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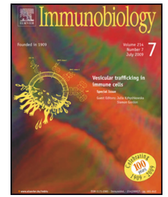

# NFAT1 transcription factor in dendritic cells is required to modulate T helper cell differentiation

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ABSTRACT

The NFAT family of transcription factors plays a central role in the regulation of cytokine gene expression during the immune response. NFAT functions have been extensively explored in lymphocyte activation and differentiation, but the involvement of NFAT proteins in dendritic cells (DCs) is still not well known. Here, we investigated the role of the NFAT1 transcription factor in murine DCs. Initially, we demonstrated by western blot that the NFAT1 protein is present in splenic DCs and is rapidly activated upon calcium influx. We then used NFAT1-deficient mice (NFAT1−/−) to investigate whether NFAT1 influences the ability of DCs to induce Th differentiation. Our data demonstrated that NFAT1−/− DCs showed an increased capacity to differentiate CD4 T cells to the Th1 phenotype. CD4 cells that were primed *in vitro* with NFAT1−/− DCs had increased IFN-γ production. The same results were observed when the CD4 cells were primed *in vivo* through the sensitization of NFAT1−/− mice with ovalbumin. Furthermore, our results demonstrated that the cytokine IL-12 is one of the factors involved in this process because its production is increased in NFAT1−/− mice, and neutralizing anti-IL-12 antibodies almost completely eliminated the IFN-γ production. These results demonstrated that the NFAT1 transcription factor regulates specific functions in DCs that are involved in CD4 differentiation, suggesting that the inhibition of NFAT1 in DCs may be used as a therapy to modulate specific immune responses.

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## Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that are present in many tissues of the body, including lymphoid and non-lymphoid organs (Steinman et al. 1997). DCs from lymphoid organs are involved in self-antigen presentation and tolerance, while the major function of non-lymphoid tissue DCs is to capture large amounts of antigen and deliver them to the T-cell areas of secondary lymphoid organs (Itano and Jenkins 2003; Banchereau and Steinman 1998). Among other functions, migratory DCs express high levels of peptide-MHC II complexes on their surfaces and are responsible for antigen-specific CD4 T lymphocyte activation (Itano and Jenkins 2003; Banchereau and Steinman 1998; Kapsenberg 2003; Moser and Murphy 2000). Once activated, these CD4 lymphocytes can differentiate into different arrays of T helper cells (Th). Th1, Th2 and Th17 are involved in the resistance against a variety of infections, while Tregs (regulatory T cells) silence the immunity to harmless self and environmental antigens (Zhu and Paul 2008). Th1 cells produce large amounts of IFN-γ and are extremely important for macrophage activation, playing a role in the defense against bacteria and viruses (Zhu and Paul 2008; Constant and Bottomly 1997). On the other hand, Th2 cells produce IL-4, IL-5 and IL-13, and they are crucial in the response against helminthes and in allergic processes (Zhu and Paul 2008; Constant and Bottomly 1997).

Many molecular mechanisms orchestrate Th differentiation. Some transcription factors, such as T-bet for Th1 differentiation and GATA-3 for Th2, are central in this process (Szabo et al. 2003). Moreover, the NFAT family of transcription factors is also important in Th differentiation (Macian 2005). This family of transcription factors has five members: NFAT1 (also called NFATp or NFATc2), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4), NFAT4 (NFAT*x* or NFATc3) and NFAT5 (TonE-PB) (Macian 2005). With the exception of NFAT5, all NFAT members are regulated by calcium-dependent phosphatase

*Abbreviations:* Ab, antibody; APC, antigen-presenting cell; CFA, Complete Freund's Adjuvant; CsA, cyclosporin A; DC, dendritic cell; Iono, ionomycin; OVA, ovalbumin; RPA, RNase protection assay; Th, T helper cell.

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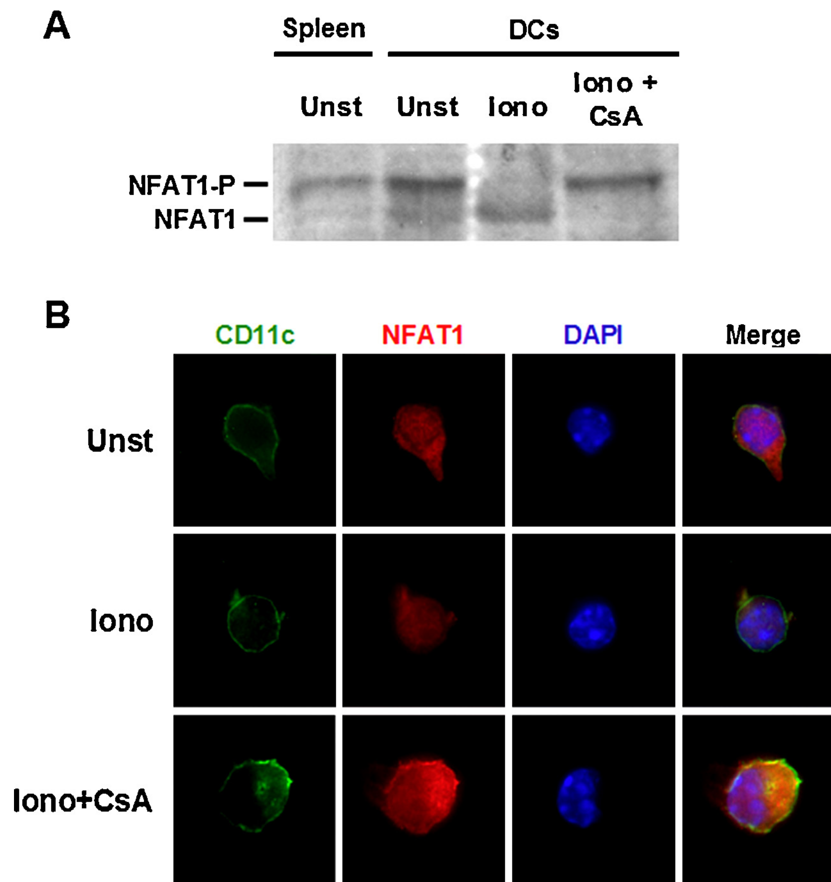


**Fig. 1.** Dendritic cells present a functional NFAT1. (A) $1 \times 10^6$ cells (total spleen or spleen-purified DCs) from C57BL/6 mice were left unstimulated (Unst) or stimulated *in vitro* with Iono (5 μM) for 10 min. CsA (2 μM) was added to the cells 15 min before the Iono stimulus. Total protein cell extracts were analyzed by western blot using a rabbit polyclonal anti-NFAT1. NFAT1-P refers to the phosphorylated inactive form of NFAT1. (B) Spleen-purified DCs from C57BL/6 mice were adhered to coverslips previously coated with 2% gelatin. After adhesion, the cells were left unstimulated or were stimulated with Iono (5 μM) for 10 min at 37 °C. CsA (2 μM) was added to the cells 15 min before stimulation. The cells were then fixed in 4% paraformaldehyde. The DC surface was stained with anti-CD11c-biotinylated and streptavidin-FITC (first column). The cells were then permeabilized and stained with a rabbit polyclonal anti-NFAT1 and an anti-rabbit Rhodamine-labeled Ab (second column) and lastly with DAPI (third column). Results are representative of 3 independent experiments.

calcineurin. In lymphocytes, NFAT1 is constitutively expressed in the cytoplasm of naive cells in a phosphorylated form. The TCR signaling pathway leads to an increase in the calcium influx that activates calcineurin. This enzyme dephosphorylates the NFAT that translocates to the nucleus and binds to a variety of gene promoters, including NFAT2 and cytokines genes (Macian 2005). It has been demonstrated that NFAT1 and NFAT2 play a major role in Th differentiation. In the absence of NFAT1, lymphocytes showed increased production of Th2 cytokines, such as IL-4, IL-5 and IL-13 (Kiani et al. 1997), while NFAT2−/− lymphocytes presented a diminished production of IL-4 (Yoshida et al. 1998).

Although the role of the NFAT family was greatly studied in T-cell activation and differentiation, these transcription factors are also expressed in other cells of the immune system, including macrophages and DCs (Wang et al. 1995; Goodridge et al. 2007). However, its role in these other cell types is poorly understood. Herein, we show that NFAT1 is functional in DCs and plays an important role in the ability of DCs to induce Th differentiation. We demonstrated that NFAT1−/− DCs lead CD4 T cells to preferentially differentiate toward a Th1 phenotype, which was dependent on high IL-12 production by NFAT1−/− DCs during the priming of CD4 lymphocytes. These results demonstrated that the NFAT1 transcription factor plays a central role in modulating the function of DCs, acting as a negative regulator during the priming of Th1 differentiation.

## Materials and methods

### *Mice, reagents and antibodies*

C57BL/6, NFAT1+/+ and NFAT1−/− (Xanthoudakis et al. 1996), and OT-II 8- to 12-week old mice were used for the experiments. The animals were bred in the Brazilian National Cancer Institute (INCA) animal facility and maintained according to the guide for the care and use of laboratory animals (National Institutes of Health, USA). All animal experiments were performed in accordance to Brazilian Government's ethical and animal experiment regulations. The experiments were approved and conducted according to animal welfare guidelines of the Ethics Committee of Animal Experimentation from the Brazilian National Cancer Institute (INCA).

All cells 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). The cytokine IL-2 and the neutralizing affinity purified polyclonal antibody (Ab) anti-murine IL-12 were purchased from PeproTech. The rabbit polyclonal Ab 67.1 (Dr. A. Rao, Harvard University, Boston, MA) was used to detect the NFAT1 protein. Anti-rabbit IgG rhodamine-labeled and peroxidase-labeled Abs were obtained from KPL (Kirkegaard & Perry Laboratories, Inc.). Anti-CD3 Ab (clone 2C11) and anti-CD16/32 Ab (clone 2.4G2) were purified from hybridoma supernatants by chromatography over

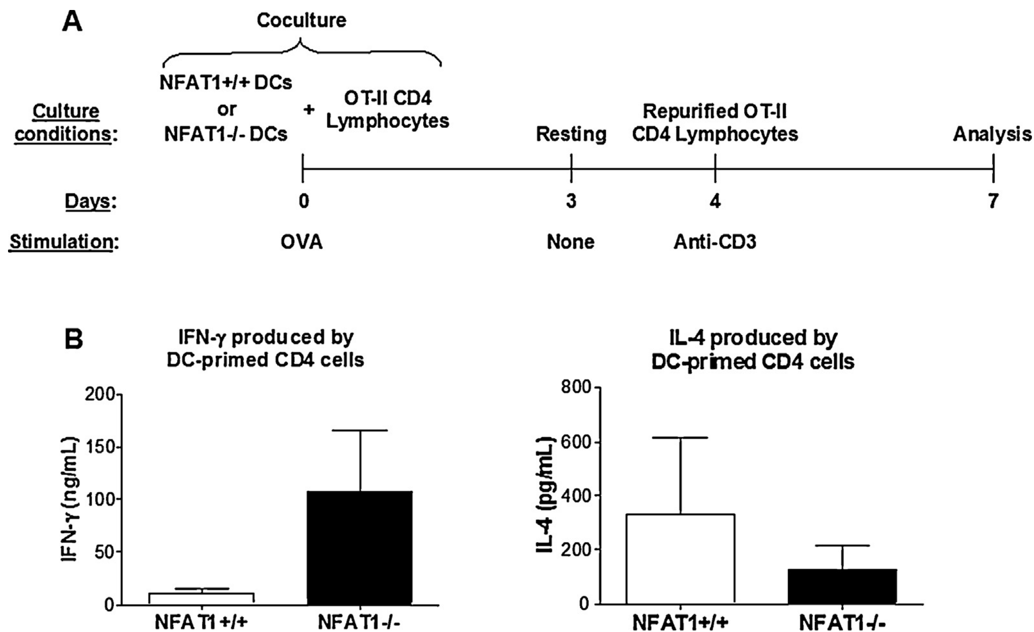


**Fig. 2.** NFAT1−/− DCs induce Th1 differentiation of OT-II CD4 lymphocytes. (A) A representative scheme of the coculture protocol, in which NFAT1+/+ and NFAT1−/− mice were subcutaneously sensitized in a hind footpad with 200 μg of OVA emulsified in CFA, and fifteen days later, the spleen was harvested and dendritic cells purified. CD4 T lymphocytes were purified from the lymph nodes of OT-II mice and cocultured in a ratio of 1:10 with dendritic cells (DCs) from NFAT1+/+ or NFAT1−/− sensitized mice in the presence of OVA (0.5 mg/ml). After 3 days, total cells were rested in only the presence of IL-2 (20 U/mL) for 24 h. After that, the CD4 cells were repurified and restimulated with plate-bound anti-CD3 (1 μg/mL) for 72 h. (B) Analysis of IFN-γ and IL-4 levels by ELISA in the cell-free supernatants of DC-primed CD4 cells restimulated with anti-CD3 for 3 days. The results are expressed as mean ± SEM of 3 independent experiments.

protein G (Amersham Biosciences), and their activity was functionally tested by cellular proliferation and complement-dependent depletion. All other labeled mAbs were obtained from BD Pharmingen. Ionomycin (Iono) was obtained from Calbiochem, and the immunosuppressive drug Cyclosporin A (CsA) was obtained from LC Laboratories. Complete Freund's Adjuvant (CFA) and chicken ovalbumin (OVA) were purchased from Sigma–Aldrich.

*Mice sensitization and cell isolation*

The NFAT1+/+ and NFAT1−/− mice were sensitized subcutaneously in the hind footpad with 200 μg (0.1 ml) of OVA emulsified in CFA. Fifteen days later, the draining lymph nodes (inguinal and popliteal) and spleen were harvested. All draining lymph node cells ($1 \times 10^6$ cells/ml) were stimulated *in vitro* with different doses of OVA (0.3, 0.5 or 1.0 mg/ml). Anti-IL-12 Ab (1 μg/ml) was added to the culture to block this particular cytokine's action. CD4 T lymphocyte purification and cell depletions were performed by negative selection with magnetic beads, according to the manufacturer's instructions (MACS, Miltenyi Biotech). Streptavidin magnetic beads were conjugated to specific biotinylated Ab (anti-CD4, anti-CD8, and anti-B220/CD45R) and used to eliminate undesired cell populations. For splenic DC purification, the spleens were cut into small pieces and incubated for 45 min in DMEM containing collagenase I (2 mg/ml; Gibco Invitrogen Corporation). The tissue pieces were then passed through a nylon mesh. To eliminate the red blood cells, an ACK lysis buffer was used. After a wash, EDTA (2 mM) was added to the cell suspension and incubated for 5 min. The cell suspension was added to a 54% Percoll (Amersham Biosciences) gradient and centrifuged at 2000 g for 10 min. The cells on the interface were collected, washed and stained with anti-CD11c for column purification. For DC culture, cells were purified by positive selection with magnetic beads (MACS, Miltenyi Biotech). For this purification, the cells were incubated with anti-CD16/32 for 15 min and then stained with anti-CD11c-FITC Ab and anti-FITC magnetic beads. For the immunofluorescence experiments, the cells were purified using the Dynabeads® Mouse DC Enrichment Kit (Invitrogen), and the resulting preparations were evaluated by flow cytometry. Cell population was isolated to 70–90% purity, $CD11c^-B220^+MHCII^+$ were the major contaminating cells (data not shown).

*CD4 Th differentiation*

CD4 T cells were *in vitro* differentiated as described (Teixeira et al. 2005). Shortly, the CD4 T lymphocytes purified from the lymph nodes of NFAT1+/+ or NFAT1−/− naive mice were differentiated *in vitro* using default conditions. The cells ($1 \times 10^6$ cells/ml) were stimulated with 1 μg/ml of plate-bound anti-CD3. Twenty-four hours after stimulation, IL-2 (20 U/ml) was added to the cultures. On the third day, the cells were harvested, washed, and rested for 48 h in the presence of IL-2 (20 U/ml). After resting, the CD4 T cells were restimulated *in vitro* with plate-bound anti-CD3 (1 μg/ml). Cell-free supernatants were harvested 24, 48 and 72 h after restimulation and assessed for IFN-γ and IL-4 levels by ELISA according to the manufacturer's instructions (BD Pharmingen).

*DC cultures*

NFAT1+/+ or NFAT1−/− DCs were cocultured with OT-II CD4 lymphocytes (DC:CD4 ratio; 1:10) and stimulated with OVA (0.5 mg/ml) for 3 days. On the third day, the cells were harvested, washed, and rested for 24 h in the presence of IL-2 (20 U/ml). After resting, the CD4 T cells were purified again and stimulated *in vitro* with plate-bound anti-CD3 (1 μg/ml). Cell-free supernatants were harvested 72 h after stimulation. IFN-γ and IL-4 levels were assessed by ELISA according to the manufacturer's instructions (BD Pharmingen).

*RNase protection assay, western blot and immunofluorescence*

For the RNase protection assay (RPA) analysis, total RNA was extracted using TRIzol reagent according to the manufacturer's

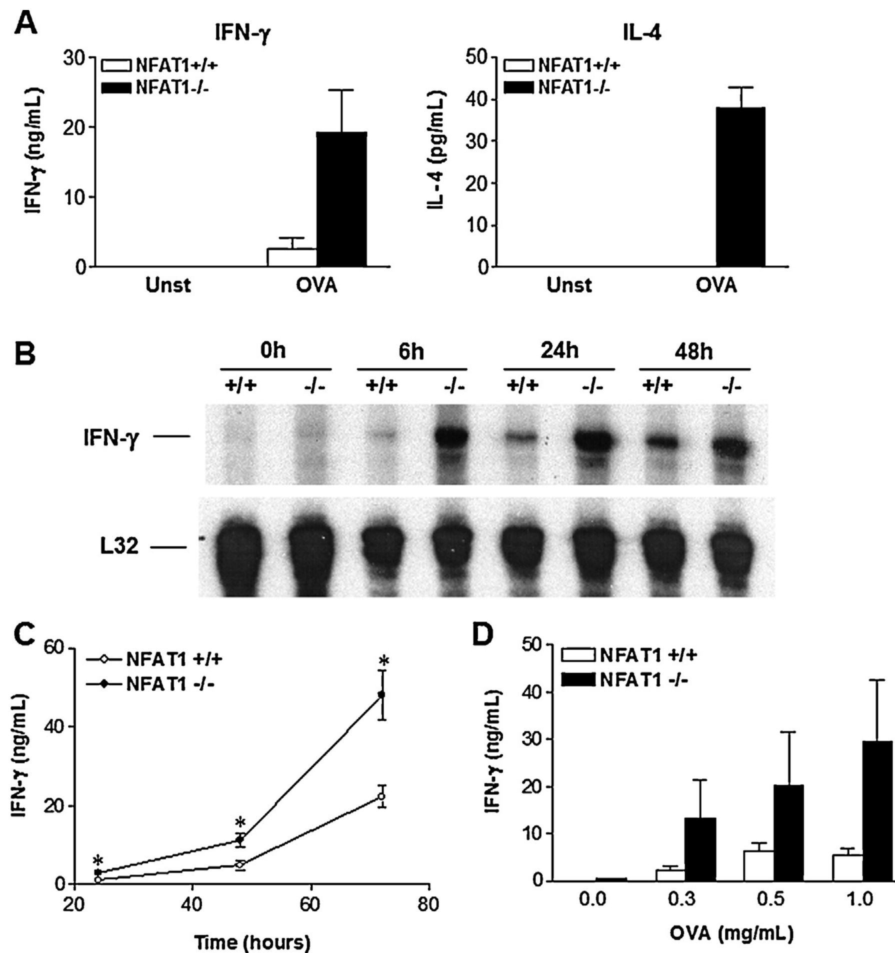


**Fig. 3.** Lymph node cells from NFAT1−/− mice produce more IFN-γ than NFAT1+/+ mice. (A)–(D) The animals were sensitized as described in Fig. 2. Fifteen days later, the draining lymph nodes were harvested, and the cells were restimulated *in vitro* with OVA (0.5 mg/ml) or left unstimulated (Unst) for 3 days. After that, the cell-free supernatants were harvested. (A) Analysis of IFN-γ and IL-4 levels in the supernatants of OVA-stimulated cells by ELISA. (B) The lymph node cells were restimulated *in vitro* with OVA (0.5 mg/mL) or left unstimulated (0 h). Total cellular RNA was analyzed by RPA for IFN-γ mRNA levels at different time points as indicated. (C) The lymph node cells were restimulated *in vitro* with OVA (0.5 mg/mL), and the cell-free supernatants were assessed for IFN-γ levels at three different time points. (D) The cells were stimulated *in vitro* with different doses of OVA (0.0, 0.3, 0.5, 1.0 mg/mL) for 72 h and cell supernatant was assessed by ELISA for IFN-γ detection. These results are representative of 3 independent experiments. The results are expressed as mean ± SEM ($n$ = 3). An asterisk (*) indicates significant differences compared with the NFAT1+/+ mice ($p < 0.05$).

instructions (Invitrogen Life Technologies). The mRNA expression was analyzed with a multiprobe RNase protection assay kit (RiboQuant; BD Pharmingen). For IFN-γ and IL-12 expression analysis, the mCK-2 multiprobe set was used, and RNA loading was estimated by the L32 housekeeping gene expression.

To detect the presence of the NFAT1 protein by western blot, purified DCs from C57BL/6 mice ($1 \times 10^6$ cells) were left unstimulated or were stimulated *in vitro* with ionomycin (5 μM) for 10 min at 37 °C. CsA (2 μM) was added to the cells 15 min before stimulation. To obtain the total protein lysates, the cells were resuspended in buffer containing 40 mM Tris (pH 7.5), 60 mM sodium pyrophosphate, 10 mM EDTA, and lysed with 5% SDS, followed by an incubation of 20 min at 100 °C. The cell extracts were analyzed by electrophoresis on 6% SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane, and the NFAT1 protein was detected by a rabbit polyclonal 67.1 Ab and anti-rabbit peroxidase-labeled Ab.

The intracellular localization of the NFAT1 protein was assessed in purified DCs from C57BL/6 mice by immunofluorescence staining. Briefly, the cells were adhered to coverslips previously coated with 2% gelatin during 4 h of incubation. After adhesion, the cells were left unstimulated or were stimulated with ionomycin (5 μM) for 10 min at 37 °C. CsA (2 μM) was added to the cells 15 min before stimulation. The cells were then fixed in 4% paraformaldehyde. The DC surface was stained with anti-CD11c-biotinylated and streptavidin-FITC for 15 min. The cells were then permeabilized with 0.1% NP-40, incubated with anti-NFAT1 67.1 Ab for 2 h, and stained with Rhodamine-labeled Ab for 30 min and DAPI (Molecular Probes) for 1 min. Representative cell images were captured using a fluorescence microscope (Olympus BX60).

### *Statistical analysis*

Statistically significant differences between the wild type (+/+) and knockout (−/−) mice, and between the control and treated groups, were determined using unpaired Student' $t$ tests. $P < 0.05$ was considered to be statistically significant.

## Results

### *NFAT1 is present in dendritic cells and can be activated by calcium influx*

To study the role of NFAT1 in DCs, we first analyzed the expression of this transcription factor and its activation in this cell type. We purified splenic DCs from C57BL/6 mice and evaluated NFAT1 expression by western blot and immunofluorescence microscopy. We observed that the DCs presented most of its endogenous NFAT1

in a phosphorylated form (inactive) (Fig. 1A). To induce NFAT1 activation, we stimulated DCs with ionomycin (Iono), a calcium ionophore. The ionomycin-induced calcium influx dephosphorylated NFAT1, while CsA completely blocked the dephosphorylation (Fig. 1A). 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 the immunofluorescence staining of DCs (Fig. 1B).

*NFAT1-deficient dendritic cells induce Th1 differentiation in vitro*

DCs present antigens to CD4 T lymphocytes, and induce its activation and differentiation. However, DCs need to maturate in order to produce an effective CD4 T cell response (Banchereau and Steinman 1998). To investigate whether NFAT1 could influence the ability of DCs to differentiate CD4 T cells, we used NFAT1-deficient mice (NFAT1−/−). For that, NFAT1+/+ or NFAT1−/− mice were sensitized with OVA plus CFA to induce DCs maturation *in vivo*. Then, splenic purified DCs were cocultured with naive CD4 T lymphocytes from OT-II OVA-TCR transgenic mice, in the presence of OVA, for 3 days (Fig. 2A). After one day of resting, the OT-II CD4 cells were restimulated with anti-CD3 for 72 h, and the CD4 cell differentiation were evaluated by the production of IFN-γ and IL-4. Then, we observed that the OT-II CD4 cells produced more IFN-γ and less IL-4 when they were cultured with the NFAT1−/− DCs, unlike OT-II CD4 cells that were cultured with NFAT1+/+ DCs, that produced less IFN-γ and more IL-4 (Fig. 2B). These results demonstrated that the absence of NFAT1 in DCs increased their ability to differentiate CD4 T lymphocytes to a Th1 phenotype.

*NFAT1-deficient CD4 cells produce increased IFN-γ levels in vivo*

To determine whether NFAT1 from DCs would have the same effect in an *in vivo* immune response, we evaluated the CD4 Th differentiation from the draining lymph nodes of sensitized NFAT1−/− and NFAT1+/+ mice. Total cells were restimulated *in vitro* with the same antigen used in the sensitization (OVA) for 3 days. As we observed in the vitro differentiation, the NFAT1−/− cells produced higher concentrations of IFN-γ when compared with the NFAT1+/+ cells (Fig. 3A). The increased production of IFN-γ by the NFAT1−/− cells was also observed at the mRNA expression levels. This result showed that the IFN-γ mRNA was expressed only after OVA stimulation and that the NFAT1−/− cells expressed more IFN-γ mRNAs than NFAT1+/+ cells at all of the times analyzed (Fig. 3B). The same pattern was observed in the analysis using the supernatants, where the OVA stimulation induced IFN-γ production, and the NFAT1−/− supernatant had a higher concentration of IFN-γ compared with the NFAT1+/+ supernatant, especially after 3 days of stimulation (Fig. 3C). This increased IFN-γ production by NFAT1−/− lymphocytes was also observed with increasing doses of OVA (Fig. 3D), showing that this phenomenon is dependent on antigenic stimulation.

Because we used all of the lymph nodes cells, we analyzed the IFN-γ mRNA expression of the purified CD4 cells. Total lymph nodes cells were restimulated *in vitro* with OVA for 24 h, and then the CD4 cells were purified, and the IFN-γ mRNA expression was analyzed by RPA. We found that the NFAT1−/− CD4 cells expressed higher levels of IFN-γ mRNA than the NFAT1+/+ CD4 cells (Fig. 4A). To confirm the contribution of each lymphocyte population in the IFN-γ production; CD4, CD8 or B cells were depleted from total lymph node cells. Then, the remaining cells were *in vitro* restimulated with OVA and the IFN-γ production was assessed. We observed a great decrease in the IFN-γ production only when CD4 cells were depleted, with no differences in B or CD8 cells depletion (Fig. 4B). These results together show that CD4 cells are the major source of

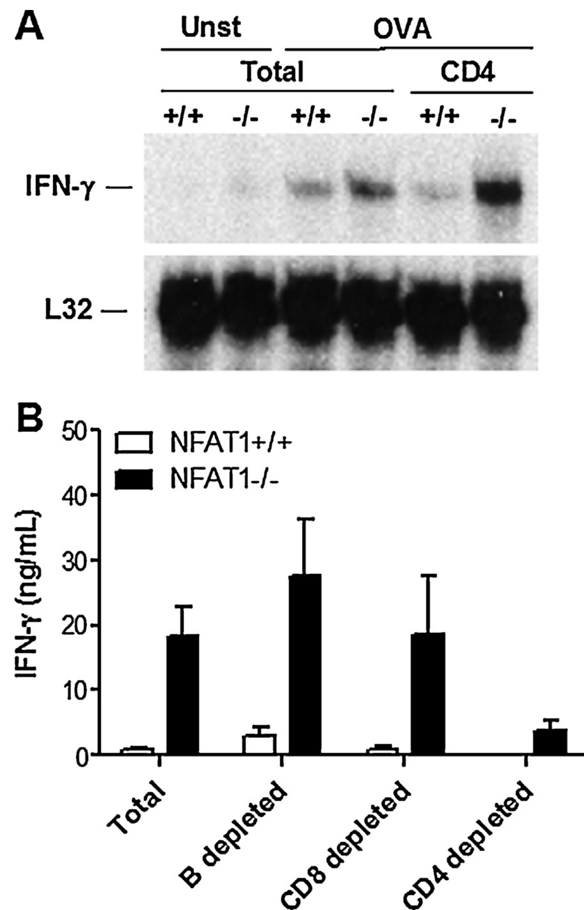


**Fig. 4.** NFAT1−/− CD4 cells express more IFN-γ than NFAT1+/+ CD4 cells. (A) and (B) The animals were sensitized as described in Fig. 2. Fifteen days later, the draining lymph nodes were harvested. (A) The lymph node cells were restimulated *in vitro* with OVA (0.5 mg/mL) or left unstimulated (Unst) for 24 h. CD4 T cells were then purified, and total cellular RNA was analyzed by RPA for IFN-γ mRNA levels. (B) The total lymph node cells were depleted of B lymphocytes, CD8T lymphocytes or CD4 T lymphocytes and the remaining cells were restimulated *in vitro* with OVA (0.5 mg/mL) for 72 h. IFN-γ levels were assessed by ELISA in cell-free supernatant. The results are expressed as mean ± SEM of 3 independent experiments.

IFN-γ production upon OVA restimulation, and indicate that DCs might be involved in this CD4 differentiation.

By contrast, we also observed that the NFAT1−/− cells produced higher levels of IL-4 compared with the NFAT1+/+ cells (Fig. 3A). However, the transcription factor NFAT1 has already been described as a negative regulator of Th2 differentiation, directly regulating IL-4 gene expression (Kiani et al. 1997), which may explain the increased IL-4 production of the NFAT1−/− cells seen in our model. Indeed, when purified CD4 T lymphocytes from naive NFAT1+/+ and NFAT1−/− mice were differentiated *in vitro* with plate-bound anti-CD3, in the absence of DCs or any added cytokines or antibodies (Fig. 5A), the NFAT1−/− CD4 cells produced more IL-4 than the NFAT1+/+ CD4 cells after restimulation with plate-bound anti-CD3 (Fig. 5B). These results suggest that the increased IL-4 production by NFAT1−/− cells might not have the involvement of DCs, indicating that NFAT1 differently influences the outcome of Th differentiation depending on the cell type upon which this transcription factor acts.

*IL-12 is the main factor involved in the production of IFN-γ by NFAT1-deficient CD4 cells*

DCs can influence a Th differentiation in a number of forms, and for that, DCs need to go through many changes, called maturation

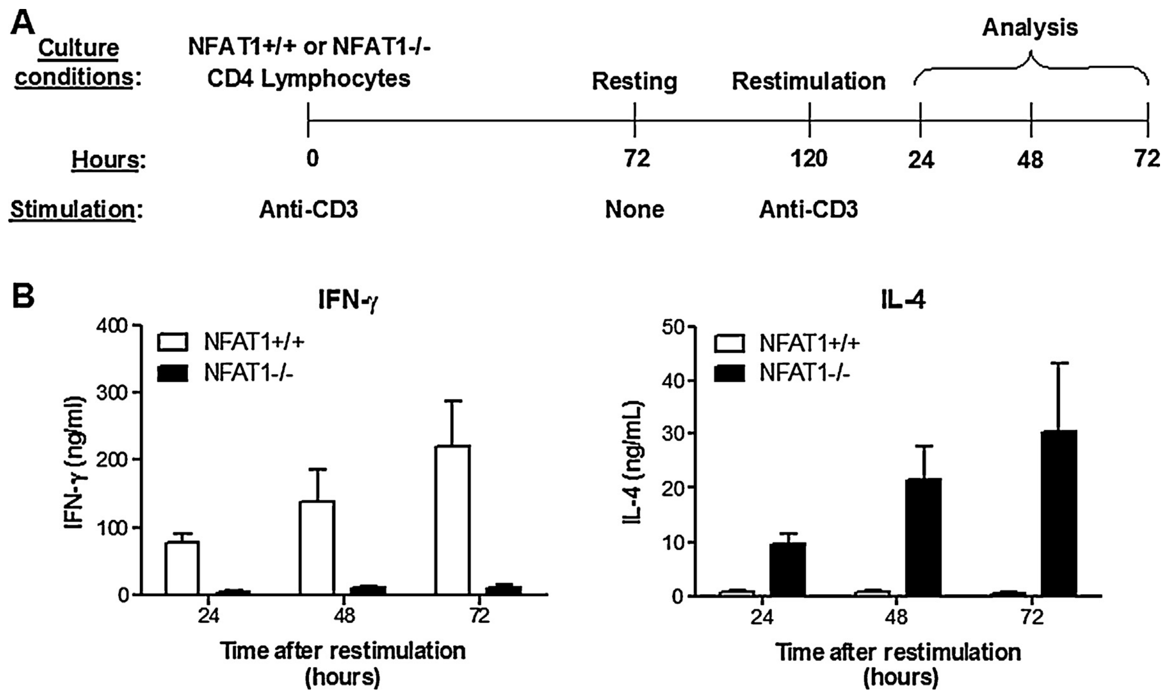


**Fig. 5.** Purified NFAT1−/− CD4 T lymphocytes differentiate into Th2 phenotype when stimulated *in vitro* in the absence of DCs. (A) A representative scheme of the CD4 Th differentiation protocol, in which CD4 T lymphocytes purified from lymph nodes of NFAT1+/+ or NFAT1−/− naive mice were stimulated with plate-bound anti-CD3 (1 μg/ml). On the third day, the cells were rested for 48 h in only the presence of IL-2 (20 U/ml). After resting, the CD4 T cells were restimulated *in vitro* with plate-bound anti-CD3 (1 μg/ml), and the cell-free supernatants were harvested at three different times. (B) Analysis of IFN-γ and IL-4 levels in the supernatants of restimulated CD4 T cells by ELISA. The results are expressed as mean ± SEM of 3 independent experiments.

(Kapsenberg 2003). To investigate whether NFAT1 is involved in DC maturation process, splenic DCs from sensitized mice with OVA plus CFA were analyzed by the expression of CD80 (B7.1), CD86 (B7.2), CD40 and MHC class II by flow cytometry. We did not find any difference in the CD80 and CD40 expression, and only a slightly reduction in the CD86 and MHC II expression when NFAT1−/− DCs were compared to NFAT1+/+ DCs (Fig. 6), suggesting that NFAT1 is not involved in DC maturation.

IL-12 is a key inducer of IFN-γ production and Th1 differentiation, and DCs are the main source of this cytokine (Macatonia et al. 1995; Trinchieri 2003). Therefore, we investigated whether IL-12 was involved in the increased IFN-γ production seen in the NFAT1−/− CD4 cells. IL-12 is a heterodimer cytokine composed of one p35 and one p40 subunit (Trinchieri 2003). When we analyzed the mRNA expression of IL-12 subunits by RPA, we observed that the NFAT1−/− cells had higher levels of p35 expression compared with the NFAT1+/+ cells (Fig. 7A). We also observed an enhanced production of IL-12 protein (p70) in the NFAT1−/− cell supernatants (Fig. 7B). Furthermore, the addition of a neutralizing anti-IL-12 Ab at the start of the culture drastically reduced the IFN-γ production of the NFAT1−/− cells (Fig. 7C), suggesting that IL-12 is the main factor involved in this process. These results indicate that NFAT1−/− DCs induce increased Th1 differentiation through augmented IL-12 production.

## Discussion

The transcription factor NFAT1 is present in the majority of cell types within the immune system, including T and B lymphocytes, mast cells, and macrophages (Wang et al. 1995). In NFAT1-expressing cells, NFAT1 is present constitutively as a cytoplasmatic protein and only translocates to the nucleus after the activation of a calcium-calcineurin-dependent pathway (Ruff and Leach 1995). Here, we demonstrate that most of the NFAT1 protein of freshly isolated splenic DCs is localized in the cytoplasm

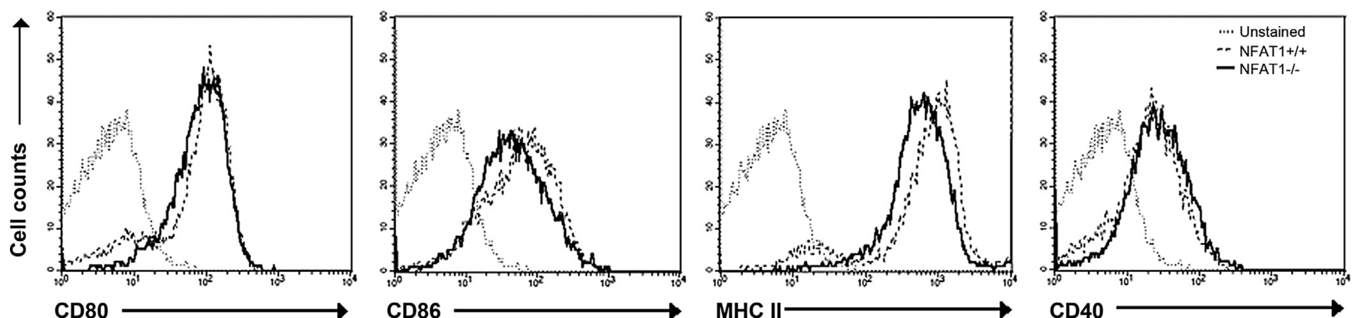


**Fig. 6.** NFAT1+/+ and NFAT1−/− DCs present similar maturation profiles. The animals were sensitized as described in Fig. 2. Fifteen days later, the spleen was harvested and dendritic cells were purified. The total NFAT1+/+ and NFAT1−/− DC population were stained for CD80, CD86, I-Ad/I-Ed (MHC II) or CD40 and assessed by flow cytometry. Results are representative of 3 independent experiments.

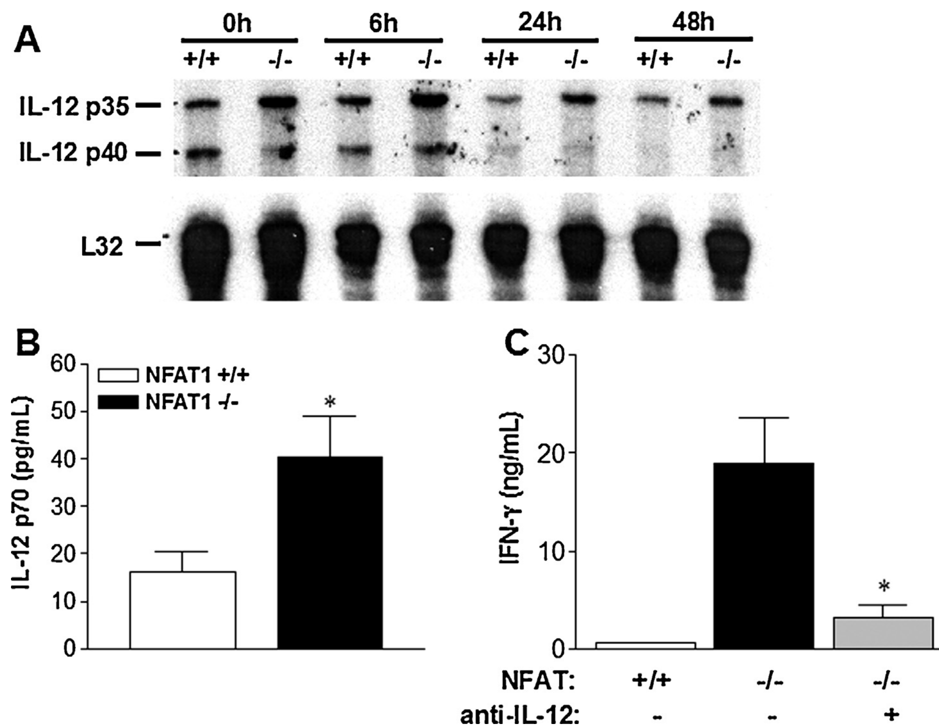


**Fig. 7.** IL-12 is an important factor in the increased IFN-γ production of NFAT1−/− cells. (A)–(C) The mice were sensitized as described in Fig. 2. Fifteen days later, the draining lymph nodes were harvested. (A) The lymph node cells were restimulated *in vitro* with OVA (0.5 mg/mL) or left unstimulated (0 h). Total cellular RNA was analyzed on different time points by RPA for IL-12 p35 and IL-12 p40 mRNA levels. (B) The lymph node cells were restimulated *in vitro* with OVA (0.5 mg/mL) for 24 h, and IL-12 p70 levels were assessed by ELISA in cell-free supernatants. The results are expressed as mean ± SEM ($n = 5$). An asterisk (*) indicates significant differences compared with the NFAT1+/+ mice ($p < 0.05$). (C) The lymph node cells were restimulated *in vitro* with OVA (0.5 mg/mL) and treated or not with anti-IL-12 (1 μg/mL) neutralizing antibodies for 72 h as indicated. IFN-γ levels were assessed by ELISA in the cell-free supernatants. Results are expressed as mean ± SEM ($n = 5$). An asterisk (*) indicates values significantly higher than in the NFAT1−/− mice not treated with anti-IL-12 ($p < 0.05$). Results are representative of 3 independent experiments.

(Fig. 1). Even without stimulation, a small amount of the NFAT1 protein can be found in the nucleus, suggesting that a pathway that induces calcium influx is constitutively activated in these cells. In fact, immature DCs may have spontaneous calcium oscillations that activate NFAT, suggesting a role for NFAT in maintaining these cells in the immature stage (Vukcevic et al. 2010). By contrast, previous research showed that calcium signaling induces DCs maturation (Czerniecki et al. 1997; Bagley et al. 2004). However, our analysis of surface molecules indicated that NFAT1 is not involved in the induction of stimulatory molecules expression, as we did not find significant differences in the expression of CD80, CD86, CD40 or MHC class II on NFAT1−/− DCs compared with control DCs (Fig. 6).

NFAT is activated in T cells through their antigen receptor (Loh et al. 1996), whereas in DCs its activation can occur through Pattern Recognition Receptors (PRRs), such as Dectin-1 and CLEC-2 (Goodridge et al. 2007; Mourão-Sá et al. 2011). Dectin-1 ligands, like Zymosan or Curdlan, can trigger Src-family kinases and phospholipase Cγ2 (PLCγ) activation, which initiates a $Ca^{2+}$ influx and culminates in NFAT activation (Zanoni et al. 2009; Xu et al. 2009). In fact, we observed that *in vitro* stimulation of DCs with zymosan induced NFAT1 dephosphorylation (data not shown). Additionally, it had been demonstrated that LPS can also activate NFAT1 through CD14 in a TLR4-independent manner (Zanoni et al. 2009). In addition to fungal and bacterial PRRs, protozoan PRRs can also trigger NFAT activation. *Trypanosoma cruzi* can induce NFAT2 activation in bone marrow DCs (BM-DCs) through a Toll-like receptor-independent pathway (Kayama et al. 2009), although the specific receptor and ligand involved in this process are still unknown. In our model, by sensitizing mice with CFA, we took advantage of heat-killed *M. tuberculosis* to induce *in vivo* DC maturation and Th differentiation (Fig. 3). Although the details of the NFAT1 activation through *M. tuberculosis* PRRs are unknown, it may occur through a Dectin-1-TLR2 pathway (Yadav and Schorey 2006; Geijtenbeek and Gringhuis 2009).

Once in the nucleus, NFAT can bind to different gene promoters and positively or negatively regulate gene expression. NFAT positively regulates the production of particular cytokines in BM-DCs, such as IL-2, IL-10, and IL-12, but not others, such as TNF-α and IL-6, which are regulated by the NF-κB transcription factor (Loh et al. 1996; Zanoni et al. 2009). However, the specific contribution of individual NFAT members on those cytokine expressions has not yet been determined. NFAT2 can positively regulate IL-12p40, which is the common subunit for the cytokines IL-12 and IL-23 (Zhu et al. 2003; Trinchieri 2003). Our results suggest that NFAT1 can negatively regulate IL-12p35 expression in DCs that would decrease IL-12 production and consequently diminish Th1-induced responses (Fig. 7). We did not observe any difference on IL-12p40 expression or production by NFAT1−/− cells (Fig. 7, and data not shown). The differential NFAT member expression in specific DCs subsets may explain the potential of DCs to induce different pathways for T helper differentiation.

Although it had been shown that zymosan can induce IL-12 production through dectin-1 receptor and NFAT (Goodridge et al. 2007), it was not determined which member of the NFAT family was involved. Furthermore, it had been also demonstrated that LPS can activate NFAT1 through CD14 in a TLR4-independent manner (Zanoni et al. 2009). However, we did not observe any difference in IL-12 production when isolated NFAT1+/+ and NFAT1−/− DCs were stimulated *in vitro* with different TLR ligands, including LPS, Pam3Cys and CpG (data not shown). These results suggest that the regulation of IL-12 expression in DCs may depend on cellular

contact in our model and an additional pathway might be related to NFAT1 activation. Further studies would be necessary to determine through which pathways NFAT1 are activated in DCs to regulate IL-12 expression.

As for T cells, NFAT1 positively regulates IFN-γ expression, both in CD4 and CD8 cells (Sweetser et al. 1998; Kiani et al. 2001; Teixeira et al. 2005). However, in this present work, we demonstrated that NFAT1−/− CD4 cells produced higher levels of IFN-γ specifically when stimulated with DCs (Figs. 3 and 4), suggesting a negative role for NFAT1 on IFN-γ expression. This result was not observed when NFAT1−/− CD4 cells were stimulated with plate-bound anti-CD3 alone (Fig. 5), suggesting that signals from APCs, including costimulation and IL-12, may activate a different combination of pathways and differentially modulate IFN-γ expression. Corroborating our findings, Rafiq et al. (1998) showed that CsA-treated CD4 cells had increased IFN-γ production only when the cells were co-stimulated with CD80. Wittmann et al. also showed that CsA enhances the production of IFN-γ by T cells and was dependent on TCR and CD28 signaling as well as the presence of IL-12 (Wittmann et al. 2006). These results together suggest that in an *in vivo* immune response, with the involvement of pathogen-maturated DCs, NFAT1 may negatively regulate Th1 differentiation. Moreover, van Rietschoten et al. showed that NFAT1 also negatively regulates the IL-12 receptor-$\beta 2$ on T cells (van Rietschoten et al. 2001), suggesting that NFAT1 may act through different mechanisms in different cell types to reduce Th1 differentiation and may prevent hypersensitive reactions and autoimmune responses.

Finally, all of our results together showed that the transcription factor NFAT1 is active, functional and has an important role in DCs during Th differentiation. However, more studies are still needed to establish how NFAT is activated and the other genes that NFAT may regulate in DCs. DCs and NFAT activation may become ideal therapeutic targets (Hackstein and Thomson 2004), and a better understanding of the function of this transcription factor in these cells will advance the therapeutic field.

## Conflict of interest

The authors have no financial conflicts of interest.

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