺ T cells could modulate the Th1/Th2 immune response and control allergic inflammation in vivo. We performed a pleurisy model of allergic inflammation to evaluate the influence of IFN- γ on eosinophil infiltration and cytokine production profile. As previously demonstrated, we show in this study that NFAT1^{-/-} mice present more eosinophils in the pleural cavity than wild-type mice after Ag challenge (Fig. 6) (36, 39). Reinforcing our proposal, in vivo depletion of CD8⁺ T cells from wild-type mice (NFAT1^{+/+} plus anti-CD8) led to eosinophilia in the pleural cavity similar to levels in NFAT1^{-/-} mice (Fig. 6). Consistently, CD8 α ^{-/-} and IFN- γ ^{-/-} mice also presented more pleural eosinophils compared with respective wild-type mice (Fig. 6). To better understand the immune response generated in vivo in this model, we analyzed the cytokine production profile after restimulation ex vivo. As shown in Fig. 7, NFAT1^{-/-} mice presented an enhanced Th2 phenotype, including higher levels of IL-4 production and lower levels of IFN- γ compared with wild-type mice. Surprisingly, CD8⁺ T cell-depleted NFAT1^{+/+} mice, which presented eosinophilia similar to NFAT1^{-/-} mice, did not show high levels of IL-4 production (Fig. 7). However, IFN- γ production was strikingly decreased to levels similar to those in NFAT1^{-/-} mice (Fig. 7). Consistently, the frequency of IFN- γ -competent CD4⁺ T cells in CD8⁺ T cell-depleted mice was also decreased to the level in NFAT1^{-/-} mice as analyzed by intracellular cytokine staining (data not shown). Interestingly, CD8 α ^{-/-} mice also presented a highly decreased IFN- γ response compared with respective wild-type mice, but showed a mild increase in IL-4, which was observed in IFN- γ ^{-/-} mice (Fig. 7). These results suggest that NFAT1 transcription factor-dependent IFN- γ production by CD8⁺ T cells plays a crucial role in eosinophil migration in this model of allergic inflammation

FIGURE 5. NFAT1 is crucial for IFN- γ production in CD8⁺ T lymphocytes. *A*, CD3⁺ T lymphocytes were purified from naive NFAT1^{+/+} and NFAT1^{-/-} mice as described and were stimulated in vitro with anti-CD3 (1 μ g/ml). After stimulation, CD4⁺ and CD8⁺ T cells were analyzed for intracellular IFN- γ production at 72 h as described. *B–D*, CD8⁺ T lymphocytes were purified as described from naive NFAT1^{+/+} and NFAT1^{-/-} mice and were stimulated in vitro with anti-CD3 (1 μ g/ml). *B*, Cell-free supernatants of stimulated cells were assessed for IFN- γ by ELISA at the indicated time points. *C*, Cells were transduced with either pLIRE-EGFP-empty or -NFAT1 vector after 24 h of stimulation. Then, EGFP⁺ cells were analyzed by flow cytometry for intracellular IFN- γ production at 96 h as described. *D*, Eomes gene expression was analyzed by semiquantitative RT-PCR at the indicated time points. RNA loading was estimated by measuring the intensity of the GAPDH housekeeping gene. All results are from a pool of three mice and are representative of at least two independent experiments.



and may be important to control Th immune responses and allergic diseases in vivo.

Discussion

In this study, we have demonstrated that IFN- γ production by CD8⁺ T cells during the primary response is dependent on NFAT1 transcription factor. Little is known about the molecular mechanisms that regulate IFN- γ production in CD8⁺ T cells. T-bet, the master switch of the Th1 response, is a key regulator of IFN- γ expression in CD4⁺ T cells (19). Nonetheless, it is noteworthy that T-bet expression is induced through the IFN- γ signaling pathway, and thus its effects are dependent on an initial source of this cytokine (10, 20). Pearce et al. (37) have shown that the transcription factor Eomes, a T-bet paralog, controls effector functions of CD8⁺ T cells, including IFN- γ

production. However, this transcription factor is highly induced in activated CD8⁺ T lymphocytes, but moderately detectable in naive CD8⁺ T cells (37). Also, the regulatory mechanisms that dictate Eomes gene expression in CD8⁺ T lymphocytes are not completely elucidated. In fact, Eomes expression was induced in CD8⁺ T cells early after TCR triggering (Fig. 5D), but this does not seem to explain the striking differences observed in IFN- γ production by NFAT1^{+/+} and NFAT1^{-/-} CD8⁺ T lymphocytes.

By contrast, the NFAT family of transcription factors is largely known to be activated soon after TCR stimulation. Within minutes after Ca²⁺ influx, NFAT translocates to the nucleus and binds to regulatory sequences of the IFN- γ promoter region, regulating its expression (23, 27, 28). In this study we have shown that ionomycin-induced calcium influx was sufficient for IFN- γ production

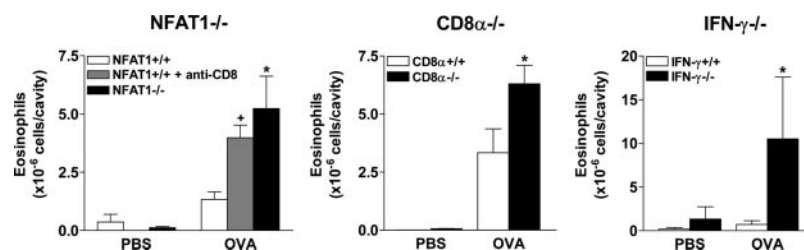
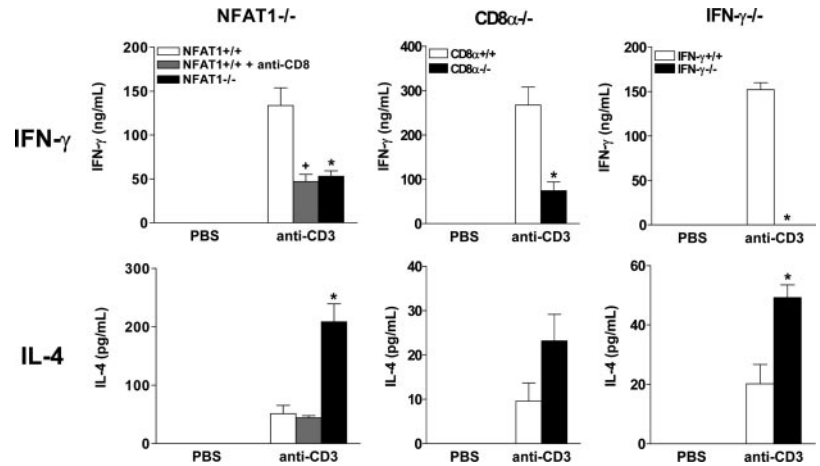


FIGURE 6. NFAT1-dependent IFN- γ production by CD8⁺ T lymphocytes controls eosinophilia in vivo. Naive animals (NFAT1^{+/+}, NFAT1^{-/-}, CD8 α ^{+/+}, CD8 α ^{-/-}, IFN- γ ^{+/+}, and IFN- γ ^{-/-}) or CD8⁺ T cell-depleted mice (NFAT1^{+/+} plus anti-CD8) were s.c. sensitized with OVA (200 μ g) emulsified in CFA in a hind footpad as described. Fifteen days later, animals were intrathoracically challenged with PBS or OVA (12 μ g) as indicated. After 24 h, the thoracic cavity was assessed for differential leukocyte analysis. The total number of pleural eosinophils is shown in control (PBS) and treated (OVA) groups. Data are expressed as the mean \pm SEM of values from five mice and are representative of two independent experiments. *, Significantly different from wild-type, OVA-challenged mice ($p < 0.05$); +, significantly different from non-CD8⁺ T cell-depleted, wild-type, OVA-challenged mice ($p < 0.05$).

FIGURE 7. IFN- γ production by CD8⁺ T cells regulates the cytokine profile in vivo. Naive animals were treated as described in Fig. 6. One day after challenge, the draining lymph nodes (popliteal and inguinal) of the indicated animals were stimulated in vitro for 48 h with anti-CD3 (1 μ g/ml). Then cell-free supernatants were assessed for IFN- γ and IL-4 by ELISA. Data are expressed as the mean \pm SEM values from five mice and are representative of two independent experiments. *, Significantly different from wild-type, OVA-challenged mice ($p < 0.05$); +, significantly different from non-CD8⁺ T cell-depleted, wild-type, OVA-challenged mice ($p < 0.05$).



in naive CD8⁺ T lymphocytes. In fact, the three NFAT-binding sites identified in the proximal regulatory region of the IFN- γ promoter are required for maximum inducibility of this gene in Jurkat T cells and primary murine splenocytes (27, 28). Consistently, CD4⁺ T lymphocytes lacking NFAT1 display a substantial defect in IFN- γ gene expression, independent of the down-regulatory effects of IL-4 and GATA-3 (33). It has also been proposed that NFAT transcription factors may act as candidates to drive early transcription of cytokine genes in T cells, because they can recruit histone acetyltransferases and thus initiate localized histone modification in the IFN- γ promoter region (40–42). Thus, TCR-inducible transcription factors, such as NFAT1, may represent the very first switch on IFN- γ production in CD8⁺ T cells.

The local cytokine microenvironment is fundamental to define the Th1/Th2 balance that CD4⁺ T cells may undergo during Ag recognition (1, 2). The cytokine IFN- γ induces IL-12 production by APCs and also up-regulates the expression of IL-12R β 2 on CD4⁺ T cells through the activation of T-bet (7–10). Both mechanisms are widely known to promote/enhance CD4⁺ Th1 differentiation (3). Myeloid cells, such as dendritic cells and macrophages, represent an early source of IFN- γ and IL-12 in the innate arm of the immune system (3, 4). In fact, CD8a⁺ dendritic cells are able to prime CD4⁺ T lymphocytes toward the Th1 phenotype (43, 44). Furthermore, it has been recently demonstrated that NK cells may also represent an initial source of IFN- γ during Th1 polarization of naive CD4⁺ T cells (21). Our data indicate that upon TCR stimulation of primary T cells, CD8⁺, but not CD4⁺, T lymphocytes, are excellent producers of IFN- γ , which is crucial to enhance CD4⁺ Th1 differentiation (Fig. 1). We thus suggest that CD8⁺ T cells also function as another source of IFN- γ that may reinforce and amplify an adaptive Th1-specific response. In accordance, it has been argued that the IFN- γ secreted by CD8⁺ T cells acts directly on CD4⁺ Th1 priming and also stimulates APCs to secrete IL-12 (22). In that work, in vivo injections of anti-CD3 in various MHC gene knockout mice have clearly demonstrated that IFN- γ is rapidly produced by a distinct population of CD8⁺ T cells and polarizes CD4⁺ T cells toward the Th1 phenotype (22). It has also been shown that the presence of IFN- γ during the early phase of CD4⁺ Th priming is essential for Th1 phenotype stabilization, because CD4⁺ T cells lacking the IFN- γ gene or its receptor do not mount an efficient Th1 response and retain the capacity to produce IL-4 (6). We thus suggest that CD8⁺ T lymphocytes represent an early source of NFAT1-dependent IFN- γ production during the initial adaptive response, which may account for the consolidation of Th1 immunity.

Altering the cytokine-producing profile of Th cells by inducing Th1 responses has been proposed to be protective against Th2-related dis-

orders, such as allergy (11–13). Our findings show that IFN- γ and also CD8⁺ T cells are regulators of eosinophil recruitment and Th immune responses in vivo. Because the IFN- γ produced by CD8⁺ T lymphocytes was highly dependent on NFAT1, we suggest that mice lacking this transcription factor could not counteract a Th2 response and presented overexpression of Th2 cytokines and eosinophilia in vivo. Thus, the impaired NFAT1-dependent IFN- γ production by CD8⁺ T cells could be an alternative explanation for the allergic phenotype described in the NFAT1-deficient mice (36, 39). However, we cannot rule out the hypothesis that NFAT1^{-/-} mice present a Th2-biased phenotype in consequence of an intrinsic defect of CD4⁺ T cells to silence IL-4. This hypothesis could explain the low levels of IL-4 production observed in CD8⁺ T cell-depleted mice and the only moderate increase in CD8 α - and IFN- γ -deficient mice, rather than the profound increase in IL-4 levels observed in NFAT1^{-/-} mice (Fig. 7).

In vivo studies of airway allergic inflammation have demonstrated that IFN- γ , CD8⁺ T cells, and also CD4⁺ Th1 cells are able to regulate Ag-induced eosinophil infiltration by inhibiting Th2 responses (11–15). Interestingly, T-bet-deficient mice had impaired IFN- γ production and also developed spontaneous airway hyper-responsiveness similar to asthma patients, who revealed deficient T-bet expression of the lungs and significantly lower IFN- γ secretion by PBMC compared with healthy individuals (45, 46). In accordance, it has been recently shown in a model of *Leishmania major* infection that the IFN- γ produced by CD8⁺ T cells directly promotes Th1 differentiation and down-regulates initial Th2 immune responses (47). Although depletion of CD8⁺ T lymphocytes in NFAT1^{+/+} mice induced eosinophil infiltration in our model of allergic inflammation (Fig. 6), we cannot exclude the involvement of other cell types in this phenomenon. In addition to CD8⁺ T cells, in vivo treatment with anti-CD8 Ab could deplete CD8a⁺ dendritic cells, which have been implicated in Th1 differentiation as well (43, 44). Thus, it is still possible that the absence of both CD8⁺ T cells and CD8a⁺ dendritic cells might explain the enhanced allergic inflammation observed in CD8-depleted NFAT1^{+/+} mice. Nevertheless, conflicting results have been documented regarding the protective action of CD8⁺ T and CD4⁺ Th1 cells against allergic diseases (48–50).

The suppressive effects of IFN- γ on allergic inflammation may be explained by several mechanisms. It is most likely that IFN- γ directly induces the differentiation of naive T cells toward the Th1 phenotype and/or represses Th2 cell recruitment/differentiation rather than acting on eosinophils itself (4–6). It is also possible that IFN- γ suppresses the release of Th2 cytokines from activated T cells (51, 52) and thus inhibits the following Th2-dependent

eosinophil recruitment (11, 38). However, other reports have shown inhibitory properties of IFN- γ directly on eosinophil infiltration into inflammatory tissues (53, 54). Our results support the idea that NFAT1 plays a positive regulatory role in IFN- γ production by CD8⁺ T cells and may control allergic inflammation in vivo. In contrast, a recent report have shown that inhibition of all NFAT family members in T cells prevents allergic pulmonary inflammation, early eosinophil recruitment to the lungs, and Th2 response development (55). These results support the idea that different NFAT members may play specific roles during immune responses in vivo, because this family consists of five proteins with distinct properties in the regulation of cytokine genes.

In conclusion, we demonstrate in this study that IFN- γ production by naive CD8⁺ T cells during primary stimulation is highly dependent on NFAT1 transcription factor. We also indicate that CD8⁺ T cells and IFN- γ are essential to control allergic inflammation. Finally, we suggest that NFAT1 protein plays a positive regulatory role on IFN- γ production in CD8⁺ T cells, which is central to the generation of Th1 immune responses in vivo.

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Disclosures

The authors have no financial conflict of interest.

References

- Seder, R. A., and W. E. Paul. 1994. Acquisition of lymphokine-producing phenotype by CD4⁺ T cells. *Annu. Rev. Immunol.* 12: 635–673.
- Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383: 787–793.
- Szabo, S. J., B. M. Sullivan, S. L. Peng, and L. H. Glimcher. 2003. Molecular mechanisms regulating Th1 immune responses. *Annu. Rev. Immunol.* 21: 713–758.
- Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon- γ . *Annu. Rev. Immunol.* 15: 749–758.
- Bradley, L. M., D. K. Dalton, and M. Croft. 1996. A direct role for IFN- γ in regulation of Th1 cell development. *J. Immunol.* 157: 1350–1358.
- Zhang, Y., R. Apilado, J. Coleman, S. Ben-Sasson, S. Tsang, J. Hu-Li, W. E. Paul, and H. Huang. 2001. Interferon γ stabilizes the T helper cell type 1 phenotype. *J. Exp. Med.* 194: 165–172.
- Ma, X., J. M. Chow, G. Gri, G. Carra, F. Gerosa, S. F. Wolf, R. Dzialo, and G. Trinchieri. 1996. The interleukin 12 p40 gene promoter is primed by interferon γ in monocytic cells. *J. Exp. Med.* 183: 147–157.
- Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul. 1993. Interleukin 12 acts directly on CD4⁺ T cells to enhance priming for interferon γ production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* 90: 10188–10192.
- Mullen, A. C., F. A. High, A. S. Hutchins, H. W. Lee, A. V. Villarino, D. M. Livingston, A. L. Kung, N. Cereb, T. P. Yao, S. Y. Yang, et al. 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 292: 1907–1910.
- Afkarian, M., J. R. Sedy, J. Yang, N. G. Jacobson, N. Cereb, S. Y. Yang, T. L. Murphy, and K. M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4⁺ T cells. *Nat. Immunol.* 3: 549–557.
- Iwamoto, I., H. Nakajima, H. Endo, and S. Yoshida. 1993. Interferon γ regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4⁺ T cells. *J. Exp. Med.* 177: 573–576.
- Dow, S. W., J. Schwarze, T. D. Heath, T. A. Potter, and E. W. Gelfand. 1999. Systemic and local interferon γ gene delivery to the lungs for treatment of allergen-induced airway hyperresponsiveness in mice. *Hum. Gene Ther.* 10: 1905–1914.
- Cohn, L., R. J. Homer, N. Niu, and K. Bottomly. 1999. T helper 1 cells and interferon γ regulate allergic airway inflammation and mucus production. *J. Exp. Med.* 190: 1309–1318.
- Suzuki, M., R. Taha, D. Ihaku, Q. Hamid, and J. G. Martin. 1999. CD8⁺ T cells modulate late allergic airway responses in Brown Norway rats. *J. Immunol.* 163: 5574–5581.
- Suzuki, M., K. Maghni, S. Molet, A. Shimbara, Q. A. Hamid, and J. G. Martin. 2002. IFN- γ secretion by CD8 T cells inhibits allergen-induced airway eosinophilia but not late airway responses. *J. Allergy Clin. Immunol.* 109: 803–809.
- Leonard, C., V. Tormey, C. Burke, and L. W. Poulter. 1997. Allergen-induced cytokine production in atopic disease and its relationship to disease severity. *Am. J. Respir. Cell Mol. Biol.* 17: 368–375.
- Renzi, P. M., J. P. Turgeon, J. E. Marcotte, S. P. Drblik, D. Bérubé, M. F. Gagnon, and S. Spier. 1999. Reduced interferon- γ production in infants with bronchiolitis and asthma. *Am. J. Respir. Crit. Care Med.* 159: 1417–1422.
- Murphy, K. M., W. Ouyang, J. D. Farrar, J. Yang, S. Ranganath, H. Asnagli, M. Afkarian, and T. L. Murphy. 2000. Signaling and transcription in T helper development. *Annu. Rev. Immunol.* 18: 451–494.
- Szabo, S. J., B. M. Sullivan, C. Stemann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in Th1 lineage commitment and IFN- γ production in CD4 and CD8 T cells. *Science* 295: 338–342.
- Lighvani, A. A., D. M. Frucht, D. Jankovic, H. Yamane, J. Aliberti, B. D. Hissong, B. V. Nguyen, M. Gadina, A. Sher, W. E. Paul, et al. 2001. T. bet is rapidly induced by interferon- γ in lymphoid and myeloid cells. *Proc. Natl. Acad. Sci. USA* 98: 15137–15142.
- Martin-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN- γ for Th1 priming. *Nat. Immunol.* 5: 1260–1265.
- Das, G., S. Sheridan, and C. A. Janeway, Jr. 2001. The source of early IFN- γ that plays a role in Th1 priming. *J. Immunol.* 167: 2004–2010.
- Rao, A., C. Luo, and P. G. Hogan. 1997. Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* 15: 707–747.
- Lewis, R. S. 2001. Calcium signaling mechanisms in T lymphocytes. *Annu. Rev. Immunol.* 19: 497–521.
- Shaw, K. T. Y., A. M. Ho, A. Raghavan, J. Kim, J. Jain, J. Park, S. Sharma, A. Rao, and P. G. Hogan. 1995. Immunosuppressive drugs prevent a rapid dephosphorylation of transcription factor NFAT1 in stimulated immune cells. *Proc. Natl. Acad. Sci. USA* 92: 11205–11209.
- Loh, C., K. T. Y. Shaw, J. Carew, J. P. B. Viola, C. Luo, B. A. Perrino, and A. Rao. 1996. Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. *J. Biol. Chem.* 271: 10884–10891.
- Sica, A., L. Dorman, V. Viggiano, M. Cippitelli, P. Ghosh, N. Rice, and H. A. Young. 1997. Interaction of NF- κ B and NFAT with the interferon- γ promoter. *J. Biol. Chem.* 272: 30412–30420.
- Sweetser, M. T., T. Hoey, Y. L. Sun, W. M. Weaver, G. A. Price, and C. B. Wilson. 1998. The roles of nuclear factor of activated T cells and ying-yang 1 in activation-induced expression of the interferon- γ promoter in T cells. *J. Biol. Chem.* 273: 34775–34783.
- Liu, J., J. D. Farmer, Jr., W. S. Lane, J. Friedman, I. Weissman, and S. L. Schreiber. 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 66: 807–815.
- Clipstone, N. A., and G. R. Crabtree. 1992. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* 357: 695–697.
- 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: 397–405.
- Kiani, A., J. P. B. Viola, A. H. Lichtman, and A. Rao. 1997. Down-regulation of IL-4 gene transcription and control of Th2 cell differentiation by a mechanism involving NFAT1. *Immunity* 7: 849–860.
- Kiani, A., F. J. Garcia-Cózar, I. Habermann, S. Laforsch, T. Aebischer, G. Ehninger, and A. Rao. 2001. Regulation of interferon- γ gene by nuclear factor of activated T cells. *Blood* 98: 1480–1488.
- Jain, J., P. G. McCaffrey, V. E. Valge-Archer, and A. Rao. 1992. Nuclear factor of activated T cells contains Fos and Jun. *Nature* 365: 801–804.
- McMillin, D. W., N. Landazuri, B. Gangadharan, B. Hewes, D. R. Archer, H. T. Spencer, and J. M. Le Doux. 2005. Highly efficient transduction of repopulating bone marrow cells using rapidly concentrated polymer-complexed retrovirus. *Biochem. Biophys. Res. Commun.* 330: 768–775.
- Viola, J. P. B., A. Kiani, P. T. Bozza, and A. Rao. 1998. Regulation of allergic inflammation and eosinophil recruitment in mice lacking the transcription factor NFAT1: role of interleukin-4 (IL-4) and IL-5. *Blood* 7: 2223–2230.
- Pearce, E. L., A. C. Mullen, G. A. Martins, C. M. Krawczyk, A. S. Hutchins, V. P. Zediak, M. Banica, C. B. DiCioccio, D. A. Gross, C. A. Mao, et al. 2003. Control of effector CD8⁺ T cell function by the transcription factor eomesodermin. *Science* 302: 1041–1043.
- Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17: 255–281.
- Xanthoudakis, S., J. P. B. Viola, K. T. Shaw, C. Luo, J. D. Wallace, P. T. Bozza, D. C. Luk, T. Curran, and A. Rao. 1996. An enhanced immune response in mice lacking the transcription factor NFAT1. *Science* 272: 892–895.
- García-Rodríguez, C., and A. Rao. 1998. Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP). *J. Exp. Med.* 187: 2031–2036.
- Avots, A., M. Buttman, S. Chuvpilo, C. Escher, U. Smola, A. J. Bannister, U. R. Rapp, T. Kouzarides, and E. Serfling. 1999. CBP/p300 integrates Raf/Rac-signaling pathways in the transcriptional induction of NF-ATc during T cell activation. *Immunity* 10: 515–524.
- Avni, O., D. Lee, F. Macian, S. J. Szabo, L. H. Glimcher, and A. Rao. 2002. T_H cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nat. Immunol.* 3: 643–651.
- Maldonado-Lopez, R., T. De Smedt, P. Michel, J. Godfroid, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain, and M. Moser. 1999. CD8 α^+ and CD8 α^- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J. Exp. Med.* 189: 587–592.
- Maldonado-Lopez, R., C. Maliszewski, J. Urbain, and M. Moser. 2001. Cytokines regulate the capacity of CD8 α^+ and CD8 α^- dendritic cells to prime Th1/Th2 cells in vivo. *J. Immunol.* 167: 4345–4350.
- Finotto, S., M. F. Neurath, J. N. Glickman, S. Qin, H. A. Lehr, F. H. Y. Green, K. Ackerman, K. Haley, P. R. Galle, S. J. Szabo, et al. 2002. Development of

- spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 295: 336–338.
46. Nurse, B., M. Haus, A. S. Puterman, E. G. Weinberg, and P. C. Potter. 1997. Reduced interferon- γ but normal IL-4 and IL-5 release by peripheral blood mononuclear cells from Xhosa children with atopic asthma. *J. Allergy Clin. Immunol.* 100: 662–668.
 47. Uzonna, J. E., K. L. Joyce, and P. Scott. 2004. Low dose *Leishmania major* promotes a transient T helper cell type 2 response that is down-regulated by interferon γ -producing CD8⁺ T cells. *J. Exp. Med.* 199: 1559–1566.
 48. Hamelmann, E., A. Oshiba, J. Paluh, K. Bradley, J. Loader, T. A. Potter, G. L. Larsen, and E. W. Gelfand. 1996. Requirement for CD8⁺ T cells in the development of airway hyperresponsiveness in a murine model of airway sensitization. *J. Exp. Med.* 183: 1719–1729.
 49. Hansen, G., G. Berry, R. H. DeKruyff, and D. T. Umetsu. 1999. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* 103: 175–183.
 50. Miyahara, N., K. Takeda, T. Kodama, A. Joetham, C. Taube, J. W. Park, S. Miyahara, A. Balhorn, A. Dakhama, and E. W. Gelfand. 2004. Contribution of antigen-primed CD8⁺ T cells to the development of airway hyperresponsiveness and inflammation is associated with IL-13. *J. Immunol.* 172: 2549–2558.
 51. Coyle, A. J., S. Tsuyuki, C. Bertrand, S. Huang, M. Aguet, S. S. Alkan, and G. P. Anderson. 1996. Mice lacking the IFN- γ receptor have impaired ability to resolve a lung eosinophilic inflammatory response associated with a prolonged capacity of T cells to exhibit a Th2 cytokine profile. *J. Immunol.* 156: 2680–2685.
 52. Elser, B., M. Lohoff, S. Kock, M. Giaisi, S. Kirchhoff, P. H. Krammer, and M. Li-Weber. 2002. IFN- γ represses IL-4 expression via IRF-1 and IRF-2. *Immunity* 17: 703–712.
 53. Luttmann, W., E. Dauer, S. Schmidt, O. Marx, M. Hossfeld, H. Matthys, and J. C. Virchow, Jr. 2000. Effects of interferon- γ and tumor necrosis factor- α on CD95/Fas ligand-mediated apoptosis in human blood eosinophils. *Scand. J. Immunol.* 51: 54–59.
 54. Fulkerson, P. C., N. Zimmermann, E. B. Brandt, E. E. Muntel, M. P. Doecker, J. L. Kavanaugh, A. Mishra, D. P. Witte, H. Zhang, J. M. Farber, et al. 2004. Negative regulation of eosinophil recruitment to the lung by the chemokine monokine induced by IFN- γ (Mig, CXCL9). *Proc. Natl. Acad. Sci. USA* 101: 1987–1992.
 55. Diehl, S., T. Krahl, L. Rinaldi, R. Norton, C. G. Irvin, and M. Rincón. 2004. Inhibition of NFAT specifically in T cells prevents allergic pulmonary inflammation. *J. Immunol.* 172: 3597–3603.