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Constitutive Nuclear Localization of NFAT in Foxp3+ Regulatory T Cells Independent of Calcineurin Activity

Qiuxia Li,*† Arvind Shakya,*,† Xiaohua Guo,*,† Hongbo Zhang, † Dean Tantin, * Peter E. Jensen,* and Xinjian Chen*

Foxp3 plays an essential role in conferring suppressive functionality to CD4+/Foxp3+ regulatory T cells (Tregs). Although studies showed that Foxp3 has to form cooperative complexes with NFAT to bind to target genes, it remains unclear whether NFAT is available in the nucleus of primary Tregs for Foxp3 access. It is generally believed that NFAT in resting cells resides in the cytoplasm, and its nuclear translocation depends on calcineurin (CN) activation. We report that a fraction of NFAT protein constitutively localizes in the nucleus of primary Tregs, where it selectively binds to Foxp3 target genes. Treating Tregs with CN inhibitor does not induce export of NFAT from the nucleus, indicating that its nuclear translocation is independent of CN activity. Consistently, Tregs are resistant to CN inhibitors in the presence of IL-2 and continue to proliferate in response to anti-CD3 stimulation, whereas proliferation of non-Tregs is abrogated by CN inhibitors. In addition, PMA, which activates other transcription factors required for T cell activation but not NFAT, selectively induces Treg proliferation in the absence of ionomycin.

The finding of a requirement for in vitro activation to induce Foxp3 binding to its target genes has raised a fundamental question regarding the role of Foxp3 in conferring the Treg phenotype (27, 28). The phenotype of the naturally occurring Tregs, such as expression of high levels of CD25 and CTLA-4 and inability to produce IL-2, is constitutive, requiring no in vitro activation. If Foxp3 plays an essential role in conferring the Treg phenotype (28, 29), Foxp3 binding to at least some targets should be constitutive. In addition, studies suggested that NFAT is required for Foxp3 expression (30). Consistent with these observations, Zheng et al. (10) found that Foxp3 is constitutively bound to ~700 target sequences in nuclear Foxp3 complexes. However, it remains unclear how NFAT becomes accessible to Foxp3 in the nucleus of Tregs; as in resting cells, NFAT proteins are located in the cytoplasm where they are heavily phosphorylated through synergistic actions of three kinase families: casein kinase 1, glycogen synthase kinase 3, and dual-specificity tyrosine phosphorylation-regulated kinase (17–19). Phosphorylation results in masking of a nuclear localization sequence, exposure of a nuclear export sequence, and NFAT cytoplasmic localization. When cells are stimulated, intracellular Ca2+ concentrations increase, and the calmodulin-dependent phosphatase calcineurin (CN) is activated, which dephosphorylates NFAT, exposing the nuclear localization sequence and masking the nuclear export sequence, resulting in nuclear translocation (20, 21). When the stimulus is removed, nuclear NFAT is rapidly exported back to the cytoplasm as a result of the constitutive kinase activity that rephosphorylates NFAT (22–24). Thus, in resting/nonstimulated cells, the equilibrium of the dephosphorylation/rephosphorylation and nuclear import/export favors rephosphorylation and cytoplasmic NFAT localization (25). Consistent with this notion, it was reported that, in Foxp3-transduced or -cultured Tregs, the binding of Foxp3/NFAT complexes to Foxp3 target genes is observed only upon stimulation with PMA plus ionomycin or ionomycin alone (16). Other studies using Foxp3-transfected T cells or T cell lines also found that changes in expression of Foxp3-bound genes, such as Il2, Ctla4, and Il2ra, occurred only upon TCR stimulation in a CN-dependent manner (11, 26).

The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; CN, calcineurin; CsA, cyclosporine A; GVHD, graft-versus-host disease; MHCII, MHC class II; nTreg, naturally occurring regulatory T cell; Treg, regulatory T cell.

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genes in freshly isolated, nonstimulated naturally occurring Tregs. These latter findings suggest that Foxp3-transduced cells or cell lines may differ from primary naturally occurring Tregs in terms of the status of Foxp3 binding to its target genes. Given the Foxp3-containing cooperative complexes that require NFAT for DNA binding, the constitutive binding of Foxp3 suggests that NFAT might be present in the nucleus of primary, naturally occurring Tregs.

In this study, we examined NFAT localization in freshly isolated primary Tregs and found that a fraction of NFAT was constitutively localized in the nucleus where it selectively binds to Foxp3 target genes. NFAT nuclear translocation was independent of CN activity, and Tregs are consistently resistant to CN inhibition. In addition, Tregs can be selectively activated and induced to proliferate from a mixed cell population, dominated by non-Tregs, by phorbol ester in the absence of CN activator ionomycin. Our findings provide important insight into Treg biology.

Materials and Methods

Western blot analysis

CD25 and CD25− CD4 T cells were isolated from splenocytes using the CD25+CD4+ Regulatory T cell Isolation Kit (Miltenyi Biotec), following the manufacturer’s instructions, as previously described (31). To isolate Foxp3+GFP+ cells, CD4+ T cells were first isolated from splenocytes of Foxp3EGFP mice (32) by negative selection using a CD4 T Cell Isolation Kit (Miltenyi Biotec), followed by sorting of GFP+ cells by FACS (33). All of the isolated cells were rested in culture with cyclosporine A (CsA) for 30 min before being processed for protein extracts. For whole-cell lysates, isolated cells were lysed with radioligand immunoprecipitation assay buffer (50 mM NaCl, 25 mM Tris [pH 7.5], 1 mM EDTA, 0.1% SDS, 1% w/v deoxycholate, and 1% Nonidet P-40) 0.5 μM phenylmethylsulfonyl fluoride or phenylmethylsulfonyl fluoride plus protease inhibitor Complete Mini Pill (Roche) per 10 ml lysis buffer. Nuclear and cytoplasmic extracts were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific), following the manufacturer’s instructions, except that CER I and CER II were diluted with PBS at 1:1 and 1:4 ratios, respectively, to avoid overlysis. A total of 0.5–2 × 106 cell equivalents of protein was loaded per lane. Western blots were revealed with Abs specific for NFATc1, NFATc2 (both from Thermo Scientific), c-Rel, and c-Fos (both from Santa Cruz Biotechnology).

Chromatin immunoprecipitation–PCR analysis

Foxp3+GFP+ Tregs and Foxp3− GFP− non-Treg CD4 T cells were isolated from Foxp3EGFP mice by FACS sorting and rested in culture. A portion of the non-Tregs was stimulated with PMA plus ionomycin for 30 min completely exports the nuclear NFAT to the cytoplasm (23, 25). Resting the Foxp3+/GFP+ cells did not export nuclear protein (77), were used as markers for cytoplasmic and nuclear fractions, respectively. Foxp3+/GFP+ CD4 T cells were rested (Fig. 1, lanes 2), or treated with 1 μM CsA in culture for 30 min (lane 8), and their nuclear extracts were blotted for NFATc1, tubulin, and MCM2. The results are representative of multiple experiments.

Results

A fraction of NFAT protein is constitutively localized in the nucleus of primary Tregs independent of CN activity

To examine subcellular NFAT localization in primary Tregs, whole-cell lysates and cytoplasmic and nuclear fractions were prepared from CD25+ and CD25− CD4 T cells freshly isolated from spleens and lymph nodes of B6 or BALB/c mice. The samples were analyzed by Western blot for NFATc1 and NFATc2, along with c-Fos and c-Rel, which are the components of two other major transcription factors AP-1 and NF-κB (Fig. 1). The total quantities of NFATc1, NFATc2, and c-Fos (AP-1) in whole-cell lysates were similar between the CD25+ and CD25− T cell subsets, but ~2–3-fold more c-Rel protein was detected in CD25+ cells compared with CD25− cells in multiple experiments (Fig. 1, lanes 1 and 2). In CD25+ cells, all four transcription factors were detected in the cytoplasm but not the nucleus (Fig. 1, lanes 3 and 6), as previously reported (23). Similarly, in the CD25− cells, NFATc1, NFATc2, c-Rel, and c-Fos proteins were also largely present in the cytoplasm (Fig. 1, lane 4). More c-Rel protein was identified in the cytoplasm in the CD25+ CD4 T cells compared with the CD25− CD4 T cells, consistent with the results from whole-cell lysates (Fig. 1, lane 2). However, a trace amount of c-Rel protein was repeatedly detected in the nucleus of the CD25+, but not the CD25−, cells (Fig. 1, lanes 5 and 6). In addition, a fraction of NFATc1 (20–40%) and NFATc2 (10–30%) proteins was also detected in the nucleus of the CD25+, but not the CD25−, CD4 T cells in multiple experiments (Fig. 1, lanes 5 and 6). The presence of NFAT and c-Rel protein in the nuclear extracts was not the result of cytoplasmic contamination, because tubulin, a cytoplasmic protein, was not detected in the nuclear extracts, although it was detected in the cytoplasm extracts and whole-cell lysates (Fig. 1).

To rule out the possibility that the nuclear translocation of NFAT in CD25+ cells was the result of inadvertent activation of the cells by the anti-CD25 Ab used during cell isolation, Western blot analysis was also performed with Tregs isolated from the Foxp3+/ GFP+ mice (32) using a fluorescent cell sorter and no anti-CD25 Abs. The flow-sorted Foxp3+/GFP+ cells were rested in culture for 30 min before being processed for preparation of nuclear extracts. Previous studies showed that resting activated non-Treg cells in culture for 30 min completely exports the nuclear NFAT to the cytoplasm (23, 25). Resting the Foxp3+/GFP− cells did not export nuclear NFAT.

FIGURE 1. Western blot analysis of transcription factors in CD25+ and CD25− CD4 T cells. Whole-cell lysates (lanes 1 and 2), cytoplasm (lanes 3 and 4), and nuclear extract (lanes 5 and 6) of isolated CD25+ (neg) and CD25+ (pos) CD4 T cells were analyzed by Western blot for the presence of NFATc1, NFATc2, c-Rel, and c-Fos. Tubulin and MCM2, a constitutive nuclear protein (77), were used as markers for cytoplasmic and nuclear proteins, respectively. Foxp3+/GFP+ CD4 T cells were rested (lane 7) or treated with 1 μM CsA in culture for 30 min (lane 8), and their nuclear extracts were blotted for NFATc1, tubulin, and MCM2. The results are representative of multiple experiments.
NFAT from the nucleus (Fig. 1, lane 7, Supplemental Fig. 1a), suggesting that the nuclear localization of a fraction of the NFAT in the Tregs is unlikely the result of inadvertent activation of the cells during cell isolation.

Calcineurin (CN) plays an essential role in dephosphorylating NFAT to induce nuclear translocation in non-Tregs (25). To determine whether NFAT nuclear localization in the Tregs was driven by continuous CN activity, the Foxp3+/GFP+ cells were treated with the CN inhibitor CsA for 30 min before nuclear extract preparation. Previous studies demonstrated that CsA completely abrogates CN activity and induces the export of NFAT from the nucleus, even when the T cells are in culture with the Ca²⁺ ionophore, ionomycin (23). However, treating Foxp3+/GFP+ Tregs did not mobilize the nuclear NFAT proteins out of the nucleus (Fig. 1, lane 8, Supplemental Fig. 1b). These results demonstrate that in Tregs, a fraction of NFAT, as well as a trace amount of c-Rel protein, is constitutively translocated in the nucleus and that the constitutive nuclear localization of NFAT is independent of continuous CN activity.

To directly visualize NFAT subcellular localization, confocal microscopy was performed on FACS-sorted Foxp3+/GFP+ and Foxp3+/GFP+ CD4 T cells (Supplemental Fig. 2). In Foxp3+/GFP+ CD4 T cells, NFAT was visualized only in the cytoplasm (Supplemental Fig. 2b, 2c). However, in Foxp3+/GFP+ Tregs, NFAT was seen both in the cytoplasm and nucleus (Supplemental Fig. 2d). Treating Foxp3+/GFP+ cells with CsA did not induce export of nuclear NFAT (Supplemental Fig. 2e), indicating that its nuclear translocation is independent of CN activity. Culturing Foxp3+/GFP+ CD4 T cells with ionomycin resulted in NFAT nuclear translocation (Supplemental Fig. 2f). However, when CsA was added to the ionomycin-containing culture, all NFAT protein was seen only in the cytoplasm (Supplemental Fig. 2g), indicating that CsA abrogates CN-dependent NFAT nuclear translocation in non-Tregs, despite the continuous presence of a CN-activating signal. Therefore, the results of confocal microscopy are consistent with the results of Western blots, demonstrating that, in Tregs, a fraction of NFAT proteins is constitutively localized in the nucleus in a CN-independent manner.

NFAT is selectively bound to Foxp3 target genes in Tregs

In activated T cells, NFAT complexes with AP-1 to bind to composite NFAT–AP-1 DNA sites (22, 42). Because no c-Fos (AP-1) protein was detected in the nucleus of Tregs (Fig. 1), we examined whether the nuclear NFAT in Tregs was bound to any DNA targets. ChIP was performed with anti-NFATc1 and anti-NFATc2 Abs to obtain NFAT-bound DNA from freshly isolated and rested Foxp3+/GFP+ CD4 Tregs. For positive and negative controls, anti-NFAT ChIP DNAs were obtained from freshly isolated Foxp3+/GFP+ CD4 non-Tregs, with and without phorbol ester PMA/ionomycin activation. The three ChIP DNA samples were then subjected to PCR amplification with 10 primer sets: 5 specific for the NFAT binding sites of genes previously identified as Foxp3 targets [the promoters of Il2, Irf4f, mir155, Fasl, and Pde3b-20 (first intron of Pde3b) (10)] (Fig. 2A–E) and 5 specific for genes not identified as Foxp3 targets (10) [the promoters of Il4 (35, 36), Il12a (37–39), Il17 (40), Pde3b, and an enhancer of Il4] (Fig. 2F–J). The PCR products were analyzed by gel electrophoresis. No PCR products were generated with any of the primer sets from ChIP DNA samples prepared with the isotype control Abs (except for Pde3b-20, for which weak background PCR products were generated from control Ab-precipitated DNA), ensuring the validity of the assay (Fig. 2, left column of each nine-unit panel). In contrast, positive PCR products were obtained with all primer sets using input DNA samples, which serve as positive controls for the primers (Fig. 2, right column of each nine-unit panel).

No PCR product was detected from ChIP DNA isolated from rested Foxp3+/GFP+ CD4 T cells with any of the 10 primer sets (Fig. 2, top middle grid of each nine-unit panel), consistent with the Western blot results (Fig. 1) and previous reports (23) that, in resting non-Tregs, NFAT is localized in the cytoplasm and not bound to DNA. In contrast to the resting non-Tregs, robust PCR amplimers were generated from ChIP DNA isolated from rested Foxp3+/GFP+ CD4 Tregs using primer sets specific to Foxp3 target genes, including Il2, Irf4, mir155, and Pde3b-20 (Fig. 2A–C, 2E, middle row), indicating that NFAT is constitutively bound to these four genes. No amplimers were generated from the Fasl promoter, although it was also identified as a Foxp3 target in a previous study (10). Despite prevalent binding to the Foxp3 targets, NFAT was not bound to any of the five tested non-Foxp3 target genes in Tregs, as indicated by the lack of DNA amplimers using the primer sets specific for these five NFAT binding sites (Fig. 2F–J, middle row), which were Il4, Il12a, Il17, and Pde3b promoters and the Il4 enhancer. The lack of PCR products from the non-Foxp3 binding sites was not due to the inability of NFAT to bind these sites or of the PCR primers to amplify these regions from ChIP DNA, because all five primer sets generated robust PCR products.
from ChIP DNA samples isolated from PMA/ionomycin-activated non-Tregs (Fig. 2F–J, bottom row). In addition to the non-Foxp3 targets, NFAT bound to three of the Foxp3 targets, including the promoters of Il-2, Irf4, and Pde3b-20 in PMA/ionomycin-activated non-Tregs (Fig. 2A, 2B, 2E, bottom row). NFAT binding to Foxp3 targets in activated non-Tregs is consistent with the previous report showing NFAT/Foxp3 binding to the Il2 promoter in Tregs (16) but NFAT/AP-1 binding in activated T cells (25). The forkhead protein-binding sequence resembles the consensus AP-1 binding site (43). In activated non-Tregs, NFAT and AP-1, along with other transcription factors, are translocated into the nucleus; as a result, NFAT can form a cooperative complex with AP-1 to bind to Foxp3 target sites in the absence of Foxp3. ChIP analysis was also performed using anti-NFATc1 or anti-NFATc2 Ab separately on resting Foxp3/GFP+ and Foxp3/GFP− CD4 T cells using PMA/ionomycin-activated Foxp3/GFP− CD4 T cells as control. The ChIP-DNA samples were analyzed by quantitative PCR, and the results (Supplemental Fig. 3) were in complete agreement with those obtained with the two Abs combined. Both NFATc1 and NFATc2 in Foxp3/GFP+ Tregs bound to three of the four tested Foxp3 targets (Il2, Irf4, and mrl155 but not Fast or the non-Foxp3 target Pde3b). In contrast, neither NFATc1 nor NFATc2 in Foxp3/GFP− non-Tregs bound to any of the tested genes before activation. Only after activation did NFATc1 and NFATc2 differentially bind to the DNA targets (Supplemental Fig. 3). These results further confirm constitutive NFAT nuclear localization and selective binding to Foxp3 targets in primary Tregs.

Taken together, the results of ChIP-PCR analysis demonstrate that NFAT in primary Tregs is constitutively bound to some Foxp3 targets but none of the tested non-Foxp3 targets. These results are consistent with the Western blot results (Fig. 1), indicating that a fraction of NFAT is constitutively localized in the nucleus of primary Tregs. The differential binding of NFAT to the Foxp3 targets in Tregs is consistent with the idea that nuclear localization of NFAT in Tregs is not the result of inadvertent activation, because NFAT in activated cells binds selectively to different DNA targets. Our finding of constitutive NFAT binding to Foxp3 targets in primary Tregs suggests that primary Tregs are different Foxp3+ GFP− non-Tregs transduced T cells or cultured cell lines, and significant binding of Foxp3 to the DNA targets is detected only after the cells are stimulated in vitro (11, 16, 26).

**Tregs are resistant to CN inhibition in the presence of IL-2**

Full T cell activation is the result of nuclear translocation of a variety of transcription factors, including NFAT, AP-1, and NF-kB (44). Given the results of constitutive NFAT nuclear localization in Tregs independent of CN activity, we next examined whether Treg activation is resistant to CN inhibitors. Foxp3+ GFP+ cells (Fig. 3B) were stimulated with anti-CD3 (2C11) and IL-2 in the absence or presence of the CN inhibitor CsA. CD44+ naive and CD44+ memory non-Tregs (Fig. 3A) were treated similarly and served as controls. In the absence of CsA, all three subsets of T cells proliferated extensively (Fig 3F–H), with the intensity of nuclear NFAT in Tregs independent of CN activity, given the essential role of NFAT in T cell activation and proliferation, these results also suggest a CN-independent mechanism to drive nuclear translocation of NFAT in Tregs to allow the cells to undergo activation and proliferation in the presence of IL-2.

**Tregs can be selectively activated by the phorbol ester PMA in the absence of ionomycin**

Although TCR stimulation is a classic way to activate T cells, T cells can also be activated by the synergistic activity of the Ca2+ channel ionophore ionomycin and a phorbol ester, such as PMA (50). PMA mimics diacylglycerol to activate protein kinase C θ and other protein kinase C θ-unrelated target proteins (51), resulting in activation of transcription factors necessary for T cell
activation other than NFAT. In contrast, ionomycin increases the intracellular Ca\(^{2+}\) concentration to activate NFAT through CN, complementing PMA-induced signaling to give rise to full T cell activation and proliferation (50, 52). PMA alone is incapable of activating primary T cells (53, 54).

The constitutive nuclear localization of NFAT in Tregs and their insensitivity to inhibition by CN inhibitor raised the possibility that Tregs might be selectively activated by stimuli that do not activate NFAT. To test this possibility, unfractionated splenocytes containing \(\sim 5\%\) Foxp3\(^+\) Tregs (Fig. 4A) were cultured with PMA in the presence or absence of ionomycin. As expected, PMA plus ionomycin induced vigorous T cell proliferation composed of Foxp3\(^-\) CD4 and CD8 non-Tregs as early as day 3 (Fig. 4C), whereas PMA alone elicited mild Foxp3\(^+\) CD4 T cell proliferation (Fig. 4B). However, by culture day 5, robust T cell proliferation was observed, deriving primarily from the proliferation of Foxp3\(^+\) CD4 cells, with few proliferative Foxp3\(^-\) non-Treg CD4 T cells (Fig. 4D). Not only did the non-Treg CD4 and CD8 T cells fail to proliferate (Fig. 4D, 4G), they also died during culture, resulting in a continuous decline in the number of Foxp3\(^-\) CD4 and CD8 T cells, whereas the number of Foxp3\(^+\) CD4 cells continued to grow: by 10-fold on culture day 5 (Fig. 4H) and up to 800-fold in 2 wk (data not shown). Conversion of Foxp3\(^-\) cells (inducible Tregs) did not contribute to the expanded Foxp3\(^+\) T cell pool. Congenic cultures of low-frequency CD45.1\(^+\)CD4\(^+\) T cells with a dominant population of CD25-depleted CD45.2\(^+\) splenocytes (Fig. 4I) cultured with PMA resulted in an expanded pool of Foxp3\(^+\) CD4 T cells that were almost exclusively CD45.1\(^+\) (Fig. 4J). Similar to experiments with CsA (Fig. 3O), PMA-induced Treg proliferation depended on paracrine IL-2 produced by the few proliferative non-Treg CD4 T cells (Fig. 4D), because CsA in the absence of exogenous IL-2 diminished Treg proliferation (Fig. 4E). Supplementing the CsA-containing cultures with exogenous IL-2 restored Treg proliferation comparable to cultures without CsA (Fig. 4F). These results further support the findings that PMA-induced Treg activation and proliferation are independent of CN activity but require IL-2.

Some studies reported that the TCRs expressed by Tregs are more self-reactive than are those of non-Tregs (55–57). Because TCR signaling could increase intracellular Ca\(^{2+}\) to activate CN, the chronic TCR interaction of Tregs with self-MHC class II (MHCII) might cause continuous NFAT activation and nuclear...
translocation, enabling Tregs to selectively respond to PMA stimulation in the absence of ionomycin. To determine whether PMA-induced Treg proliferation depends on Treg interaction with self-MHCII, Foxp3⁺GFP⁺ or CD25⁺CD4⁺ T cells were isolated and cultured with PMA and IL-2 in the presence or absence of wild-type or MHCII-deficient APCs. In the absence of APCs, PMA/IL-2 induced only minimal Treg proliferation (Fig. 4K). In contrast, an ∼30-fold expansion of Tregs was obtained when either wild-type or MHCII-deficient APCs were added to the culture (Fig. 4K). These findings indicate that, although a Treg–APC interaction is necessary, MHCII-mediated TCR signaling is not required for PMA-induced Treg proliferation, consistent with the above results that Tregs could be activated in the presence of CN inhibitors (Fig. 3). Because APCs could provide costimulation to Tregs through CD28 to synergize PMA stimulatory effect on Tregs, stimulatory anti-CD28 Ab was added to the culture in place of APCs. This combination of PMA and CD28 stimulation resulted in an ∼18-fold Treg expansion (Fig. 4K), suggesting that part of the APC-derived synergistic stimulatory effect can be delivered through CD28 but independently of TCR stimulation. To examine the role of costimulation, B7.1- and B7.2-blocking Abs were added individually or together to the PMA-stimulated splenocyte culture (Fig. 4L–P). The two blocking Abs nearly abrogated Treg proliferation when added to the culture together (Fig. 4Q, 4R).

**Tregs expanded in the absence of CN activation maintain Treg phenotype and suppressive capacity**

Studies showed that structure-guided mutations of Foxp3 predicted to disrupt its interaction with NFAT disrupt Foxp3 function to upregulate the expression of the Treg markers CTLA4 and CD25 and to confer suppressive function (16). If constitutive NFAT nuclear localization in Tregs depends on CN activity, Tregs expanded in the presence of CN inhibitors or expanded by PMA should lose expression of CTLA-4 and CD25, as well as suppressive function due to interruption of Foxp3–NFAT interaction, because CN inhibitors prevent NFAT nuclear translocation, and PMA does not activate CN. To examine this possibility, freshly isolated primary naturally occurring Foxp3⁺GFP⁺ cells (nTregs) and Tregs expanded by anti-CD3/CD28 in the absence or presence of CsA (Treg/CsA) or expanded by PMA (Treg/PMA) were compared. Expression levels of CTLA-4, CD25, and Foxp3, as well as the suppressive functions, were measured. Both Treg/CsA and Treg/PMA cells expressed slightly higher levels of CTLA-4 (Fig. 5A), CD25 (Fig. 5B), and Foxp3 (Fig. 5C) compared with freshly isolated primary Tregs. The level of CD25 on Treg/CsA cells was also higher than

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FIGURE 4. Flow cytometric analysis of the impact of PMA on Treg and non-Treg proliferation. CFSE-labeled unfractionated splenocytes (A) were cultured with PMA or PMA plus ionomycin for 3 d (B, C), or with PMA (D), PMA plus CsA (E), or PMA plus CsA and IL-2 (F) for 5 d and analyzed by flow cytometry. Cells in (A)–(F) were gated on CD4⁺ cells. (G) CD8⁺ cells in day-5 culture of splenocytes with PMA. (H) Average cell counts of Foxp3⁻ CD4 and CD8 T cells and Foxp3⁺ CD4 cells in splenocyte culture with PMA in triplicates. Data are representative of more than three experiments. CD25⁺ CD4 T cells isolated from CD45.1⁺ B6 mice were added at a 1:10 ratio to CD25-depleted CD45.2⁺ splenocytes (I) and cultured for 5 d (J). Isolated Foxp3⁺GFP⁺ CD4 T cells were cultured with PMA plus IL-2, PMA plus IL-2 and syngeneic APCs, PMA plus IL-2 and MHCII-deficient APCs, or PMA plus IL-2 and anti-CD28 for 5 d. Data represent the average Treg counts in each culture condition in triplicates from three independent experiments. Unfractionated splenocytes (L) were cultured with PMA (M), control (anti-DR) (N), or anti-B7.1/CD80 (1G10) blocking Ab (O), anti-B7.2/CD86 (GL-1) blocking Ab (P), or the two blocking Abs combined (Q) for 5 d and analyzed by flow cytometry. The displayed cells were derived from CD4⁺ gates. (R) Summary of the average absolute number of proliferative Foxp3⁺ cells from culture triplicates.
that of Treg/CD3/CD28 cells, whereas CTLA-4 levels were similar. However, the level of CTLA-4 expressed by Treg/PMA cells was lower than that of Treg/CD3/CD28 cells, whereas the CD25 levels were similar. In contrast, CsA inhibited CD25 upregulation on expanded non-Tregs (Fig. 5P). Consistent with the high-level expression of the Treg markers, both Treg/CsA and Treg/PMA cells were able to suppress anti-CD3-elicited proliferation of non-Treg CD4 and CD8 responder cells with similar efficacy as freshly isolated nTregs (Fig. 5C, 5D). However, Treg/CD3/CD28 cells were much more potent in suppression than were the nTregs and Treg/CsA and Treg/PMA cells (Fig. 5C, 5D). These results demonstrate that Tregs expanded in the absence of CN activation do not lose the prototypic Treg phenotype or suppressive function of primary Tregs, suggesting that the Foxp3–NFAT interaction in Treg/CsA and Treg/PMA cells is not interrupted by CN inhibitors or the lack of CN-mediated activation of NFAT. These results are consistent with the finding that treating Tregs with CsA did not result in the export of NFAT from the nucleus (Fig. 1). Nevertheless, further activation of NFAT through activating CN can enhance Treg suppressive capacity.

Discussion

We show that a fraction of NFATc1 and NFATc2, as well as c-Rel protein, is present in the nucleus of freshly isolated primary Tregs but not non-Tregs. Consistent with this result, ChIP analysis reveals that NFAT in primary Tregs is constitutively bound to genes that are

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**FIGURE 5.** Characterization of Tregs expanded in the presence or absence of CN activation. Foxp3+/GFP+ CD4 T cells expanded with anti-CD3/28 in the presence (black) or absence of CsA (green) or with PMA (red) were compared with primary Tregs (nTregs; blue) for their levels of expression of CTLA-4 (A), CD25 (B), or Foxp3 (C). CFSE-labeled CD25-depleted splenocytes were stimulated with 2C11 in the absence of Tregs (D, E), with nTregs (F, G), with Tregs expanded in the presence of 1 μM CsA (H, I), with PMA-expanded Tregs (J, K), or with Tregs expanded in the absence of CsA (L, M) at a 1:1 Treg/non-Treg CD4 T cell ratio, cultured for 3 d, and analyzed for proliferation of non-Treg CD4 and CD8 T cells by flow cytometry. Proliferation of non-Treg CD4 (N) and CD8 (O) T cells stimulated by 2C11 in the presence of each expanded Treg preparation at various Treg/responder T cell ratios is displayed as fractions relative to the number of proliferative cells in the absence of Tregs (proliferation index). (P) CD25 levels on non-Treg CD4 cells expanded in the presence of absence or absence CsA.
known to be Foxp3 targets, whereas the binding of NFAT to DNA in non-Tregs only occurs after the cells are stimulated. Resting or treating Tregs with CN inhibitor CsA did not induce export of NFAT from the nucleus of the Tregs, suggesting that NFAT nuclear localization in primary Tregs is independent of CN activity. In line with this finding, Tregs are resistant to CN inhibition in the presence of IL-2. Similarly, PMA, which activates transcription factors other than NFAT, selectively activates Tregs to proliferate.

In addition, the Tregs expanded by PMA or by anti-CD3 in the presence of CN inhibitors maintain phenotype and functionality similar to primary Tregs, suggesting that the Foxp3–NFAT interaction is not interrupted by lack of CN activation during proliferation. Taken together, these results indicate that a fraction of NFAT proteins is constitutively present in the nucleus of primary Tregs, independent of CN activity.

The finding of constitutive nuclear NFAT localization is consistent with the reports that, in primary Tregs, Foxp3 is constitutively bound to hundreds of target genes (10) and that the binding of Foxp3 to I22, Cita4, and I22a (CD25) requires Foxp3 to form a cooperative complex with NFATc2 (13, 16). In addition to NFATc2, we detected NFATc1 in the Treg nucleus. It is known that NFATc1, NFATc2, and NFATc3 are coexpressed in T cells, and are all activated in response to TCR stimulation. Although we only examined NFATc1 and NFATc2, given the high degree of similarity among the Rel-homology region of the different NFAT family members and the fact that the Rel-homology region confers common DNA-binding specificities and partner interactions (58), it is possible that multiple members of the NFAT family, including NFATc3, are involved in partnership with Foxp3.

Interestingly, the ChIP analysis revealed that NFAT in Tregs selectively binds to Foxp3 target genes but not to the binding of Foxp3 to Il2, Cita4, and I22a (CD25) requires Foxp3 to form a cooperative complex with NFATc2 (13, 16). In addition to NFATc2, we detected NFATc1 in the Treg nucleus. It is known that NFATc1, NFATc2, and NFATc3 are coexpressed in T cells, and are all activated in response to TCR stimulation. Although we only examined NFATc1 and NFATc2, given the high degree of similarity among the Rel-homology region of the different NFAT family members and the fact that the Rel-homology region confers common DNA-binding specificities and partner interactions (58), it is possible that multiple members of the NFAT family, including NFATc3, are involved in partnership with Foxp3.

In addition to the Foxp3–NFAT interaction, c-Rel is also critical in the maintenance of Treg properties. Our finding that Tregs are resistant to CN inhibitors in the presence of exogenous IL-2 could have important clinical implications. CN inhibitors, such as CsA and tacrolimus, are classic drugs used for the treatment and prophylaxis of allograft rejection and graft-versus-host disease (GVHD). Although some patients respond to CsA well, a high percentage (up to 50%) of patients, especially those receiving unrelated or mismatched bone marrow grafts, develop severe GVHD when treated with CsA (64, 65). The reason for the lack of therapeutic effect in these patients is not well understood. Our results (Fig. 3), along with the data from other investigators (66, 67), suggest that CsA may harm Tregs by IL-2 deprivation. Loss of Tregs in bone marrow recipients is reported to increase the risk for GVHD (68, 69). These findings raise the possibility that CN inhibitors might be more efficacious if used in conjunction with IL-2, because IL-2 selectively allows Treg cells to survive and propagate in the presence of CN inhibitors (Fig. 3). Early studies reported a positive impact of IL-2 on protection against GVHD (70, 71).

Clearly, a fundamental question that has arisen from this study is how NFAT becomes translocated into the nucleus in primary Tregs. It has been generally believed that CN is the only phosphatase that dephosphorylates NFAT (72, 73). Our results show that CN activity is not required for constitutive NFAT nuclear translocation in Tregs, suggesting that other mechanisms might activate NFAT. Studies suggested the potential role for CsA-resistant protein phosphatases in dephosphorylating NFAT (74, 75). Another possibility is that the activity of NFAT kinases, such as glycogen synthase kinase 3β, which dephosphorylates NFAT in Tregs is attenuated, shifting the dynamic equilibrium between CN and kinase activities toward formation of dephosphorylated NFAT and NFAT nuclear retention. A recent study reported that inhibition of glycogen synthase kinase 3β activity enhances Tregs functionality (76). It is not clear whether NFAT proteins can remain in the nucleus of Tregs, regardless of its phosphorylation status. Given the extensive protein–protein interaction interface between NFAT and Foxp3 (13, 16), there is a possibility that the interaction with Foxp3 facilitates NFAT nuclear retention in Tregs in the form of Foxp3–NFAT complexes. All of these possibilities are yet to be investigated.
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Disclosures
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