NFATc1 deficiency in T cells protects mice from experimental autoimmune encephalomyelitis

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NFATc1 is a member of the nuclear factor of activated T cells (NFAT) family of transcription factors. NFAT is activated upon T-cell receptor activation followed by intracytoplasmatic calcium influx where calmodulin, a calcium sensor protein, activates the phosphatase calcineurin that dephosphorylates NFAT proteins and results in NFAT nuclear import. Here, we show the analysis of conditional NFATc1-deficient mice bearing a deletion of NFATc1 in CD4+ and CD8+ T cells. NFATc1-deficient CD4+ T cells polarized under Th17 conditions express reduced levels of the Th17-associated transcription factor RORγT (where ROR is RAR-related orphan receptor) as well as the Th17-associated cytokines IL-17A, IL-17F, IL-21, and IL-10. In the murine model of experimental EAE, we found a strong reduction of the disease outcome in conditional NFATc1-deficient mice, as compared with control littermates. This was accompanied by a diminished inflammation in the brain and spinal cord and reduced IL-17A and IFN-γ expression by antigen-specific spleen, spinal cord, and brain cells. Altogether, these results reveal an important role of NFATc1 in inducing Th17-cell responses and IFN-γ, both being relevant for the EAE development.

Keywords: CD4+ T cells · EAE · NFAT · RORγT · Th17

Introduction

Nuclear factor of activated T cells (NFAT) was originally described as a transcription factor inducing the expression of interleukin 2 (IL-2) [1]. The NFAT family of transcription factors consists of five members, named NFAT1-5, and the main forms expressed in T cells are NFATc1 and NFATc2 [2]. NFATc2 is constitutively expressed in T cells [3], whereas NFATc1 is activated upon T-cell receptor stimulation [4]. NFATc1 is the only NFAT protein family member which acts in a positive autoregulatory loop [5] and thereby augments NFATc1 expression. NFAT proteins reside phosphorylated in the cytoplasm. Upon T-cell receptor activation the phospholipase C signaling pathway is activated, which leads to an induced influx of calcium into the cell. Intracytoplasmatic calcium signaling activates calmodulin, a calcium sensor protein that activates the phosphatase calcineurin. These phosphatase dephosphorylates NFAT proteins, so that they can shuttle into the nucleus where they act as transcription factors on the promoter of target
genes such as IL-2 [6]. Previous studies showed that NFAT proteins play regulatory roles during T-cell differentiation and effector functions. NFATc2 deficiency in T cells diminished Th1 differentiation and induced IL-4 production [7, 8]. NFATc2 was also shown to contribute to IL-21 expression and to limit the immunosuppressive function of CD4⁺CD25⁺Foxp3⁺GITR⁺ T regulatory (Treg) cells [8, 9].

The role of NFATc1 in T-cell differentiation processes is not fully understood, partially because NFATc1 total knockout mice die at the embryonic stage [10]. Previous analysis on Th1- and Th2-skewed T cells isolated from NFATc1⁻/⁻/Rag1⁻/⁻ chimeric mice revealed an involvement of NFATc1 in the induction of the Th2-cytokines IL-4 and IL-6, whereas it had no effect on interferon gamma (IFN-γ) and IL-2 expression in Th1 cells [11, 12]. Other studies showed that NFATc1 induces IL-4 expression. In Th17 cells, NFAT transcription factors have been shown to be involved in the gene expression of RORγT (where ROR is RAR-related orphan receptor), IL-17, IL-21, IL-22, IL-10, and IL-6 [13]. This has been shown mostly for other NFAT transcription factor family members than NFATc1. Within the IL17a promoter, but not the IL17f promoter, binding sites for NFATc1 were found [14]. Additionally, NFATc1 binding sites were also found within the Il10 promoter [15]. NFATc1-deficient TGF-β-induced iTreg cells showed a slight reduction of CD25 and Foxp3 expression as compared with WT cells [16], indicating no essential role for NFATc1 in iTreg cell development.

EAE is the murine model for the early inflammatory phase of human multiple sclerosis (MS). In EAE, autoreactive T cells cause severe nerve demyelination and nerve loss, leading to physical dysfunctions such as paralysis of the extremities [17, 18]. EAE is characterized by enhanced IFN-γ and IL-2 expression in Th1 cells [11, 12]. The transcription factor T-bet, which is the main transcription factor for IFN-γ in CD4⁺ T cells [22, 23], has been shown to be involved in EAE development [24]. Previous studies have shown that mice with an NFATc1 inactivation in splenic B cells have a mild EAE disease outcome, and this was accompanied by a threefold IL-10 reduction in NFATc1-deficient B cells [25].

In this study, we analyzed conditional NFATc1-deficient mice (NFATc1^ΔT/ΔT^) that were generated by crossing NFATc1^ΔiΔB^ mice to CD4-Cre mice. These mice reveal a functional NFATc1 deficiency only in CD4-expressing cells. Thus, NFATc1 inactivation in CD4⁺ cells includes, beyond CD4⁺ T cells, also CD8⁺ T cells, because these cells are also expressing CD4 during T-cell development in the thymus. The generation of NFATc1^ΔiΔB^ mice has been described earlier [10]. Here, we analyzed the role of NFATc1 during Th17 cell differentiation and EAE development in mice. Our results indicate that NFATc1 plays an important role in Th17 cells by participating in the induction of the transcription factor RORγT and Th17-associated cytokines. We further demonstrate that conditional inactivation of NFATc1 in T cells almost completely abrogated EAE-associated paralyses by inhibiting Th17- and Th1-cell responses.

Results

**NFATc1 regulates RORγT and Th17 cytokines during TGF-β-mediated Th17-cell differentiation**

In previous studies using Rag1-deficient/NFATc1-deficient chimeric mice, it was shown that NFATc1 is involved in the induction of Th2 responses [11, 12]. The role of NFATc1 in Th17-cell differentiation processes was not fully investigated so far. To analyze the effects of an NFATc1-deficiency on Th17 differentiation, we used naive T cells from conditional NFATc1-deficient mice (NFATc1^ΔT/ΔT^). These conditional NFATc1-deficient mice were generated by crossing NFATc1^ΔiΔB^ mice to CD4-cre mice, thereby resulting in mice with a functional NFATc1-deficiency in CD4-expressing cells, including CD8⁺ T cells since these cells express also CD4 during their development. NFATc1^ΔiΔB^ mice were generated and described by Aliprantis et al. in 2008 [10] and were used as littermate control mice. Th17 cells can be divided into pathogenic and nonpathogenic Th17 cells. Nonpathogenic cells are differentiated in the presence of TGF-β1 and IL-6, whereas pathogenic cells are those differentiated in the absence of TGF-β1, but in the presence of IL-23 and IL-1β [15, 26]. To investigate the role of NFATc1 in Th17 cells, we first analyzed nonpathogenic Th17 cells. For the generation of these Th17 cells, naive CD4⁺ spleen T cells were cultured for 40 h either with IL-6 and TGF-β1 and antibodies against CD3, CD28, IL-4, and IFN-γ (Th17), or anti-CD3 and anti-CD28 antibodies alone (Th0) (Fig. 1A). To investigate if NFATc1 or rather NFATc2 plays a role in TGF-β-induced Th17 cells, we first assessed their mRNA expression. In addition, the expression of those genes was measured in Th1 cells. We found that in Th17 cells as well as in Th1 cells Nfatc1 expression was higher compared with Nfatc2 (Fig. 1B). Our analysis on Th17 cells revealed that NFATc1-deficient CD4⁺ T cells cultured under TGF-β1-mediated Th17 skewing conditions have reduced IL-17A and IL-17F protein and Il17a and Il17f mRNA expression compared with control cells (Fig. 1C to F). Furthermore, the expression of Rorc mRNA and RORγT protein was found reduced in NFATc1-deficient Th17 cells compared with control cells (Fig. 1G and H).

**NFATc1-deficient Th17 cells polarized by TGF-β1 express lower levels of IL-21, IL-10, and IL-23R**

IL-21 and IL-10 are additional cytokines known to be highly induced under Th17 cell conditions [27]. The expression of both cytokines was downregulated in NFATc1-deficient Th17 cells compared with control cells (Fig. 2A to C). These data indicate that NFATc1 is important for the induction of the main transcription factor RORγT, as well as for most of the cytokines in nonpathogenic Th17 cells. The IL-23R is also highly expressed on pathogenic cells are those differentiated in the absence of TGF-β1, but in the presence of IL-23 and IL-1β [15, 26]. To investigate the role of NFATc1 in Th17 cells, we first analyzed nonpathogenic Th17 cells. For the generation of these Th17 cells, naive CD4⁺ spleen T cells were cultured for 40 h either with IL-6 and TGF-β1 and antibodies against CD3, CD28, IL-4, and IFN-γ (Th17), or anti-CD3 and anti-CD28 antibodies alone (Th0) (Fig. 1A). To investigate if NFATc1 or rather NFATc2 plays a role in TGF-β-induced Th17 cells, we first assessed their mRNA expression. In addition, the expression of those genes was measured in Th1 cells. We found that in Th17 cells as well as in Th1 cells Nfatc1 expression was higher compared with Nfatc2 (Fig. 1B). Our analysis on Th17 cells revealed that NFATc1-deficient CD4⁺ T cells cultured under TGF-β1-mediated Th17 skewing conditions have reduced IL-17A and IL-17F protein and Il17a and Il17f mRNA expression compared with control cells (Fig. 1C to F). Furthermore, the expression of Rorc mRNA and RORγT protein was found reduced in NFATc1-deficient Th17 cells compared with control cells (Fig. 1G and H).
Expression of RORγT and the cytokines IL-17A and IL-17F in Th17 cells polarized through IL-6 and TGF-β depends on NFATc1. (A) Experimental design of Th17 skewing conditions. Naïve CD4+CD62L+ cells were separated from the spleen of NFATc1fl/fl and NFATc1ΔΔΔΔT mice and cultured for 40 h either under Th0 skewing conditions with anti-CD3/anti-CD28, or under Th17 skewing conditions with additional application of IL-6 and TGF-β and antiIFN-γ and antiIL-4 antibodies (Th17). (B) Naïve CD4+CD62L+ spleen cells from NFATc1fl/fl mice were cultured for 40 h either under Th1-inducing or Th17-inducing conditions. Nfatc1 and Nfatc2 mRNA expression in Th1 (left) and Th17 (right) was measured by qRT-PCR. Data are representative of two independent experiments, with four to five mice per group. (C) The cell supernatants from Th0 and Th17 cells generated in (A) were analyzed for IL-17A expression by ELISA. Data are pooled from six independent experiments, with 12–16 mice per group. (D) Th0 and Th17 skewed cells were harvested and Il17a mRNA expression was analyzed by qPCR-PCR. Data are representative of five independent experiments, with three to seven mice/group. (E) IL-17F ELISA and (F) Il17f mRNA expression analysis in the supernatants and cells of 40 h Th0 and Th17 skewed cells, respectively. (G and H) Th0 and Th17 cells were analyzed for Rorc mRNA expression by qPCR (G) and for RORγT protein expression by flow cytometry analysis. (H) The frequency of RORγT-positive cells among CD4+ T cells is shown. (B to H) Data are shown as mean ± SEM. (B) Shows one representative of two independent experiments with 4-5 mice per group. (C) Shows a pool from six independent experiments with 12–16 mice per group. One representative of five independent experiments are shown (D); 3–7 mice/group. (E and G) Data are pooled from three independent experiments, with five to nine mice per group. (F and H) Data are representative of three independent experiments, with three to seven mice per group. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.01; Student’s two-tailed t-test.

Impact of NFATc1 on Th17 cells polarized in the absence of TGF-β1

Th17 cells can be generated not only by IL-6 and TGF-β1, but also in the absence of TGF-β1, then they are called pathogenic
**Figure 2.** NFATc1 deficiency decreases IL-21 and IL-10 expression in IL-6/TGF-β-induced Th17 cells. (A) Naïve CD4+CD62L+ T cells were cultured for 40 h under Th0 and Th17 skewing conditions as depicted in Figure 1A. (A) Th0 and Th17 skewed cells were analyzed for Il21 mRNA expression by qPCR (n = 12–17 samples/group). (B and C) Il10 qPCR and IL-10 ELISA were performed in Th0 and Th17 cell supernatants and cells, respectively. (D) Il23r mRNA expression in Th0 and Th17 skewed CD4+ T cells (n = 6 samples/group). (E) Eomes qPCR was performed in Th0 and Th17 cells. (A to E) Data are shown as mean ± SEM. (A and D) Data are pooled from (A) six or (D) three independent experiments. (B and C) Data are representative of four independent experiments, with (B) four to five mice/group or (C) two replicates per group from a pool of eight mice per group. *p < 0.05; **p < 0.01; ***p < 0.01; Student’s two-tailed t-test.

**Figure 3.** Impact of NFATc1 on gene expression in Th17 cells in vitro polarized without TGF-β. (A) Experimental design of Th17 skewing conditions used in this figure. Naïve CD4+CD62L+ spleen cells from conditional NFATc1-deficient mice (NFATc1^Δ/Δ; T/D) or NFATc1^fl/fl control littermates were cultured in vitro with anti-CD3/anti-CD28 antibodies, IL-6, IL-23, and IL-1β (Th17), or anti-CD3/anti-CD28 antibodies alone (Th0) for 40 h and cells were analyzed for the expression of different cytokines and transcription factors. (B) Th0 and Th17 cells were analyzed for RORγT protein expression via flow cytometry. The frequency of RORγT-expressing cells among CD4+ T cells is shown. (C to J) Th0 and Th17 cells were analyzed for (C) Rorc, (D) Il21, (E) Il17f, (F) Il17a, and (I) Il10 mRNA expression by qPCR as well as. (F) IL-17F, (H) IL-17A, and (J) IL-10 protein levels measured by ELISA. (B to J) Data are shown as mean ± SEM from two independent experiments, with n = 2–4 mice/group. *p < 0.05; **p < 0.01; ***p < 0.01; Student’s two-tailed t-test.
Th17 cells. We performed Th17 skewing conditions culturing naïve CD4+ spleen cells with IL-6, IL-23, and IL-1β (Th17), and with anti-CD3/anti-CD28 antibodies alone (Th0; Fig. 3A). Our results showed that NFATc1-deficient pathogenic Th17 cells express lower levels of Rorc mRNA and RORγT protein as compared with control cells (Fig. 3B and C). Additionally, the expression of Il21 was diminished in the absence of NFATc1 in these cells (Fig. 3D). In contrast to nonpathogenic Th17 cells, pathogenic Th17 cells showed no differences in the expression of Il17f and Il10 mRNA and IL-17F and IL-10 protein in the absence of NFATc1 (Fig. 3E, F, I, and J). Furthermore, the expression of Il23r showed no differences in NFATc1-deficient Th17 cells (Supporting Information Fig. 1G). The expression of Il17a mRNA and IL-17A protein was enhanced in the absence of NFATc1 in pathogenic Th17 cells (Fig. 3G and H). Analysis of the frequency of IFN-γ+ cells among CD4+ T cells showed that in pathogenic as well as in nonpathogenic Th17 cells, these cell population was enhanced in NFATc1-deficient skewed cells as compared with control cells. Generally, the frequency of IFN-γ+CD4+ T cells was diminished in Th17 cells compared with Th0 cells (Supporting Information Fig. 1H).

NFATc1 deficiency in CD4+ T cells attenuates EAE disease symptoms and splenic cytokine secretion

To further analyze the influence of NFATc1 in Th17 cells, we next used the murine EAE model that resembles the early inflammatory phase of MS in humans [17, 29]. EAE is an autoimmune disease in which the nerves of the brain and spinal cord are affected by loss of function. It is characterized by an infiltration of autoreactive T cells in the brain, especially Th17 cells and also Th1 cells. To induce EAE development in mice, we immunized mice with myelin oligodendrocyte glycoprotein (MOG)-peptide in the presence of complete Freund’s adjuvant (CFA) and pertussis toxin. EAE scoring of the mice was performed until day 32 after EAE immunization (Fig. 4A). To investigate if NFATc1 plays a role during EAE development, we analyzed spleen cells from WT naive and EAE mice on day 32 of the disease. In WT mice, we found Nfjtc1 expression to be upregulated under EAE conditions (Fig. 4B), indicating an important role for this transcription factor in EAE. Thus, we next analyzed conditional NFATc1-deficient (NFATc1Δ/Δ) mice and NFATc1Δ/Δ mice as control mice in a murine model of EAE. Conditional NFATc1-deficient mice have an NFATc1-deficiency in CD4+ T cells as well as CD8+ T cells. We confirmed the NFATc1-deletion in CD8+ T cells via qPCR for Nfatc1 (Supporting Information Fig. 4A). Conditional NFATc1-deficient mice revealed only very mild EAE disease characteristics, when compared with control mice (Fig. 4C). Histological analysis of brain and spinal cord tissue, at day 17 after EAE induction, demonstrated almost no CD45+ T-cell infiltrates in conditional NFATc1-deficient mice when compared with control mice (Fig. 4D). Spleen cells from conditional NFATc1-deficient mice, which were isolated on day 32 after EAE induction and restimulated with different doses of MOG peptide, showed a reduced proliferation capacity in comparison to cells derived from control mice (Fig. 4E). This indicates that NFATc1 is important for the proliferation of MOG-specific cells in the spleen. To determine the involvement of NFATc1 in Th17-associated genes, we analyzed IL-17A expression levels in the supernatants of MOG-restimulated spleen cells and found reduced secretion of IL-17A in these cell cultures (Fig. 4F). Beyond Th17 cell infiltration, EAE is also characterized by an enhanced IFN-γ expression. In this respect, we observed reduced IFN-γ protein levels in the supernatants of MOG restimulated spleen cells derived from conditional NFATc1-deficient mice (Fig. 4G). In addition, the cytokines IL-6 and IL-2, known to support EAE development, were reduced in spleen cells derived from conditional NFATc1-deficient mice (Fig. 4H and I). IL-4 was very low (<20 pg) or not detectable (data not shown).

NFATc1 deficiency does not influence IL-2 responses during antigen-specific restimulation of splenic cells

To exclude that the reduction of proliferation in MOG-specific spleen cells from NFATc1-deficient mice depends on low IL-2 levels caused by the loss of NFATc1 itself, we also cultured spleen cells with different concentrations of MOG peptide together with recombinant IL-2 at day 32 after EAE induction. We found that NFATc1-deficient spleen cells were able to proliferate in the presence of recombinant IL-2 and antigen-specific MOG peptide, but by trend toward a reduced proliferation capacity in MOG cultures with NFATc1-deficient spleen cells compared with control mice (Fig. 5A), which indicates that the proliferative defect is not only due to autocrine IL-2 levels and that NFATc1 deficiency does not induce a complete deletion of T cells in general. The cytokine levels of IL-17A were significantly diminished in MOG-specific NFATc1-deficient cells (Fig. 5B), while IFN-γ and IL-6 only showed a reduced trend (Fig. 5C and D). Again IL-4 was very low (<20 pg) or not detectable (data not shown).

NFATc1 deficiency inhibits Th1 and Th17 genes in the CNS of mice with EAE

EAE is characterized by an infiltration of T cells in the brain and in the spinal cord. To further investigate the CNS in the EAE model, we first had a look at the spinal cord tissue at day 17 after EAE induction to investigate the expression of Ifng in the spinal cord at the peak of disease. We found a reduced Ifng mRNA expression in spinal cord from conditional NFATc1-deficient mice as compared with control mice (Fig. 6A). For analysis of CNS-infiltrating cells in these mice, we prepared brain and spinal cord and isolated infiltrating cells from these two organs on day 17 after EAE induction and cultured them with anti-CD3/anti-CD28 antibodies. We found reduced IFN-γ protein and Ifng mRNA as well as Il17a mRNA and IL-17A protein expression by CNS-infiltrating cells.
Figure 4. NFATc1 deficiency protects mice from EAE. (A) Experimental design of EAE induction in NFATc1^{fl/fl} and NFATc1^{ΔT/ΔT} mice. On day 0, mice were immunized with MOG peptide s.c. and pertussis toxin was injected i.p. On day 2, additional i.p. application of pertussis toxin was done. (B) On day 32 after EAE induction, spleen cells from WT naive (n = 2) and WT EAE mice (n = 5) were analyzed for the expression of Nfatc1 mRNA by qPCR. (C) EAE scoring was performed in NFATc1^{fl/fl} (n = 14) and NFATc1^{ΔT/ΔT} EAE mice (n = 19) on the indicated days. (D) CD4+ cell infiltrates in the brain (upper panel, scale bar = 100 μM) as well as in the spinal cord (lower panel, scale bar = 10 μm) of NFATc1^{fl/fl} and NFATc1^{ΔT/ΔT} EAE mice. Mice were analyzed on day 17 after EAE induction. These experiments were performed at least three times. Data presented here represent a typical experiment. (E) After EAE induction, spleen cells from NFATc1^{fl/fl} (n = 6) and NFATc1^{ΔT/ΔT} mice (n = 6) were restimulated with MOG peptide for 4 days. The proliferation of the cells was measured by thymidine incorporation. (F to I) Cell supernatants of MOG-stimulated spleen cells from Figure 3E were analyzed for the protein expression of IL-17A (F), IFN-γ (G), IL-6 (H), and IL-2 (I) with NFATc1^{fl/fl} (white bars) and NFATc1^{ΔT/ΔT} (black bars) mice. (F to I) Data are shown as mean ± SEM and are pooled from two independent experiments, with n = 2–3 mice/group. (B and F to I) Student’s two-tailed t-test, (C) Man–Whitney U-test and (E) two-way ANOVA were used for statistical analysis. *p < 0.05; **p < 0.01; ***p < 0.001. EAE scoring significances: day 12, n.s., days 13, 24, 25, 27, 29; *, days 17, 18, 26, 32; **, days 14, 15, 16, 17, 19, 20, 21, 22, ***.

Cells from NFATc1-deficient mice as compared with cells from control mice (Fig. 6B and C). Furthermore, IL-10 secretion and IL21 expression in cultured cells were found reduced in the absence of NFATc1 (Fig. 6D and E). In addition to anti-CD3/anti-CD28 stimulated CNS cells, we also analyzed directly frozen CNS cells and found diminished expression of Ifng and Tbx21 (Fig. 6F and G) as well as Rorc, Il10, and Il22 (Fig. 6H to J). The expression of Il17a and Maf in untouched CNS cells was unchanged (Supporting Information Fig. 4E and F). These data indicate that in conditional NFATc1-deficient mice, there is a reduced expression of some Th17- and Th1-associated genes in the CNS, which is in accordance with the reduced disease outcome in these mice.

Reduced frequency of IL-17A/IFN-γ expressing CD4+ T cells in the CNS of conditional NFATc1^{ΔT/ΔT} mice

To further investigate the effect of a conditional NFATc1 deficiency especially in the CD4+ T populations, we analyzed CNS cells via flow cytometry staining. Here, we found a reduced frequency of CD4+IFN-γ^+ T lymphocytes as well as IFN-γ^+ cells among CD4+ T cells in the absence of NFATc1 (Fig 7A and B). Additionally, the frequency of CD4^+IL-17A^+ T cells was reduced (Fig. 7C). The number of CD4^+ T lymphocytes in general was also diminished in the CNS of conditional NFATc1-deficient mice (Fig. 7D) as compared with control cells. Furthermore, the frequency of B cells (Supporting Information Fig. 4B), CD8^+ T cells...
Figure 5. Proliferation defect of NFATc1-deficient spleen cells does not depend solely on IL-2. (A) On day 32 after EAE induction splenic cells were separated from NFATc1^fl/fl and NFATc1^fl/D^mice and restimulated with MOG peptide and rec. IL-2 for 4 days. Proliferation of the cells was measured by thymidine incorporation. Ex vivo cytokine expression of culture supernatants from MOG peptide and IL-2 restimulation assays from (A) were measured with cytometric bead array as described in the Materials and methods. (B to D) Levels of (B) IL-17A, (C) IFN-γ, and (D) IL-6 from NFATc1^fl/fl (white bars) and NFATc1^fl/D^mice were measured by cytometric bead array. (A to D) Data are shown as mean ± SEM and are pooled from two independent experiments, with n = 2–3 mice per group. Student’s one-tailed t-test (B, C, D) and two-way ANOVA (A) were used for statistical analysis; *p < 0.05.

Reduced proliferation and secretion of Th1 cytokines in NFATc1-deficient CD4^+ T cells

To directly analyze CD4^+ T cells, we isolated the cells from spleens of mice 30 days after EAE induction. CD4^+ T cells isolated from the spleen of NFATc1-deficient mice showed an impaired proliferation capacity after restimulation with MOG peptide (Fig. 8A). This was accompanied by a reduced secretion of the cytokines IFN-γ, TNF-α, and IL-2 (Fig. 8B, D, and E). The expression of IL-17A was only slightly reduced (Fig. 8C). These data indicate that NFATc1 is involved in the proliferation of MOG-specific CD4^+ T cells and their secretion of cytokines known to be characteristic for EAE.

Discussion

In this study, we have identified NFATc1 as a positive regulator of Th17 cell differentiation. Th17 cells are a subtype of CD4^+ T cells induced by different cytokines, such as IL-6, TGF-β1, IL-23, IL-21, and IL-1β [30, 31]. They play an important role in the pathogenesis of several diseases, such as asthma, MS, and cancer [32–34]. Th17 cells are involved in the induction of EAE, which resembles the early inflammatory phase of MS in humans.
**Figure 6.** Decreased expression of IL-17A and IFN-γ in the CNS of conditional NFATc1-deficient EAE mice. EAE mice were sacrificed at days 17–19, at peak of disease, and spinal cord and brain was taken for analysis. (A) Expression of *Ifng* in spinal cord tissue from WT (*n* = 11) and EAE mice (*n* = 16). (B to E) CNS-infiltrating cells were cultured with anti-CD3/anti-CD28. Data are pooled from two independent experiments, with 11–16 mice per group. (B) IFN-γ, (C) IL-17A, and (D) IL-10 levels in stimulated CNS cells were measured by ELISA. Data are representative of two independent experiments, with *n* = 2–6 mice per group. (B) *Ifng*, (C) *Il17a*, and (E) *Il21* mRNA expression was assessed by RT-qPCR. Data are pooled from two experiments, with *n* = 2–6 mice per group/experiment. (F to J) CNS infiltrating cells were isolated from three to five mice, directly frozen, and analyzed for the expression of *Ifng* (F), *Tbx21* (G), *Rorc* (H), *Il10*, and *Il21* mRNA. (F to J) Data are representative of two independent experiments, with *n* = 2 to 3 mice/group. (A to J) Data are shown as mean ± SEM. *p* < 0.05; **p** < 0.01; ***p*** < 0.01; Student’s two-tailed t-test.

[35] The cells are characterized by the expression of the transcription factors RORγT, RORα, Interferon regulatory factor 4, and Basic leucine zipper transcription factor, ATF-like (BATF) [36, 37]. RORγT induces the expression of IL-17A, IL-17F, and IL-23R [38, 39], the latter being important for the maintenance of the Th17 phenotype [40]. The expression of the cytokine IL-17A is also induced by RORα, STAT3, and BATF, and STAT3 additionally induces the expression of the IL-23R [41]. Binding of IL-6 to its receptor leads to the phosphorylation of STAT3 and pSTAT3 also induces the expression of IL-21, IL-23R [41, 42], RORγT [43], and BATF [44].

Depending on the cytokines being induced by, Th17 cells can be divided into pathogenic and nonpathogenic Th17 cells. If they are generated in the presence of IL-6 and TGF-β1, they are called nonpathogenic cells, whereas cells generated in the absence of TGF-β1 but in the presence of IL-23, IL-18, and IL-6 are pathogenic and can induce autoimmune encephalomyelitis in an IL-23-dependent manner [15, 26]. In our study, we showed that there seem to be differences in the role of NFATc1 in the differentiation of pathogenic and nonpathogenic Th17 cells. In both cell types, NFATc1 is involved in the induction of the main transcription factor RORγT, as well as the cytokine IL-21. In contrast to that, only in TGF-β1-induced nonpathogenic Th17 cells NFATc1 deficiency leads to a reduction of IL-17A, IL-17F, and IL-10 in vitro. The effects of NFATc1 in inducing Th17 cell differentiation processes might therefore in vitro rely partially on TGF-β1. In general, our analysis showed that in TGF-β1-induced nonpathogenic Th17 cell cultures, the expression of the corresponding signature genes was induced compared with Th0 cells and pathogenic Th17 cells cultured without TGF-β1. So one could speculate that the effects of an NFATc1 deficiency are more visible when expression of Th17-associated genes is high, which is the case in nonpathogenic Th17 cells. In NFATc1-deficient Th17 cells, only the expression of the transcription factor RORγT was...
found to be downregulated, whereas there was no influence on the expression of the above-mentioned transcription factors.

In contrast to its role in Th17 cells, NFATc1 seems to play no essential role in the differentiation of Th1 and induced iTreg cells in vitro. We and others showed that NFATc1 seems to have only a slight effect on differentiation of iTreg cells, indicating no essential role for NFATc1 [16]. Which role NFATc1 has in natural occurring Treg cells has to be further investigated. Our NFATc1-deficient Th1 skewing data are in agreement with analysis on Rag2<sup>−/−</sup>/NFATc1<sup>−/−</sup> chimeric mice, which showed no changes in the secretion of IFN-γ and TNF-α [12]. This suggests that NFATc1 is not essential for the developmental program of Th1 cells in vitro. Either it is not directly involved in the induction of Th1-specific genes or the absence of NFATc1 is compensated by other transcription factors during Th1 development in vitro. This has to be further investigated. Since we could not find differences in the expression of T-bet, other transcription factors might be involved.

In contrast to the in vitro Th1 polarizing data, using a murine model of EAE, we identified an influence of T-cell associated NFATc1 on Th17 as well as Th1 cells in vivo. In these studies, clinical EAE symptoms were almost completely reduced in conditional NFATc1-deficient mice with inactivated NFATc1 in CD4<sup>+</sup> cells. Previous studies using Rag2<sup>−/−</sup>/NFATc1<sup>−/−</sup> chimeric mice also reported impaired proliferation of NFATc1-deficient T cells. To exclude that the reduced proliferation was due to a reduced autocrine IL-2 secretion, we did cultures with MOG peptide plus IL-2. There were no differences anymore between different MOG doses regarding proliferation in general, but we could show a slide decrease of proliferation in NFATc1-deficient spleen cells in these cultures, indicating that not only autocrine IL-2 levels decrease the proliferation of NFATc1-deficient cells.

However, it is not clear if the reduction of IL-17A is a secondary effect due to the impaired expression of ROR<γT> or whether NFATc1 directly binds to the promoter of IL-17A. Previous studies showed that the promoter of Il17a but not of Il17f encodes a binding site for NFATc1 [14]. This supports our data that NFATc1

Figure 7. Decreased number of CD4<sup>+</sup> T cells and IL-17A and IFN-γ expressing CD4<sup>+</sup> T cells in the CNS of NFATc1-deficient EAE mice. CNS infiltrating cells from NFATc1<sup>fl/fl</sup> and NFATc1<sup>CΔT</sup> EAE mice were isolated on days 17–19 after EAE induction and the cell populations were analyzed by flow cytometry analysis. (A and B) CNS cells were stained for CD4 and IFN-γ and the frequency of CD4<sup>+</sup>IFN-γ<sup>+</sup> T cells among lymphocytes (A) and the frequency of IFN-γ-expressing cells among CD4<sup>+</sup> lymphocytes (B) are shown. (C and D) CNS cells were stained for CD4 and IL-17A. (C) Frequency of CD4<sup>+</sup>IL-17A<sup>+</sup> cells among lymphocytes. (D) Number of CD4<sup>+</sup> expressing cells in the CNS of NFATc1<sup>fl/fl</sup> and NFATc1<sup>CΔT</sup> EAE mice. (A to D) Data are shown as mean ± SEM (n = 8–9) and are pooled from three independent experiments. Dot plot shown are from one representative of three independent experiments. Student’s two-tailed t-test was used for statistical analysis. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.01.
might bind to the promoter of Il17a and induces the gene expression of this cytokine. Our data additionally indicate that NFATc1 is involved in the induction of Il17f expression in nonpathogenic Th17 cells although no binding site for NFATc1 was found on its promoter. The importance of the reduced IL-17A and IFN-γ expression in EAE using NFATc1-deficient mice was emphasized by the strongly reduced paralysis and diminished T-cell infiltration in the brain and the spinal cord. Since also the expression of Il21 was reduced in the absence of NFATc1, it can be assumed that the transcription factors inducing Il21 expression, such as STAT3, are not sufficient to induce IL-21 without NFATc1. It seems that no other transcription factors can compensate for the loss of IL-21 in Th17 cells. This is also true for the expression of IL-17A and IL-17F in nonpathogenic Th17 cells and EAE-specific cells. Besides proinflammatory cytokines, Th17 cells are also known to secrete the anti-inflammatory cytokine IL-10 that suppresses the functions of effector T cells [47]. We found that NFATc1-deficient cells showed lower expression levels of Il10 mRNA and IL-10 protein compared with control cells. In previous studies it has been shown that there is an NFATc1 binding site within the Il10 promoter [48]. IL-10 is known to be induced by the transcription factor complex JUN/BATF/Interferon regulatory factor 4 [37, 49, 50]. In the absence of NFATc1, we found no changes in these transcription factors. Whether NFATc1 influences the Il10 expression by directly binding to the Il10 promoter or the reduction of IL-10 protein expression is secondary to the downregulation of other transcription factors has to be further investigated. Previous studies in NFATc1-deficient B cells in an EAE model revealed an enhanced IL-10 expression in the absence of NFATc1, which is opposite to our results. Therefore, the regulation of IL-10 by NFATc1 seems to be different in B and T cells [25].

Taken together our data indicate that NFATc1 is important for the differentiation of nonpathogenic and pathogenic Th17 cells through the influence on the expression of the Th17-associated transcription factor RORγT and the cytokine IL-21. This is also true for the Th17 cytokines IL-17A, IL-17F, and IL-10 in nonpathogenic Th17 cells. Using the EAE model, we could in vivo show that NFATc1 enhances IL-17A and IFN-γ expression in MOG antigen specific cells, thereby exacerbating the EAE-associated paralyses. Inactivation of NFATc1 in T cells reduces the development and disease symptoms of EAE. Previous studies have shown that treatment of mice with 2-methoxyestradiol, which reduces NFATc1 transcriptional activity in lymphocytes, suppressed the development of EAE [51]. This, together with our findings, indicates that deletion of NFATc1 in CD4+ lymphocytes might be useful for the potential development of new immunotherapies in MS.

Materials and methods

Mice

C57BL/6 WT mice were bred in house. NFATc1fl/fl mice were initially provided by L. Glimcher, Harvard Medical School, and crossed to CD4-cre mice, which were provided by Chris Wilson,
Figure 9. Reduced proliferation and cytokine secretion of NFATc1-deficient EAE mice in LN cells at peak of disease. LN cells from NFATc1<sup>fl/fl</sup> and NFATc1<sup>Δ/Δ</sup> T/EAE mice were isolated on day 17 after EAE induction and analyzed. (A to C) LN cells were restimulated with MOG peptide for 4 days. (A) Proliferation of the cells was measured by thymidine incorporation. (B) IFN-γ and (C) IL-17A levels were measured from the supernatants of restimulated cell cultures by cytometric bead array. (D and E) LN cells were stained for CD4 and IL-10 and the (D) frequency of IL-10<sup>+</sup> T cells among CD4<sup>+</sup> T cells as well as the (E) frequency of CD4<sup>+</sup>IL-10<sup>+</sup> cells among lymphocytes is shown. (A to E) Data are shown as mean ± SEM (<i>n</i> = 2–3 mice per group) and are pooled from two independent experiments. Two ways ANOVA (A) and Student’s two-tailed t-test (B, C, D, and E) were used for statistical analysis. *<i>p</i> ≤ 0.05; **<i>p</i> ≤ 0.01; ***<i>p</i> ≤ 0.01.

Washington, to generate NFATc1<sup>Δ/Δ</sup> T mice. All mice were on C57BL/6 background and kept in house under specific pathogen-free conditions. Animals were treated according to national and institutional guidelines for animal care and use.

**T-cell separation and in vitro polarization**

For T-cell separation and differentiation total spleen cells were isolated as described earlier [52]. Naive CD4<sup>+</sup>CD62L<sup>+</sup> T cells and naive CD8<sup>+</sup>CD62L<sup>+</sup> T cells were separated using CD4<sup>+</sup>/CD8<sup>+</sup>/CD62L<sup>+</sup> T-cell isolation kits from Miltenyi Biotec GmbH. For nonpolarizing conditions, naive CD4<sup>+</sup>/CD8<sup>+</sup> were stimulated exclusively with anti-CD3 (cell culture plate coated with 5 µg/mL αCD3 in NaHCO<sub>3</sub> buffer) and anti-CD28 antibodies (Th0/Tc0). For pathogenic Th17 conditions CD4<sup>+</sup>CD62L<sup>+</sup> were additionally cultured with anti-IL-4 (10 µg/mL), anti-IFN-γ (10 µg/mL) antibodies, IL-6 (20 ng/mL), and TGF-β1 (3 ng/mL) besides anti-CD3 and anti-CD28 antibodies. Pathogenic Th17 cells were cultured with anti-IL-4 (10 µg/mL) and anti-IFN-γ (10 µg/mL) antibodies, IL-6 (20 ng/mL), IL-23 (50 ng/mL), and IL-1β (50 ng/mL) besides anti-CD3 and anti-CD28 antibodies. Tc17 cells were cultured with IL-6 (20 ng/mL), TGβ-β1 (5 ng/mL), IL-23 (50 ng/mL), IL-21 (80 ng/mL), and IL-1β (40 ng/mL). Th1 cells were cultured additionally with αIL-4 antibodies (10 µg/mL) and IL-12 (12 ng/mL). For regulatory T-cell conditions (Treg) cells were stimulated with αIL-6R antibodies (10 µg/mL; clone MR16-1; Chugai Pharmaceuticals), IL-2 (100 ng/mL), and TGβ-β1 (0,3 µg/mL). All cells were cultured with the mentioned stimuli for 40 h. Cytokines were purchased from PeproTech GmbH. Antibodies for IL-4 were obtained from hybridoma cell lines (αIL-4: 11B11; αIFN-γ: XMG 1.2).
EAE induction

NFATc1^fl/fl^ and NFATc1^3T/-T^ mice (12–16 weeks old) were immunized s.c. with MOG35-55 (Charite) at day 0 to induce EAE as described earlier [53]. Additionally, mice received 200 ng pertussis toxin (List Biochemicals) i.p. on days 0 and 2 postimmunization. The clinical evaluation was analyzed daily and scored as follows: 0, no clinical sign; 1, limp tail or waddling gait with tail toxicity; 2, waddling gait with limp tail (ataxia); 2.5, ataxia with partial limb paralysis; 3, full paralysis of one limb; 3.5, full paralysis of one limb with partial paralysis of a second limb; 4, full paralysis of two limbs; 4.5, moribund; and 5, death.

In vitro MOG restimulation

Splenocytes and LN cells were harvested from mice 32 days or 17–19 days after immunization with MOG peptide and used for restimulation assays. Cells were cultured in HL-1 serum-free medium supplemented with penicillin (100 U/mL, Sigma), streptomycin (100 μg/mL, Sigma), l-glutamine (2 mM, Sigma), and 2-ME (50 μM, Sigma). MOG-specific cells were incubated by inculating triplicates of 4 × 10^5 cells with different concentrations of MOG peptide in 200 μL HL-1/well in a 96-well tissue culture plate. Additionally, 4 × 10^5 splenic cells were stimulated together with rc-IL-2 (50 U/mL, PeproTech) and indicated amounts of MOG peptide. As a negative control, unstimulated cultures were used. In addition, CD4^+^ T cells from these cells were separated with CD4 microbeads according to the manufacturer’s protocol (Miltenyi Biotec GmbH). Triplicate aliquots of 3 × 10^5 CD4^+^ T cells in a 200 μL volume were cultured in 96-well flatbottom microtiter plates (Nunc), and stimulated with indicated concentrations of MOG peptide for 72 h. Additionally, at the peak of disease, that is, around day 17, draining LNs were removed and single cell suspensions were made. These LN cells were also restimulated with different amounts of MOG35-55 and incubated as described above, pulsed with 1 μCi/well [methyl-^3H^]thymidine (Hartmann Analytik) for 16 h, harvested onto glass fiber filters (Printed Filtermat A; Wallac, Turku, Finland) using an ICH-110 harvester (Inotech, Dottikon, Switzerland), and [methyl-^3H^]thymidine incorporation was determined using a microplate counter (Wallac). From all restimulation cultures, supernatants were harvested to determine the ex vivo cytokine production.

Isolation of CNS-infiltrating cells

Mice were sacrificed at day 17 and perfused with 20 mL PBS. Brain and spinal cord were cut into small pieces and incubated under constant stirring in RPMI 1640 (supplemented with 10% FCS, 1% l-glutamine, 1% penicillin–streptomycin, 0.1% β-ME), containing 2.5 mg/mL collagenase III (Sigma-Aldrich) and 0.5 mg/mL DNase I (Sigma-Aldrich) for 60 min at 37°C. Then, 5 mM EDTA (Applichem) was added and the tissues were passed through a 70 μm cell strainer. Cells were centrifuged, washed twice with R10, resuspended in 40% Percoll (Sigma-Aldrich), and overlaid on 70% Percoll. Gradient solutions were centrifuged at 1800 rpm for 30 min. Without braking, the interphase, containing mononuclear cells, was recovered. Cells were washed twice with R10 and used for further experiments.

RNA isolation and quantitative real-time PCR analysis

RNA from tissue and cells was isolated with phenol/chloroform using PqueGold (PqueLab GmbH) reagent according to the manufacturer’s directions. cDNA was synthesized with a reverse transcription kit (Fermentas GmbH). For amplification, the EvaGreen qPCR mix was used (Bio-Rad). Gene expression was analyzed with CFX real-time detection system (Bio-Rad). The relative gene expression levels were calculated with the comparative Ct (threshold cycle) method, where the data were expressed as 2^(-ΔΔCT). Expression was normalized to HPRT. Primer sequences were for as follows: Hprt fwd: 5’-GCC CCA AAA TGC TTA AGG TT-3’; rev: 5’-TTG GCC TCA TCT TAT GCT TT-3’. Il17a fwd: 5’-TCC AGA AGG CCC TCA GAC TA-3’, rev: 5’-AGC ATC TTC TCG ACC CTG AA-3’; Il21 fwd: 5’-CAGGAGGGGAGAAAC3-’, rev: 5’-GGGAATCTTCTCGATCATC-3’; Rorc fwd: 5’-TGC AAG ACT CAT CGA CAA GG-3’, rev: 5’AGG AGA TTC AAC ATC AGT GC-3’; Il17f fwd: 5’-GCACCCGTTGAAACAGCCATGTT-3’, rev: 5’-GGCGCTTGGTGGCAATGGGC-3’; Il23r fwd: 5’-GGGAAAAGAAGACAGCAAGC-3’, rev: 5’-CAACCCATGTC-ACCAGAG-3’; Il10 fwd: 5’-CCA AGC CTT ATC GGA AAT GA-3’, rev: 5’-TTT TCA CAG GGG AGA AAT CG-3’; Ifng fwd: 5’-GCCGCTTGGTGGCAATGGGC-3’; Il2fwd: 5’-GCCAGGGGAGAAAC3-’, rev: 5’-GGGAATCTTCTCGATCATC-3’; Nfatc1 exon3 fwd: 5’-TGC TCT TTG CGA GCA GTA TCT-3’, rev: 5’-CAG GCA AGG ATG GGC TCA TAT-3’.

Cytokine measurements from cell supernatants

Cytokine measurements from cell supernatants were performed on days 17 post-MOG immunization, mice were perfused with 30 mL PBS and then brains and spinal cords were removed, fixed in 10% formalin, and embedded in paraffin. 5 μm-thick sections were cut and stained with hematoxylin and eosin (H&E).

Histology

Histologically, mouse brains and spinal cords were stained using H&E.
liquid nitrogen and stored at –80°C. Sections were cryoprotected in Tissue-Tek® (Sakura) and slicetted sequentially with a thickness of 5 μm with a cryotome (KryoCut CM 3050S, Leica). Aceton-fixed cryostat sections were stained with hematoxylin (Sigma) and examined by light microscopy (Leica). Immunohistological staining was performed using an immunoperoxidase detection system in a humid incubation chamber. Aceton-fixed sections were incubated in PBS. Endogenous peroxidase was blocked by incubating sections in 3% H2O2. The primary antibody used was an anti-mouse-CD45 antibody (Clone 30G12, kindly provided by Lydia Sorokin, Lund, Sweden). As secondary antibody biotinylated goat-anti-rat IgG (Dako) and ABC-Mix (Vector) were used. To visualize antigens, sections were incubated with AEC Chromogene Substrate (Vector), mounted in Aqua Tex (Merck), and covered with a cover slip.

Flow cytometry

For flow cytometry analyses cells were stained with antibodies to detect surface expressed CD4 (CD4 Alexa 488; CD4 APC; CD4 Percp [BD Biosciences]) and intracellular expressed RORγ-T, IL-10, IL-17A, and IFN-γ (RORγ-T PE, IFN-γ FITC, IL-17A Percp, IL-10 PE- BD biosciences). Staining was performed with Fix/Perm and Perm/Wash from BD Biosciences according to the manufacturer’s protocols. To monitor cytokine expression by flow cytometry, cells were incubated with the corresponding stimuli and stimulated for 4 h in the presence of PMA (50 ng/mL), Ionomycin (500 ng/mL) and 1.5 μL/1 mio cells GolgiStop™ (BD Biosciences) before staining. Cells were analyzed using FACS Calibur from BD Bioscience and data analysis was performed with FlowJo software from TreeStar (San Carlos).

Statistical analysis

Results were expressed as standard error of the mean (SEM). Two-tailed Student’s t-test in case of two groups and two-way ANOVA using Bonferroni posttest in multiple groups was used to determine the statistical significances. A nonparametric Mann–Whitney U-test was used to analyze clinical EAE scoring. Differences with p values less than 0.05 were considered statistically significant. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Statistics was calculated with Excel or Prism 5.0 (GraphPad, La Jolla, CA).

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Abbreviations: BATF: basic leucine zipper transcription factor, ATF-like · Eomes: Eomesodermin · MOG: myelin oligodendrocyte glycoprotein · MS: multiple sclerosis · NFAT: nuclear factor of activated T cells · ROR: RAR-related orphan receptor

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