Dependence on nuclear factor of activated T-cells (NFAT) levels discriminates conventional T cells from Foxp3$^+$ regulatory T cells

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Several lines of evidence suggest nuclear factor of activated T-cells (NFAT) to control regulatory T cells: thymus-derived naturally occurring regulatory T cells (nTreg) depend on calcium signals, the *Foxp3* gene harbors several NFAT binding sites, and the Foxp3 (Fork head box P3) protein interacts with NFAT. Therefore, we investigated the impact of NFAT on Foxp3 expression. Indeed, the generation of peripherally induced Treg (iTreg) by TGF-β was highly dependent on NFAT expression because the ability of CD4$^+$ T cells to differentiate into iTreg diminished markedly with the number of NFAT family members missing. It can be concluded that the expression of Foxp3 in TGF-β–induced iTreg depends on the threshold value of NFAT rather than on an individual member present. This is specific for iTreg development, because frequency of nTreg remained unaltered in mice lacking NFAT1, NFAT2, or NFAT4 alone or in combination. Different from expectation, however, the function of both nTreg and iTreg was independent on robust NFAT levels, reflected by less nuclear NFAT in nTreg and iTreg. Accordingly, absence of one or two NFAT members did not alter suppressor activity in vitro or during colitis and transplantation in vivo. This scenario emphasizes an inhibition of high NFAT activity as treatment for autoimmune diseases and in transplantation, selectively targeting the proinflammatory conventional T cells, while keeping Treg functional.

gene regulation | tolerance | autoimmunity

Regulatory T cells (Treg) are T lymphocytes specialized for immune suppression. They are necessary to maintain immune homeostasis and to prevent autoimmune diseases. Treg are identified by the expression of CD4, CD25, and the key transcriptional regulator Foxp3 (Fork head box P3). Within the CD4$^+$ compartment Treg are represented as a heterogeneous population of thymus-derived (naturally occurring or nTreg) and various peripherally induced Treg (iTreg). Differentiation of nTreg requires high-affinity T cell receptor (TCR) signals as well as costimulatory signals, both provided by thymic medullary epithelial cells. In contrast, adaptive or iTreg are generated from conventional naïve CD4$^+$ T cells (Tconv) in peripheral tissue. This can be mimicked in culture by TCR (and coreceptor) engagement in the presence of the cytokines TGF-β and IL-2 (1).

Foxp3 is crucial for nTreg function (2). Mice and humans with mutations in the *Foxp3* gene suffer from a severe autoimmune disorder known as scurfy or IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, which manifests in lymphoproliferation, multiorgan lymphocytic infiltration, and systemic autoimmune inflammation. It can be prevented by the adoptive transfer of CD4$^+$CD25$^+$ T cells. Foxp3 binds DNA through a winged helix-forkhead DNA binding domain and functions as a transcriptional activator/repressor by recruiting deacetylases as well as histone acetyltransferases (3). In addition, several transcription factors, including nuclear factor of activated T-cells (NFAT), NF-κB (nuclear factor "kappa-light-chain-enhancer" of activated B-cells), and Runx1/AML1 (runt-related transcription factor1/acute myeloid leukemia1) have been identified as interaction partners of Foxp3 (4–6). Interestingly, all three transcription factors have also been reported to regulate Foxp3 expression.

Recently, several studies have demonstrated the importance of the NF-κB family member c-Rel for thymic Foxp3 induction (7). c-Rel binds directly to the *Foxp3* locus, thereby initiating chromatin opening at a newly identified *cis*-regulatory element (CNS3) (8), concomitantly binding to further enhancer regions and the *Foxp3* promoter (9).

Accumulating evidence has pointed to a role of NFAT in Treg, because the necessity of Ca^{2+} signals in nTreg development and function was emphasized (10, 11). TCR-initiated Ca^{2+} influx and subsequent calmodulin/calcineurin activation is central for the translocation of NFAT transcription factors to the nucleus, where they bind to regulatory regions of numerous genes (12), including at least one *cis*-regulatory element of *Foxp3*, namely CNS1 (13). The NFAT family comprises four calcium-regulated members: NFAT1/NFATc2, NFAT2/NFATc1, NFAT3/NFATc4, and NFAT4/NFATc3, with NFAT1, -2, and -4 being predominant in T cells.

In contrast to the established role of Ca^{2+}, previous data revealed that nTreg in mice deficient for NFAT1 plus NFAT4 were neither decreased in number nor impaired in their suppressive capacity (14). Therefore, it was concluded that NFAT2 might be the important family member for controlling nTreg development and/or function (10). Indeed, former analyses elicited mRNA encoding the long isoforms of NFAT2 being present in peripheral CD4$^+$ as well as CD4$^+$CD25$^+$ T cells. However, the activation-induced NFAT2/αA was missing in CD4$^+$CD25$^+$ nTreg, in line with less overall NFAT2 protein in nTreg (15).

In this study, we addressed the role of individual NFATs in nTreg and iTreg development and function. We analyzed and compared Treg from mice lacking only NFAT1, NFAT2, or NFAT4, as well as NFAT1 plus NFAT2 or NFAT1 plus NFAT4, in T cells. We found that decreased NFAT activity progressively impaired Foxp3 induction in TGF-β–induced iTreg. Nevertheless, in accordance with most Treg-associated markers being stationary, NFAT-deficient iTreg as well as nTreg were fully

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suppressive, suggesting that high levels of NFAT activity are not required for their regulatory function. In accordance, human and murine Treg have lower levels of both nuclear and cytoplasmic NFAT than conventional $CD4^+$ T cells. This designates specific NFAT-directed drugs as potent therapy in autoimmune disease and transplantation.

Results

NFAT Is Required for Foxp3 Expression in iTreg. NFAT2 was reported to bind CNS1 of *Foxp3* (13) [i.e., to an element that is crucial for iTreg generation in gut-associated lymphoid tissues (8)]. Here, we analyzed the dependence of Foxp3 expression on NFAT2 in comparison with NFAT1 and -4. The offspring of *Nfat2*$^{fl/fl}$ mice were crossed with *Cd4-cre* mice (16) (Fig. S1 *A–C*). Together with anti-CD3/28 and IL-2, TGF-β induced robust Foxp3 expression in WT $CD4^+CD25^-$ T cells, whereas induction was moderately diminished in the absence of NFAT2 and especially when both NFAT1 and NFAT2 were missing (Fig. 1 *A* and *B* and Fig. S1*D*). Analysis of NFAT1 single- and NFAT1NFAT4 double-deficient $CD4^+CD25^-$ T cells yielded a similar result. Whereas lack of one family member led to some reduction of Foxp3-expressing cells, loss of two members almost abrogated iTreg induction (Fig. 1 *C* and *D*). Pharmacological inhibition of all NFAT members by cyclosporine A (CsA) totally blocked Foxp3 induction in naïve $CD4^+$ T cells during stimulation with anti-CD3/28 plus TGF-β/IL-2 (Fig. S2*A*). The difference between WT and NFAT-deficient T cells was more pronounced under suboptimal doses of TGF-β (Fig. 1*D* and Fig. S2*B*). Skewing the same naïve $CD4^+$ T cells toward T helper cell subtypes demonstrated a similar dependence on the level of NFAT for IFN-γ and IL-17, as well as a dominant influence of NFAT2 on IL-17 expression (Fig. S2*C*).

CD4 is first expressed at the $CD4^+CD8^+$ double-positive stage of thymocytes, presumably ahead of Treg development. Therefore, *Nfat2*$^{fl/fl}$ × *Cd4-cre* created a thymocyte/T cell-specific *Nfat2* knockout (Fig. S1*D*) and enabled us to analyze nTreg development in the absence of NFAT2. However, the frequency of $Foxp3^+$ $CD25^+$ nTreg among the $CD4^+$ cell population in thymus, spleen, and lymph nodes (LN) were not affected by deficiency of any—alone or in combination—NFAT member (Fig. S3 *A–F*). That is not due to lack of NFAT expression, because all three NFAT members are similarly expressed in Tconv and Treg, even though up-regulation of NFAT2 does not occur in nTreg and only marginally in iTreg (Fig. S3*G*). In summary, whereas nTreg develop irrespective of NFAT expression, iTreg crucially rely on high NFAT levels with permissiveness for individual family members.

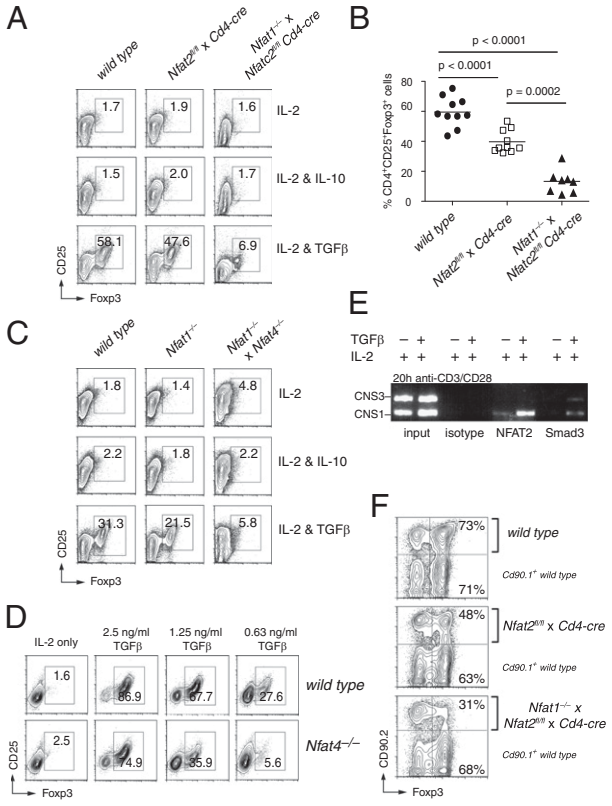

Fig. 1. Impaired induction of Foxp3 by TGF-β in NFAT-deficient $CD4^+CD25^-$Tconv. (*A* and *B*) $CD4^+CD25^-$ T cells from WT, *Nfat2*$^{fl/fl}$ × *Cd4-cre* and *Nfat1*$^{-/-}$ × *Nfat2*$^{fl/fl}$ × *Cd4-cre* mice were stimulated for 3 d with plate-bound anti-CD3/28 in the presence of IL-2 only, IL-2 plus IL10, or IL-2 plus TGF-β followed by rest of 4 d. (*A*) Representative FACS analysis of $CD4^+CD25^+Foxp3^+$ T cells and (*B*) summary of at least eight independent IL-2/TGF-β cultures. (*C*) $CD4^+CD25^-$ T cells from WT, *Nfat1*$^{-/-}$, and *Nfat1*$^{-/-}$ × *Nfat4*$^{-/-}$ mice as in *A*. (*D*) $CD4^+CD25^-$ T cells from WT and *Nfat4*$^{-/-}$mice as in *A*, but with different concentrations of TGF-β. (*E*) ChIP assay (one out of three) of NFAT2 and Smad3 binding to the *Foxp3*-enhancer elements CNS1 and CNS3 from twenty-hour stimulated $CD4^+CD25^-$ T cells. (*F*) $CD90.2^+$ $CD4^+CD25^-$ T cells from WT, *Nfat2*$^{fl/fl}$ × *Cd4-cre* and *Nfat2*$^{fl/fl}$ × *Cd4-cre* × *Nfat1*$^{-/-}$ mice were mixed 1:1 with congenic ($CD90.1^+$) T cells and processed as in *A*. Frequency of $CD4^+Foxp3^+$ T cells among genotypes (CD90.1 vs. CD90.2) is denoted.

NFAT Influences *Foxp3* Directly During iTreg Differentiation. To elucidate whether NFAT2 was capable to bind to the regulatory elements of *Foxp3*, we stimulated naïve $CD4^+CD25^-$ T cells in the presence or absence of TGF-β for 20 h, and performed ChIP assays. Specific binding of NFAT2 was observed at CNS1 in cells stimulated in the presence of TGF-β, whereas Smad3 bound to CNS1 and -3 in cells just beginning to express Foxp3 (Fig. 1*E*). Electromobility shift assays (EMSA) with extracts from human or murine T cells demonstrated some binding to their respective *Foxp3* promoters. However, mobility of those complexes was atypical, and unlabeled *Foxp3* promoter probe could not compete for NFAT binding to CNS1, whereas anti-NFAT1 or anti-NFAT2 supershifts were only found at CNS1 (Fig. S3 *H–J*). This indicates a specific, CNS1-directed, and cell-intrinsic influence of NFAT on the *Foxp3* locus during TGF-β–stimulated iTreg differentiation. To further investigate this point, we mixed congenic WT $CD4^+CD90.1^+$ T cells with $CD4^+CD90.2^+$ T cells from WT, *Nfat2*$^{fl/f}$× *Cd4-cre*, or *Nfat1*$^{-/-}$× *Nfat2*$^{fl/fl}$× *Cd4-cre* mice and induced Foxp3. Whereas NFAT-deficient $CD4^+CD90.2^+$ T cells showed reduced Foxp3 expression, congenic WT $CD4^+$ $CD90.1^+$ T cells from the same TGF-β cultures remained unaffected (Fig. 1*F*). Therefore, iTreg differentiation by TGF-β is highly dependent on the level of NFAT, from which at least NFAT2 binds to CNS1 in vivo and seems to transactivate the *Foxp3* gene for iTreg induction.

NFAT Is Essential for iTreg Induction in Vivo. Induction of iTreg occurs primarily in gut-associated lymphoid tissues, where iTreg balance Th17-driven immune responses. To explore whether NFAT deficiency also impaired induction of iTreg in vivo, we first analyzed Helios expression in $Foxp3^+$ T cells from mesenteric LNs (mLN). This allowed us to distinguish between nTreg, which are $Foxp3^+Helios^+$, and iTreg, which are $Foxp3^+$ but $Helios^-$ (17), in untreated mice lacking NFAT2 and NFAT1 plus NFAT2 in T cells. The data revealed that in vivo (*i*) the percentage of $Foxp3^+Helios^+$ nTreg remained unaffected by the deficiency of NFAT2 or NFAT1 plus NFAT2, but (*ii*) the NFAT1 plus NFAT2-deficient $Foxp3^+$ $Helios^-$ iTreg were clearly reduced (Fig. 2*A*).

Second, we addressed iTreg differentiation in a model of murine colitis by transfer of naïve $CD4^+$ T cells to lymphopenic recipients (18). $CD4^+CD62L^+$ but $CD25^-$ T cells in a 1:1 mixture of WT $CD90.1^+$ (to ensure disease onset) and $CD90.2^+$ WT or *Nfat2*$^{fl/fl}$ × *Cd4cre* were delivered into *Rag1*$^{-/-}$ mice (Fig. S4*A*). Disease was monitored by colon miniendoscopy. In both cases the transfer caused colitis (Fig. S4 *B–D*), whereas naïve $CD90.2^+$ NFAT2-deficient $CD4^+$ T cells were significantly disabled to

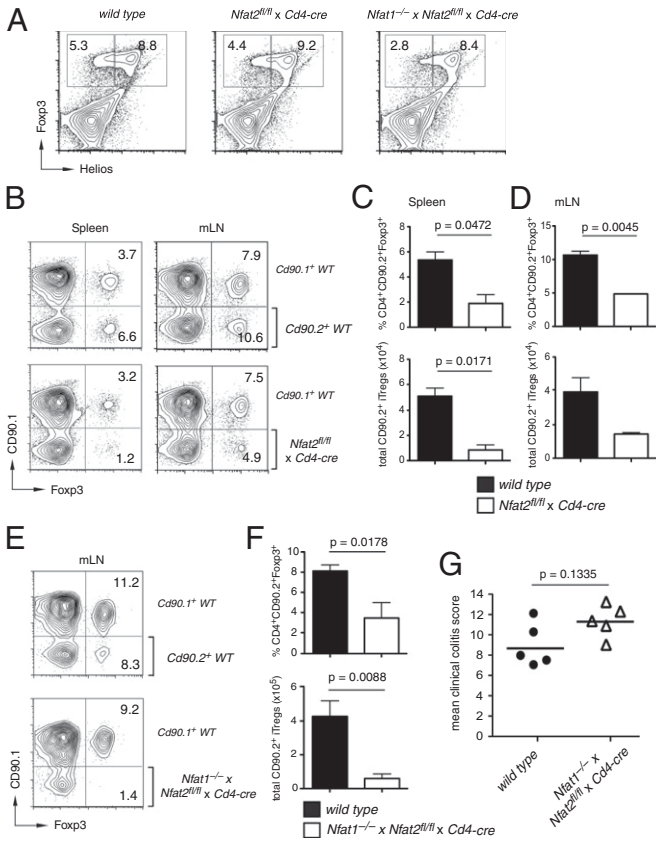

Fig. 2. Impaired iTreg generation in NFAT-deficient T cells in vivo. (*A*) Representative FACS analysis of Foxp3$^+$Helios$^-$ iTreg and Foxp3$^+$Helios$^+$ nTreg in mesenteric LN of untreated WT, *Nfat2*$^{fl/fl}$ × *Cd4-cre*, and *Nfat1*$^{-/-}$ × *Nfat2*$^{fl/fl}$ × *Cd4-cre* mice. (*B–D*) 2.5 × 10^5 *Cd90.1*$^+$ (WT) and 2.5 × 10^5 *Cd90.2*$^+$ (WT or *Nfat2*$^{fl/fl}$ × *Cd4cre*) CD4$^+$CD62L$^+$CD25$^-$ T cells were injected into *Rag1*$^{-/-}$ mice. The frequency of Foxp3$^+$ iTreg was impaired in spleen (*B*, *Left* and *C*) and mesenteric LN (*B*, *Right* and *D*) after 6 wk, analyzed by FACS (*B*) and total cell number of CD90.2$^+$Foxp3$^+$ iTreg per organ compiled from five different mice (*C* and *D*). (*E* and *F*) As in *B* and *D*, but WT compared with *Nfat1*$^{-/-}$ × *Nfat2*$^{fl/fl}$ × *Cd4-cre* T cells. (*G*) Mean clinical score of colitis by colon endoscopy from mice in *E*; each dot represents one individual mouse. The difference is not statistically significant.

develop into Foxp3$^+$ iTreg both in spleen and mLN (Fig. 2 *B–D*). When cells from *Nfat1*$^{-/-}$ × *Nfat2*$^{fl/fl}$ × *Cd4cre* were transferred, we observed even less iTreg in spleen and mLN, associated with an enhanced severity of colitis (Fig. 2 *E–G*). However, effector function of conventional cells (e.g., cytokine production derived from the identical naïve CD4$^+$ T-cell pool) was additionally impaired (Fig. S2*D*). This probably masked the significance of diminished iTreg differentiation as seen in CNS deficiency (19).

NFAT Is Dispensable for iTreg Function in Vivo and in Vitro. An altered repressor capacity of iTreg cannot be monitored, when their numbers are changed during colitis and Tconv function is additionally affected. An in vivo readout for the suppressive capacity of Treg is allograft acceptance after adoptive transfer of Tconv together with defined numbers of Treg. iTreg specific for alloantigens can be induced in vivo—and even in thymus-ectomized mice, excluding nTreg involvement—by injection of the non-depleting anti-CD4 antibody YTS177.9, together with a donor-specific blood transfusion (DST) of allogeneic blood as antigen (Fig. 3*A*) (20, 21). DST pretreatment in WT vs. *Nfat2*$^{fl/fl}$ × *Cd4-cre* mice resulted in a different total yield of CD4$^+$CD25$^+$ Treg per spleen to less than half of Treg in NFAT-deficient compared with WT mice, once more indicating the dependence of iTreg generation on NFAT expression in vivo (Fig. 3*B*). To evaluate the suppressive function of this remaining iTreg population, *Rag2*$^{-/-}$ mice were injected with 2 × 10^5 WT or NFAT2-deficient CD4$^+$CD25$^+$ T cells from DST-pretreated mice together with CD45RB^{hi} Tconv. One day after cell transfer, the mice received a BALB/c skin transplant. Both WT and NFAT2-deficient iTreg guaranteed a clear increase in graft survival (Fig. 3*C*), demonstrating no obvious impairment in suppressor function of NFAT2-deficient iTreg.

To ensure purity of iTreg and avoiding contaminating nTreg for functional tests we generated TGF-β–induced iTreg from naïve CD4$^+$ T cells in vitro. Classic suppression assays demonstrated equal capability of WT and NFAT2-deficient iTreg (Fig. S5*A*). When allo-specific iTreg were generated in vitro—hindering differentiation upon NFAT2 deficiency—and transferred in adjusted numbers, NFAT2$^{-/-}$ iTreg were at least as functional as WT iTreg in the model of skin transplantation (Fig. S5 *B–E*). This verified the functional equivalence of in vivo generated WT and *Nfat2*$^{-/-}$ iTreg (Fig. 3).

nTreg Function Independently of Individual NFAT Members. If iTreg operate with markedly reduced NFAT levels, the same might apply to nTreg. NFAT1$^{-/-}$ plus NFAT4$^{-/-}$ nTreg are functional in vitro (14). We tested whether NFAT2 is necessary for the repressor function of nTreg. Nonfunctional nTreg would lead to spontaneous autoimmunity, but peripheral lymphoid organs in 6-mo-old *Nfat2*$^{fl/fl}$ × *Cd4-cre* and *Nfat1*$^{-/-}$ × *Nfat2*$^{fl/fl}$ × *Cd4cre* mice were without any pathological findings (Fig. S6 *A–E*). To exclude that frequency and number of nTreg had recovered with aging (22, 23), we analyzed Treg development in neonates. The ratio of both thymic and splenic CD4 vs. CD8 exhibited some peculiarities, but frequency of Foxp3$^+$ T cells was unaltered upon deficiency of NFAT1 plus NFAT2 (Fig. S7). Furthermore, in vitro suppression assays revealed no functional dependence

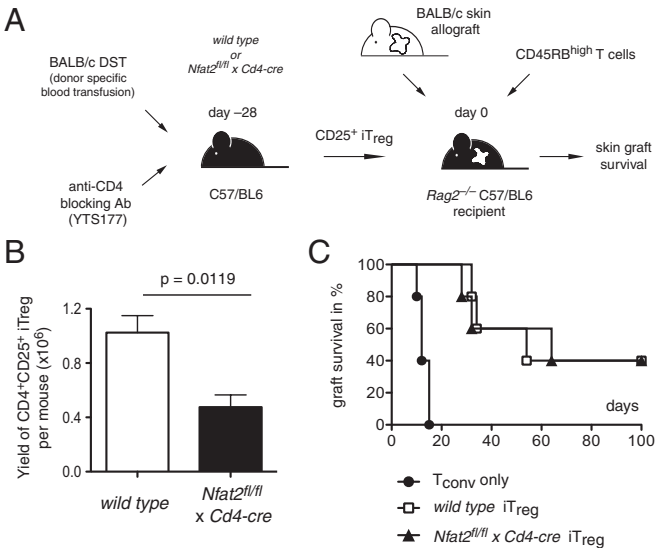

Fig. 3. Reduced iTreg generation but normal suppressive capacity in NFAT-deficient cells in vivo. (*A*) Schematic overview of adoptive transfer of B6 WT or *Nfat2*$^{fl/fl}$ × *Cd4-cre* in vivo-induced iTreg along with CD4$^+$CD45RB^{hi} cells into a BL6 *Rag2*$^{-/-}$ recipient mouse, receiving a BALB/c skin allograft. Induction of CD4$^+$CD25$^+$ iTreg by BALB/c DST along with anti-CD4 YTS177.9 in vivo, 28 d before adoptive transfer. (*B*) Yield of CD4$^+$CD25$^+$ Treg from DST/YTS177.9-treated WT or *Nfat2*$^{fl/fl}$ × *Cd4-cre* mice by FACS sorting ($n > 5$). (*C*) 2 × 10^5 Treg from WT or *Nfat2*$^{fl/fl}$ × *Cd4-cre* mice along with 2 × 10^5 CD4$^+$CD45RB^{hi} T cells were injected i.v., and BALB/c skin allograft survival in the *Rag2*$^{-/-}$ recipient mouse was measured by log–rank test. All mice receiving only CD4$^+$CD45RB^{hi} cells acutely rejected their skin transplants [$n = 5$, mean survival time (MST) = 12.8 d]. Addition of WT CD4$^+$CD25$^+$ Treg or *Nfat2*$^{fl/fl}$ × *Cd4-cre* Treg could prolong skin graft survival to the same extend (WT: $n = 5$, MST = 64.0 d; *Nfat2*$^{fl/fl}$ × *Cd4-cre*: $n = 5$, MST = 64.8 d).

on NFAT2 (Fig. S8*A*). nTreg can be activated and expanded by superagonistic CD28-specific mAb (CD28SA; D665) in mice (24, 25). Injecting CD28SA into mice with Treg-specific inactivation of NFAT2 (*Nfat2*$^{fl/fl}$ × *FIC*; Fig. S1*E*) evoked comparable expansion of nTreg to WT mice after 3 d (Fig. S8*B*). To address the functional capacity of NFAT2-deficient nTreg in vivo, serum levels of IL-2 were measured after 3 h of CD28SA injection. If Treg were functionally impaired, an increase in IL-2 production should be evident. Indeed, when Treg are completely depleted, 10 times more IL-2 is secreted (25, 26). The similar low amount of IL-2 produced from suppressed effector T cells in both genotypes of mice revealed functional equivalence between WT and NFAT2-deficient nTreg (Fig. S8*C*).

For therapeutic interest, acceptance after adoptive transfer of Tconv together with nTreg was evaluated. We transferred CD25^{hi} nTreg from WT or *Nfat2*$^{fl/fl}$ × *Cd4-cre* together with CD45RB^{hi} Tconv from WT animals into *Rag2*$^{-/-}$ mice (cells and mice C57/BL6) that received a skin graft from allogeneic BALB/c mice (Fig. S8*D*). nTreg from NFAT2-deficient mice were as efficient as WT nTreg to ensure survival of skin allografts (Fig. S8*E*). In summary, nTreg lacking NFAT2 (or NFAT1 plus NFAT2 and NFAT1 plus NFAT4) do not show any impairment in their suppressor function.

To address the question of overall functional equivalence of NFAT-deficient nTreg or iTreg, we analyzed surface markers associated with suppressor function. Subtle, but no major differences could be observed ex vivo or after 3 d of stimulation, whereas CD25 was reduced to levels of activated Tconv exclusively on NFAT1 plus NFAT2-deficient iTreg after 4 d (Fig. S9 *A–C*). Unbiased microarray experiments were carried out with RNA from nTreg and Tconv +/− TGF-β (Fig. S9*D*). Microarray data are available under the accession number E-MEXP-12345 on the ArrayExpress database, www.ebi.ac.uk/arrayexpress. To evaluate direct target genes cells had only been stimulated for 24 h. Evaluation focused on genes potentially regulated by NFAT: Foxp3-complexes (6, 27) and revealed a rather mild influence of NFAT1 plus -2 double-deficiency, although IL-2 expression was strikingly diminished (Fig. S9*E*).

Impaired Nuclear Translocation of NFAT1 and NFAT2 in Treg. Given our finding that the function of Foxp3^{+} Treg does not require high levels of NFAT activity, we compared NFAT expression and activation in Tconv vs. Treg. Our previous data from murine nTreg (15) (Fig. S3*G*) were extended to human CD4^{+} Tconv and CD4^{+}CD25^{+} nTreg, which were analyzed for nuclear and cytoplasmic proteins. Two isoforms of human FOXP3 (28) were detected in the nucleus of isolated nTreg, but also to a limited extent in Tconv (Fig. 4*A*). The strong staining for Galactin-10 verified the identity of human nTreg (29). In Tconv NFAT proteins were both cytoplasmic and nuclear, where stimulation promoted expression and nuclear transportation. However, the overall levels of both nuclear and cytoplasmic NFAT1 and especially NFAT2 were strongly diminished in human nTreg compared with Tconv. Analyzing murine Foxp3^{+} Treg, the overall levels of both nuclear and cytoplasmic NFAT1 and NFAT2 proteins appeared less in both types of Treg compared with Tconv (Fig. 4 *B* and *C*). Quantitation demonstrated reduced nucleo-cytoplasmic ratios of NFAT2 in CD4^{+}CD25^{+} compared with CD4^{+} T cells, as well as less than 20% Foxp3^{+} nTreg and only 25–30% Foxp3^{+} iTreg positive with robust nuclear NFAT2 (Fig. S10 *A–D*). Performing a time course revealed that indeed nuclear translocation of NFAT2 is less in nTreg (Fig. S10 *E* and *F*), reflected by slightly

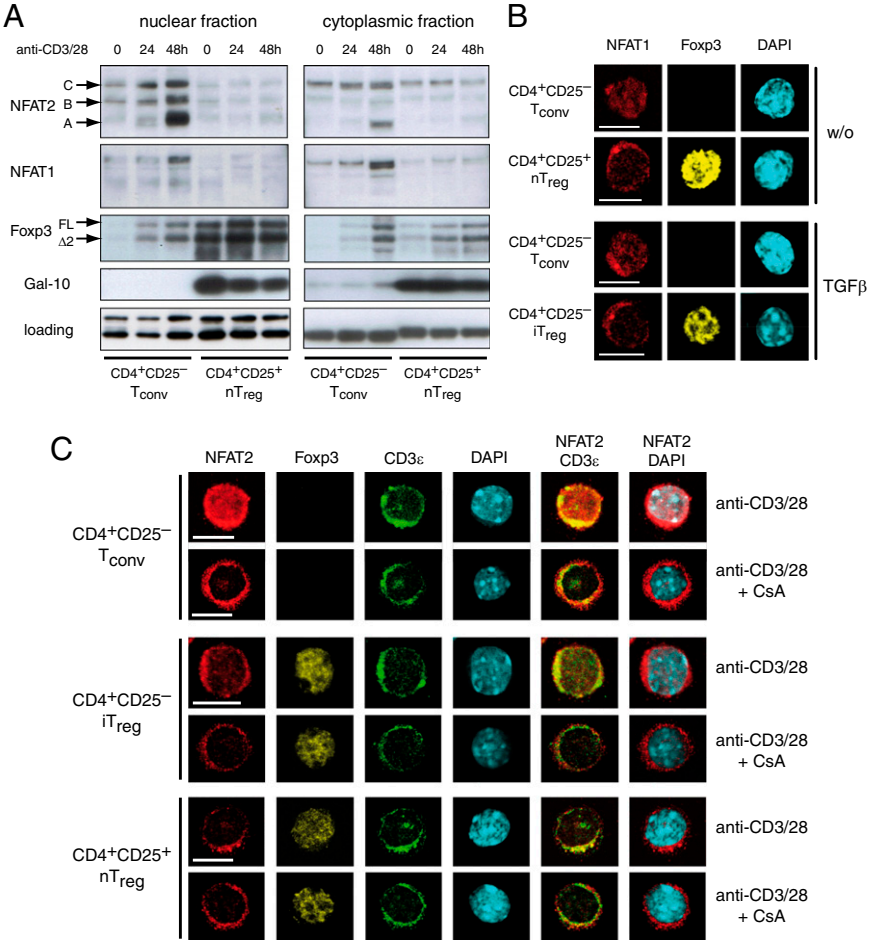

Fig. 4. Impaired nuclear translocation of NFAT in Foxp3^{+} iTreg and nTreg. (*A*) Immunoblot analysis of NFAT2 and NFAT1 in nuclear (*Left*) and cytosolic (*Right*) fractions of human CD4^{+}CD25^{-} Tconv and CD4^{+}CD25^{+} nTreg. Cells were left unstimulated (0 h) or stimulated for 24 h or 48 h with anti-CD3/28 before lysis. Arrows indicate isoforms of NFAT2 and (human) Foxp3, as loading control laminin (*Left*) and actin (*Right*) is given. (*B*) Three-color staining of NFAT1 (red), Foxp3 (yellow), and chromatin (cyan) of freshly isolated CD4^{+} Tconv and CD4^{+}CD25^{+} nTreg stimulated for 6 h with anti-CD3/28 (*Upper*). Foxp3^{-} Tconv and TGF-β–induced Foxp3^{+} iTreg generated from CD4^{+}CD25^{-} Tconv after 24 h stimulation with anti-CD3/28 (*Lower*). (*C*) Four-color staining of NFAT2 (red), Foxp3 (yellow), CD3ε (green), and chromatin (cyan) of CD4^{+}CD25^{-} Tconv, CD4^{+}CD25^{-} TGF-β–induced iTreg, and CD4^{+}CD25^{+} nTreg. Cells were stimulated with anti-CD3/28 in absence (Tconv) or presence (iTreg) of TGF-β and IL-2 for 3 d, followed by 4 d resting. Restimulation for 6 h with anti-CD3/28. As a negative control, to avoid nuclear translocation of NFAT2, 10 nM CsA was used.

less calcium flux (Fig. S10*G*) (30). Taken together, the low amount of NFAT present in nTreg and iTreg resembles their functional integrity upon loss of one or two NFAT family members.

Discussion

The data presented here document dependency of iTreg on NFAT transcription factors to develop and especially to express Foxp3. However, once differentiated, in vitro or in vivo, they can exert their suppressor function with severely reduced levels of NFAT. Accordingly, Foxp3$^+$ iTreg express and activate less NFAT compared with peripheral CD4$^+$ Tconv. The reduced level and activity of NFAT is common to both nTreg and iTreg from mice and men and is consistent with our finding that also nTreg do not primarily depend on NFAT expression for suppressor function.

TCR engagement is mandatory for all differentiation events of CD4$^+$ T cells toward lineages or subsets. It starts numerous signaling cascades, including calcium mobilization and subsequent nuclear translocation of NFAT. Together with cytokine-induced and other transcription factors, NFAT forms complexes at several loci of lineage-determining target genes encoding cytokines, cytokine receptors, and key regulatory transcription factors. In a murine thymoma cell line, cooperative binding of NFAT and Smad3 to the enhancer1/CNS1 upon anti-CD3/28 plus TGF-β treatment was demonstrated (13). This is in line with histone acetylation (13) and demethylation of CpG residues (31) at this site upon activation of CD4$^+$ T cells in the presence of TGF-β.

To address the in vivo situation, we tested different NFAT-deficient mouse lines for their ability to develop Foxp3$^+$ Treg. Our data prove that peripheral CD4$^+$ T cells are dependent on NFAT for Foxp3 induction in response to TGF-β. It is mediated by NFAT binding to enhancer1/CNS1 but not to CNS3 or the promoter of *Foxp3*. This supports the identification of CNS1 as the *cis*-regulatory element responsible for Foxp3 induction in peripheral T cells (8). Therefore, promoter occupancy of NFAT is a distinct feature of human effector T cells (32), which—unlike murine Tconv—transiently express some FOXP3 after TCR stimulation.

CNS3 has recently been recognized as being initially responsible for Foxp3 expression in nTreg via the recruitment of a c-Rel enhanceosome (8, 33). This might explain why the severe reduction in NFAT level does not hamper the development of nTreg, but not why their development is crucially disturbed, when Ca^{2+} flux or calcineurin are blocked, which are involved in NFAT activation (10). Unexpectedly, *Nfat1*$^{-/-}$ and *Nfat1*$^{-/-}$ × *Nfat4*$^{-/-}$ mice exhibited normal nTreg development (14). Likewise, using newly created conditional NFAT2-deficient mice, we demonstrate here that NFAT2 deficiency and even NFAT1 plus NFAT2 double-deficiency leaves thymic development of nTreg untouched. We conclude that no individual NFAT member is necessary for Foxp3 induction in nTreg, and even deletion of two out of the three NFAT proteins has no discernible effect. Of note, the data gathered with EL-4 cells (13) do not resemble the thymic situation. In line with a high dependency on NFAT for the generation of iTreg, but not nTreg, a mouse strain with hyperactivatable NFAT1 gives rise to more iTreg—and even less nTreg (34). The apparent contradiction between impaired nTreg development in Ca^{2+} signaling-deficient mice vs. the normal nTreg in NFAT-deficient mice can be attributed to two, not mutually exclusive, hypotheses. First, in thymocytes the threshold level of NFAT activity necessary for Foxp3 expression may be very low: in *Nfat1*$^{-/-}$ × *Nfat4*$^{-/-}$ the constitutively expressed long isoforms of NFAT2 (35) or in NFAT1 plus NFAT2 doubly-deficient T cells the amount of NFAT4 could be sufficient to support Foxp3 expression. This is at least in sharp contrast to the periphery, where the loss of two NFAT members abolishes Foxp3 induction. Second, additional pathways such as for AP1 and NF-κB induction are Ca^{2+}/calcineurin-dependent (36). For example, calcineurin controls the formation of the CARMA1/BCL10/MALT1 complex during TCR-induced NF-κB activation (37, 38), whereas mathematical calculations provide evidence that NF-κB is activated at lower Ca^{2+} oscillation frequencies than NFAT (39). This is consistent with the observation that severely reduced calcium flux as observed in *Stim1*$^{fl/fl}$ × *Cd4-cre* allows regular development of nTreg, albeit Stim1/2 double-deficiency conducting an almost complete block of calcium flux abrogates thymic Treg development (40). Furthermore, development of nTreg—in contrast to iTreg—requires high-affinity/avidity TCR interactions in conjunction with costimulatory signals through CD28. In comparison, iTreg differentiate under suboptimal TCR stimulation without the necessity of CD28 costimulation (41). Most likely, this leads to activation of NFAT, but less NF-κB, reminiscent of anergy induction in peripheral CD4$^+$ T cells (42) and in agreement with a crucial impact of NFAT on peripheral iTreg differentiation. In thymus, however, $Ca2^+$/calcineurin might result in the essential c-Rel/NF-κB activation.

Our data reveal reduced NFAT activity in established iTreg and nTreg, which has been suggested for human IL-4 induced Treg (43) and because CsA treatment does not abolish suppressive activity of nTreg (44). This is in agreement with the functional intactness of nTreg and iTreg being single or double-deficient for NFAT members. Likewise, TCR-proximal signaling like Ca^{2+} influx is impaired in nTreg (30, 45). It unravels an unexpected view of Foxp3–NFAT interactions analyzed in detail for NFAT1 (6): if Foxp3 does not depend on NFAT to build a repressesome, it might interact to inhibit NFAT actions, which would be unfavorable for Treg. In line, recent data demonstrate that only a subset of genes is dependent on the interaction of Foxp3 with NFAT, whereas Foxp3–Foxp3 homodimers predominate repression (27). On the other hand, Foxp3 might act on additional levels, like repressing translocation and NFAT2 expression (45, 46), again entailing the implication that high NFAT activity has to be avoided for Treg function.

In conclusion, combined deletion of two of the three NFAT family members expressed in T cells barely impairs Treg suppressive activity, indicating that either minimal levels of NFAT activity suffice for regulatory function or that suppressive capacity is even independent of NFAT. This is of exciting importance for transplant therapy and treatment of autoimmune diseases. Instead of the calcineurin inhibitors CsA and FK506, new therapeutics like R11-VIVIT (47) and MCV1 (48) should be clinically improved. Those would reduce NFAT activation specifically, thereby functionally inhibiting proinflammatory Tconv but not Treg suppression.

Materials and Methods

Mice and Cells. *Nfat2*$^{fl/fl}$ animals were generated in A. Rao's laboratory (Harvard Medical School, Boston, MA). Nfat1$^{-/-}$ × Nfat4$^{-/-}$, B6-Tg (Cd4-cre) 1Cwi/Cwilbcm (European mouse mutant archive, Rome, Italy) and Foxp3-IRES-cre have been described previously (16, 49, 50) (*SI Materials and Methods*).

Antibodies, Reagents, and Media. *SI Materials and Methods* gives clone numbers, provider, and concentrations used.

T-cell Subsets. Human T-cell subsets (29, 51) and murine CD4$^+$CD25$^+$ nTreg and CD4$^+$CD25$^-$ Tconv (26) were isolated and stimulated as before. Details can be found in *SI Materials and Methods*.

Immunofluorescence and Immunoblot. See refs. 15 and 26. The following primary antibodies were used: anti-NFAT2 (7A6; BD Pharmingen), anti-NFAT1 (IG-209; immunoGlobe), anti-Smad3 (ab28379; Abcam), and anti-Foxp3 (FJK-16s; eBiosciences), anti-NFAT4 (F-1; Santa Cruz Biotechnology), and anti–β-actin (C4; Santa Cruz Biotechnology). Extended protocols are given in *SI Materials and Methods*.

ChIP Analysis. ChIP-IT Express kit (Active Motif) was used with enzymatic shearing followed by additional sonication. IP-Ab: anti-NFAT2 (7A6; BD Pharmingen), anti-Foxp3 (FJK-16s, eBioscience), and anti-Smad3 (Abcam). Primers are given in *SI Materials and Methods*.

Adoptive Transfer Colitis and Endoscopy. Colitis was induced in *Rag1*$^{-/-}$ mice by injecting i.p. 2.5 × 10^5 *Cd90.1*$^+$ (WT) and 2.5 × 10^5 *Cd90.2*$^+$ (WT or *Nfat2*$^{fl/fl}$ × *Cd4cre*) CD4$^+$CD62L$^+$CD25$^-$ cells. Scoring (52) is described in *SI Materials and Methods*.

Skin Transplant Model. To test alloantigen-induced or nTreg in vivo (20, 21), mice received 200 μg of anti-CD4 YTS177.9 mAb (Bio-Xcell) i.v. on day −28/−27. On day −27 the mice also received 250 μL DST from BALB/c mice. $CD4^+CD25^+$ T cells were FACS-sorted on day 0, and C57BL/6 *Rag2*$^{-/-}$ mice were reconstituted i.v. with 2×10^5 C57BL/6 $CD4^+CD45RB^{hi}$ cells along with 5×10^5 $CD4^+CD25^+$ nTreg cells isolated from naïve or 2×10^5 $CD4^+CD25^+$ Treg cells from YTS177/DST-pretreated mice. Next day, BALB/c tail skin allografts were transplanted onto flanks of reconstituted mice.

Statistical Analysis. Groups were compared with Prism software (GraphPad) using two-tailed Student's *t* test.

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