

# NFATc2 and NFATc3 transcription factors play a crucial role in suppression of CD4<sup>+</sup> T lymphocytes by CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells

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**The phenotype of NFATc2<sup>-/-</sup> c3<sup>-/-</sup> (double knockout [DKO]) mice implies a disturbed regulation of T cell responses, evidenced by massive lymphadenopathy, splenomegaly, and autoaggressive phenomena. The population of CD4<sup>+</sup> CD25<sup>+</sup> T cells from DKO mice lacks regulatory capacity, except a small subpopulation that highly expresses glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR) and CD25. However, neither wild-type nor DKO CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (T reg cells) are able to suppress proliferation of DKO CD4<sup>+</sup> CD25<sup>-</sup> T helper cells. Therefore, combined NFATc2/c3 deficiency is compatible with the development of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells but renders conventional CD4<sup>+</sup> T cells unresponsive to suppression, underlining the importance of NFAT proteins for sustaining T cell homeostasis.**

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Homeostasis of the immune system critically depends on the balance between activating and repressing mechanisms, the latter being mainly based on the phenomena of central and peripheral tolerance. Whereas central tolerance refers to the thymic deletion of potentially autoaggressive T cells, naturally occurring CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (T reg cells) are widely accepted to serve as pivotal mediators of peripheral tolerance. T reg cells are able to suppress activation and expansion of potentially self-reactive T cells, but the underlying mechanisms are still elusive (1, 2).

The family of NFAT comprises the four genuine NFATc members NFATc1 (also designated as NFATc or NFAT2), NFATc2 (NFATp or NFAT1), NFATc3 (NFATx or NFAT4), and NFATc4 (NFAT3), and the distantly related protein NFAT5 (TonEBP). Upon elevation of intracellular Ca<sup>2+</sup> concentration and activation of the Ca<sup>2+</sup>/calmodulin-dependent phosphatase calcineurin, all four cytosolic NFATc proteins are dephosphorylated and translocated into the nucleus where they control transcription of a

plethora of genes, including those encoding a variety of cytokines and surface molecules (3–5). The immunosuppressants cyclosporin A and FK506, which suppress T cell activation and the transcription of numerous lymphokine genes, have been shown to prevent the nuclear translocation and activation of NFAT factors by targeting calcineurin. Based on these findings, it has been concluded that NFAT proteins are critical activators of the immune response (3–5). However, analyses of mice bearing inactivated *NFATc2* and/or *NFATc3* genes suggested that both factors might also have inhibitory or additional regulatory functions. Mice deficient for NFATc2 show a modest splenomegaly, hyperproliferation of T cells and B cells and dysregulated production of IL-4 (6–8), whereas mice bearing an inactive NFATc3 gene express normal cytokine levels, but show also slightly increased numbers of B and T cells with activated phenotype (9). However, double deficiency for NFATc2 and NFATc3 causes massive lymphadenopathy, splenomegaly and a strong increase in serum IgE and IgG1 levels. Such mice have a dramatic increase in peripheral T cells with an activated phenotype and develop severe allergic blepharitis and pneumonitis

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(10). In the absence of NFATc2 and NFATc3, naive CD4<sup>+</sup> T cells intrinsically differentiate into the Th2 cell direction, even in the absence of endogenous IL-4, and are hyperresponsive to TCR-mediated activation (11). Although a part of the phenotype of NFATc2/c3-deficient mice might be due to defects in lymphocyte apoptosis (12), defects in additional molecular mechanisms could also be the cause of various abnormalities. Taken together, these studies revealed the importance of both NFATc2 and NFATc3 to maintain lymphoid homeostasis.

In this study, we characterized the peripheral CD4<sup>+</sup> CD25<sup>-</sup> and CD4<sup>+</sup> CD25<sup>+</sup> T cell populations from NFATc2/c3 double deficient (double KO [DKO]) mice and found out that combined NFATc2/c3 deficiency is compatible with the development of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells but renders conventional CD4<sup>+</sup> T cells unresponsive to suppression.

## RESULTS AND DISCUSSION

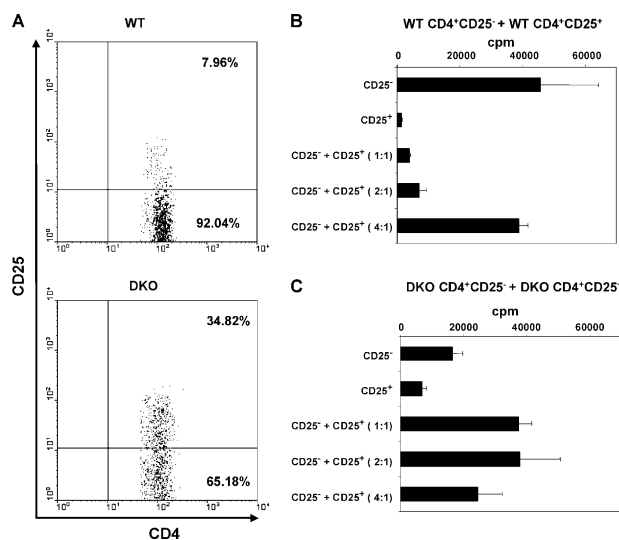
CD4<sup>+</sup> CD25<sup>+</sup> T reg cells are characterized by constitutive expression of the IL-2 receptor  $\alpha$  chain (CD25) and constitute ~5–10% of peripheral CD4<sup>+</sup> T cells in naive mice. Comparative FACS analyses of spleen cells stained for CD4 and CD25 revealed a dramatically increased proportion of CD4<sup>+</sup> CD25<sup>+</sup> T cells in DKO mice (Fig. 1 A), which constantly increased with the age of these mice (not depicted).

To characterize the peripheral CD4<sup>+</sup> CD25<sup>-</sup> and CD4<sup>+</sup> CD25<sup>+</sup> T cell populations from DKO mice, positive selection was used by MACS. CD4<sup>+</sup> CD25<sup>+</sup> T reg cells are im-

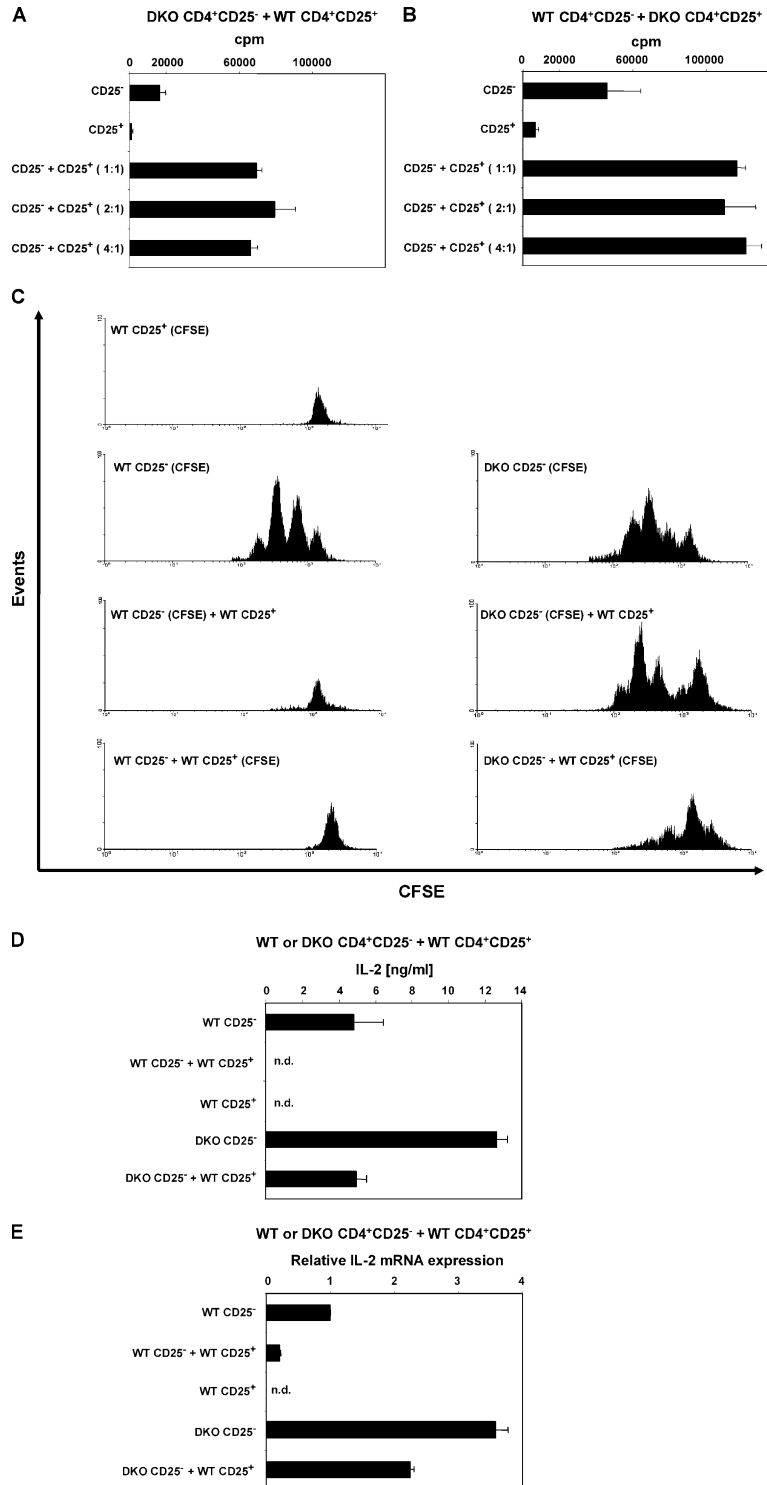
munologically inert with respect to activation-induced proliferation and cytokine production, but a hallmark is their ability to suppress the proliferation of conventional T cells in a contact-dependent manner. Therefore, we stimulated CD4<sup>+</sup> CD25<sup>-</sup> in the presence of CD4<sup>+</sup> CD25<sup>+</sup> T cells isolated from DKO mice and wild-type littermates in variable ratios and measured [<sup>3</sup>H]thymidine incorporation. As depicted in Fig. 1 B, using cells derived from littermate controls, proliferation of CD4<sup>+</sup> CD25<sup>-</sup> T cells was strongly reduced in the presence of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells. In contrast, costimulation of CD4<sup>+</sup> CD25<sup>-</sup> and CD4<sup>+</sup> CD25<sup>+</sup> T cells both derived from DKO mice did not reduce proliferation of the former population (Fig. 1 C).

This led to the conclusion that combined NFAT deficiency interferes with either the development and/or function of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells or renders CD4<sup>+</sup> CD25<sup>-</sup> T cells unresponsive to suppression. To address this issue, proliferation of CD4<sup>+</sup> CD25<sup>-</sup> T cells from DKO mice was assessed upon coculture with CD4<sup>+</sup> CD25<sup>+</sup> T reg cells derived from littermate controls. As shown in Fig. 2 A, littermate CD4<sup>+</sup> CD25<sup>+</sup> T reg cells failed to suppress the proliferative response of DKO CD4<sup>+</sup> CD25<sup>-</sup> T cells. Furthermore, DKO CD4<sup>+</sup> CD25<sup>+</sup> T cells also failed to suppress proliferation of littermate CD4<sup>+</sup> CD25<sup>-</sup> T cells (Fig. 2 B). The strong [<sup>3</sup>H]thymidine uptake in coculture (Fig. 2 A) can be explained by proliferation of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells under conditions in which suppression is abrogated (13), i.e., in the presence of DKO CD4<sup>+</sup> CD25<sup>-</sup> T cells. This was confirmed by activating carboxyfluorescein succinimidyl ester (CFSE)-labeled littermate CD4<sup>+</sup> CD25<sup>+</sup> T reg cells in the presence of unlabeled DKO CD4<sup>+</sup> CD25<sup>-</sup> T cells (Fig. 2 C). The strong proliferation of littermate CD4<sup>+</sup> CD25<sup>+</sup> T reg cells in coculture with DKO CD4<sup>+</sup> CD25<sup>-</sup> T cells might be explained by the presence of high amounts of IL-2 (Fig. 2 D). Compared with cells derived from littermates, DKO CD4<sup>+</sup> CD25<sup>-</sup> T cells produced large quantities of IL-2 (Fig. 2 D) and IL-2 mRNA (Fig. 2 E). Production of IL-2 and IL-2 mRNA was not effectively suppressed by littermate CD4<sup>+</sup> CD25<sup>+</sup> T reg cells. Obviously, combined NFATc2/c3 deficiency causes a strongly diminished response of conventional CD4<sup>+</sup> CD25<sup>-</sup> T cells to CD4<sup>+</sup> CD25<sup>+</sup> T reg cell-mediated suppression. A role for TGF- $\beta$  in suppression of responder cells by CD4<sup>+</sup> CD25<sup>+</sup> T reg cells is still a matter of debate (1, 2). However, additional experiments revealed that DKO CD4<sup>+</sup> CD25<sup>-</sup> T cells were sensitive to TGF- $\beta$  as indicated by decreased production of IL-2 (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20041538/DC1>).

Because CD25 is also expressed after stimulation of conventional CD4<sup>+</sup> CD25<sup>-</sup> T cells, it is no reliable marker for T reg cells. It has already been reported (10) that DKO mice display an increase of CD4<sup>+</sup> T cells with an activated phenotype (CD62L<sup>low</sup> CD45RB<sup>low</sup> CD44<sup>high</sup> CD69<sup>high</sup>); therefore, regarding CD25 as an additional activation marker, it is impossible to discriminate T reg cells from activated conventional CD4<sup>+</sup> T cells in such mice. This leaves the question unresolved as to whether the development and/or function

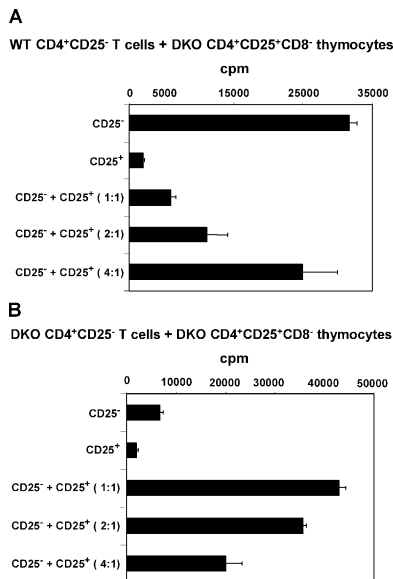


**Figure 1. Characterization of CD4<sup>+</sup> CD25<sup>+</sup> T cells from DKO mice.** (A) Spleen cells derived from either littermates (WT) or DKO mice at the age of 6 wk were stained for the expression of CD4 and CD25. FACS analyses of CD4<sup>+</sup> cells are shown. One representative of four experiments is shown. (B) CD4<sup>+</sup> CD25<sup>-</sup> and CD4<sup>+</sup> CD25<sup>+</sup> T cells were isolated from the spleens of littermate mice (WT) by magnetic cell separation. Cells were activated separately or in coculture at variable ratios with anti-CD3 mAb and A20 as accessory cells. Proliferation was assessed after 4 d. Cpm, counts per minute. (C) CD4<sup>+</sup> CD25<sup>-</sup> and CD4<sup>+</sup> CD25<sup>+</sup> T cells from DKO mice were isolated and stimulated as described in B. (B and C) Each show one representative result performed in triplicates  $\pm$  SD of three independent experiments.



**Figure 2.** CD4<sup>+</sup>CD25<sup>-</sup> T cells from DKO mice escape suppression by wild-type CD4<sup>+</sup>CD25<sup>+</sup> T cells. (A) Conventional DKO CD4<sup>+</sup>CD25<sup>-</sup> T cells were costimulated in the presence of CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from littermates (WT). (B) Conventional CD4<sup>+</sup>CD25<sup>-</sup> T cells derived from littermates (WT) were costimulated in the presence of CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from DKO mice. In A and B, proliferation was measured after 4 d. (C) Different T cell populations were labeled with CFSE and stimulated either alone or in coculture with unlabeled cells as indicated. On day 4,

CFSE fluorescence was measured by FACS analyses. Representatives of three independent experiments are shown. (D and E) T cells were stimulated either alone or in coculture (ratio 1:1) as specified. After 18 h, IL-2 in the supernatants was assayed by ELISA (D) and real-time PCR for the expression of IL-2 mRNA was performed (E). N.d., not detectable. (A–E) Each show one representative assay performed in triplicates ± SD of three independent experiments.



**Figure 3. DKO CD4<sup>+</sup> CD25<sup>+</sup> CD8<sup>-</sup> thymocytes possess regulatory capacity.** (A) Thymocytes from DKO mice were enriched for CD25<sup>+</sup> cells by MACS separation and sorted positively for CD4 and CD25 excluding CD8<sup>+</sup> cells using FACS. Cells were activated separately or in coculture with CD4<sup>+</sup> CD25<sup>-</sup> T cells from littermate spleens (WT) at variable ratios using CD3 mAb and A20 as accessory cells. Proliferation was assessed after 4 d. (B) This experiment was performed as described in A except that DKO mice were used as a source of CD4<sup>+</sup> CD25<sup>-</sup> T cells. A and B show one representative result performed in triplicates  $\pm$  SD of three independent experiments.

of T reg cells is impaired in DKO mice or whether T reg cells develop normally in the thymus and are simply overgrown and functionally masked by activated conventional CD4<sup>+</sup> T cells. Consequently, they would constitute only a minor fraction of CD4<sup>+</sup> CD25<sup>+</sup> T cells in the periphery. If the latter assumption holds true, CD4<sup>+</sup> CD25<sup>+</sup> CD8<sup>-</sup> thymocytes with suppressive capacity should develop in the thymus of DKO mice (14). In an attempt to clarify this issue, we isolated CD4<sup>+</sup> CD25<sup>+</sup> CD8<sup>-</sup> T cells from thymi of DKO mice via FACS sort and tested their suppressive capacity. As shown in Fig. 3 A, DKO CD4<sup>+</sup> CD25<sup>+</sup> CD8<sup>-</sup> thymocytes strongly suppressed proliferation of littermate CD4<sup>+</sup> CD25<sup>-</sup> T cells. The suppressive activities as well as the numbers of CD4<sup>+</sup> CD25<sup>+</sup> CD8<sup>-</sup> thymocytes derived from either DKO or littermate mice were equivalent (not depicted). However, DKO CD4<sup>+</sup> CD25<sup>+</sup> CD8<sup>-</sup> thymocytes did not inhibit proliferation of autologous peripheral CD4<sup>+</sup> CD25<sup>-</sup> T cells, corroborating our finding that NFATc2/c3 double deficiency renders CD4<sup>+</sup> CD25<sup>-</sup> T cells unresponsive to suppression (Fig. 3 B).

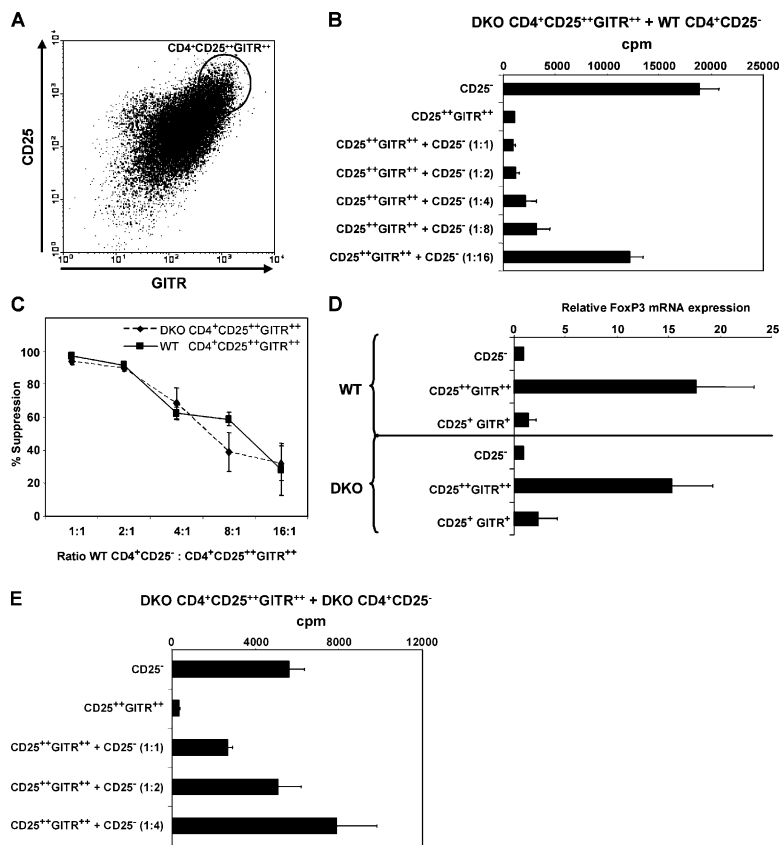
A further characterization and isolation of peripheral DKO CD4<sup>+</sup> CD25<sup>+</sup> T reg cells has been hampered by the lack of specific markers for these cells. One exception is the transcription factor FoxP3, which has been shown to be critical for the development and function of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells (15–17). Although not a unique marker for CD4<sup>+</sup> CD25<sup>+</sup> T reg cells, glucocorticoid-induced tumor necrosis factor receptor family–related gene (GITR), a member of

the tumor necrosis factor receptor family, has been found to be predominantly expressed on T reg cells (18, 19). Using a combination of MACS and FACS separations, we isolated CD4<sup>+</sup> T cells highly expressing both CD25 and GITR (CD4<sup>+</sup> CD25<sup>++</sup> GITR<sup>++</sup>) from the spleens of DKO mice (Fig. 4 A). Even in FACS density plots, these cells did not represent a distinct population within the CD4<sup>+</sup> T cell pool of DKO or littermates (not depicted). Using the setting shown in Fig. 4 A,  $\sim$ 10% of littermate and 20% of DKO CD4<sup>+</sup> CD25<sup>+</sup> T cells were sorted as CD25<sup>++</sup> GITR<sup>++</sup>, which obviously correlates with increased numbers of CD4<sup>+</sup> CD25<sup>+</sup> T cells in DKO mice (Fig. 1 A). However, CD4<sup>+</sup> CD25<sup>++</sup> GITR<sup>++</sup> cells from DKO mice showed a very strong suppressive activity on the proliferation of CD4<sup>+</sup> CD25<sup>-</sup> T cells derived from littermate controls unlike the unsorted CD4<sup>+</sup> CD25<sup>+</sup> population (compare Fig. 4 B with Fig. 1 C). Furthermore, the suppressive capacity of CD4<sup>+</sup> CD25<sup>++</sup> GITR<sup>++</sup> cells derived from either littermate or DKO mice was comparable (Fig. 4 C). Because both CD25 and GITR can also be expressed on conventional CD4<sup>+</sup> CD25<sup>-</sup> T cells after their activation, a combination of these markers is not sufficient to phenotypically define CD4<sup>+</sup> CD25<sup>+</sup> T reg cells; but, the data shown in Fig. 4, B and C, allow us to conclude that T cells with regulatory activity are highly enriched in the CD25<sup>++</sup> GITR<sup>++</sup> population. This assumption was further corroborated by the observation that enrichment of regulatory activity was accompanied by the accumulation of FoxP3 mRNA (Fig. 4 D). Therefore, NFATc2/c3 deficiency does not interfere with development and function of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells.

Coculture experiments revealed that DKO CD4<sup>+</sup> CD25<sup>++</sup> GITR<sup>++</sup> T cells only moderately reduced proliferation of autologous CD4<sup>+</sup> CD25<sup>-</sup> T cells ( $\sim$ 50% at a ratio of 1:1; Fig. 4, compare E with B and C). Hence, although CD4<sup>+</sup> CD25<sup>++</sup> GITR<sup>++</sup> T cells are potent suppressors, they are unable to effectively regulate NFATc2/c3-deficient CD4<sup>+</sup> CD25<sup>-</sup> T cells.

In general, suppression mediated by CD4<sup>+</sup> CD25<sup>+</sup> T reg cells can be overridden by using strong TCR signals or costimulation via CD28. Under such conditions, CD4<sup>+</sup> CD25<sup>+</sup> T reg cells retain their suppressive capacity, but strongly activated conventional CD4<sup>+</sup> CD25<sup>-</sup> T cells escape regulation (20–22). It has been shown that both NFATc2 and NFATc3 are involved in the modulation of TCR responsiveness because conventional CD4<sup>+</sup> T cells from DKO mice showed increased proliferation in response to anti-CD3 cross-linking in the absence of CD28 stimulation (11). Obviously, NFATc2/c3 deficiency lowers the threshold for T cell activation, which, accompanied by enhanced production of IL-2, allows CD4<sup>+</sup> CD25<sup>-</sup> T cells to escape suppression by CD4<sup>+</sup> CD25<sup>+</sup> T reg cells.

The importance of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells for the maintenance of peripheral tolerance became recently evident because mice and humans carrying loss-of-function mutations in the FOXP3 gene develop severe autoaggressive phenomena due to the absence of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells (23–25).



**Figure 4. Isolation and characterization of CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup> T cells.** (A) DKO CD25<sup>+</sup> T cells were purified using magnetic cell sorting and stained for GITR and CD25 in FACS analyses. (B) DKO CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup> T cells from the gated region indicated in A were FACS sorted and stimulated together with CD4<sup>+</sup> CD25<sup>-</sup> T cells from littermates (WT) as depicted. (C) DKO and wild-type CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup> T cells show comparable suppressive activity on the proliferation of wild-type CD4<sup>+</sup> CD25<sup>-</sup> T cells. (D) CD4<sup>+</sup> CD25<sup>-</sup> T cells were isolated by magnetic cell separation, CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup>, and CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>-</sup> T cells (the remaining population after depletion of CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup> cells shown in A) were

isolated using FACS sorting from the spleens of either littermate (WT) or DKO mice. Real-time PCR for the expression of FoxP3 mRNA was performed and data were normalized according to the expression of EF-1 $\alpha$  mRNA. Shown are the means  $\pm$  SD of three independent experiments each performed in triplicates. (E) DKO CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup> T cells were isolated as described in B and stimulated in the presence of DKO CD4<sup>+</sup> CD25<sup>-</sup> T cells. (B, C, and E) Proliferation was measured after 4 d. Shown is one representative result performed in triplicates  $\pm$  SD of three independent experiments.

On the other hand, defects in the responder T cell population, which allow them to escape regulation, can also be the cause of an imbalanced T cell homeostasis. Our results indicate that NFATc2/c3 transcription factors are critically involved in this process because their absence culminates in the development of lymphoproliferative disorder despite the existence of functionally active CD4<sup>+</sup> CD25<sup>+</sup> T reg cells in NFATc2/c3-deficient mice.

## MATERIALS AND METHODS

**Mice.** NFATc2<sup>-/-</sup> and NFATc3<sup>-/-</sup> mice on a BALB/c genetic background were provided by L. Glimcher (Harvard University, Boston, MA). NFATc2<sup>-/-</sup> c3<sup>-/-</sup> mice were obtained by intercrossing these lines accordingly. All mice were used at the age of 5–7 wk.

**Cytokines, antibodies, and reagents.** Hybridoma cells producing anti-CD4 mAb GK1.5 were obtained from the American Type Culture Collection (no. TIB 207). PE-labeled rat anti-CD25 (7D4) and APC-labeled rat anti-CD8 $\alpha$  (53–6.7) were purchased from BD Biosciences. Rat anti-GITR (DTA-1) was provided by S. Sakaguchi through R. Suttmuller (University Medical Center, Nijmegen, Netherlands). IL-2-specific ELISA was per-

formed using anti-mIL-2 (JES6-1A12) and biotinylated anti-mIL-2 (JES6-5H4), both from BD Biosciences. In addition, the following mAbs were used: anti-CD3 mAb 145-2C11. If required, mAbs were affinity purified using protein G sepharose (Amersham Biosciences) and coupled with FITC or biotin. Mitomycin C was purchased from Sigma-Aldrich (M 0503).

**Preparation of T cell populations.** Conventional CD4<sup>+</sup> CD25<sup>-</sup> T cells (GK1.5-FITC) and CD4<sup>+</sup> CD25<sup>+</sup> T cells (7D4-PE) were isolated from spleen cells by positive selection using MACS (Miltenyi Biotec) according to the manufacturer's instructions. The CD4 sort as well as the CD25 sort were performed twice. Conventional CD4<sup>+</sup> CD25<sup>-</sup> T cells were subsequently depleted from CD4<sup>+</sup> CD25<sup>+</sup> T cells using mAb PC61 and enriched >99%. CD4<sup>+</sup> CD25<sup>+</sup> enriched T cells were additionally depleted from CD8<sup>+</sup> T cells using anti-CD8 Dynabeads, and the purity of the resulting CD4<sup>+</sup> CD25<sup>+</sup> T cells was typically >95%. CD4<sup>+</sup> CD25<sup>+</sup> cells derived from wild-type mice did neither proliferate nor produce IL-2 in the presence of both soluble anti-CD3 mAb and A20 B cell tumor line as accessory cells. CD4<sup>+</sup> CD25<sup>+</sup> CD8<sup>-</sup> thymocytes were enriched by positive selection using MACS (Miltenyi Biotec), stained for CD4 (GK1.5-FITC), CD25 (7D4-PE), and CD8 (53–6.7-APC), and CD4<sup>+</sup> CD25<sup>+</sup> CD8<sup>-</sup> thymocytes were isolated using a cell sorter (FACS Vantage SE and CELLQuest Pro; BD Biosciences), with exclusion of dead cells by propidium iodide incorporation. CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup> T



cells were separated as follows. After enrichment of CD25<sup>+</sup> spleen cells using MACS as described above, these cells were stained for CD8 (53–6.7-APC), CD25 (7D4-PE), and GITR (DTA-1-FITC). Upon exclusion of CD8<sup>+</sup> T cells, CD4<sup>+</sup> CD25<sup>++</sup> GITR<sup>++</sup> T cells were separated using a cell sorter (FACS Vantage SE and CELLQuest Pro; BD Biosciences), with exclusion of dead cells by propidium iodide incorporation. Re-analyses of these cells revealed a purity of CD4<sup>+</sup> CD25<sup>++</sup> GITR<sup>++</sup> T cells >98%. Remaining CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup> T cells were sorted separately.

**T cell stimulation and proliferation assays.** Culture medium was IMDM (Life Technologies) supplemented with 2 mM L-glutamine, 5 × 10<sup>-5</sup> M β-mercaptoethanol, 10 IU penicillin, 100 μg/ml streptomycin, and 5% FCS inactivated at 56°C. 2 × 10<sup>4</sup> conventional CD4<sup>+</sup> CD25<sup>-</sup> T cells from DKO mice or from littermate controls were stimulated using 96-well round-bottom microplates (Costar) in a total volume of 0.2 ml in the presence or absence of different numbers of freshly isolated CD4<sup>+</sup> CD25<sup>+</sup> T cells, CD4<sup>+</sup> CD25<sup>++</sup> GITR<sup>++</sup> T cells, or CD4<sup>+</sup> CD25<sup>+</sup> CD8<sup>-</sup> thymocytes. Mitomycin C-treated (60 μg/ml/10<sup>7</sup> cells for 30 min) A20 B tumor cells (2 × 10<sup>3</sup>/well) as accessory cells and anti-CD3 mAbs (145-2C11; 3 μg/ml) were used as stimulus. After 96 h, [<sup>3</sup>H]thymidine was added to the cultures (0.5 μCi/well) and [<sup>3</sup>H]thymidine uptake was assessed by β scintillation counting after an additional 18 h.

**CFSE staining.** Either freshly isolated CD4<sup>+</sup> CD25<sup>-</sup> T cells from wild-type and DKO mice or CD4<sup>+</sup> CD25<sup>+</sup> T cells from wild-type mice (1–2 × 10<sup>7</sup>) were labeled with the vital dye CFSE (CFDASE; Molecular Probes). After washing cells twice in 10 ml PBS, pH 7.4, they were incubated with 2.5 μM CFSE in 4 ml PBS at 37°C in 5% CO<sub>2</sub> for 4 min. To stop the staining reaction, 8 ml IMDM plus 10% FCS was added. The cells were then washed three times in 10 ml IMDM and stimulated alone or in coculture as described above. On day four, proliferation of the CFSE-labeled cells was analyzed by flow cytometry on a FACScan (BD Biosciences).

**mRNA detection.** RNA was isolated using TRIzol (Invitrogen) and cDNA was synthesized with RevertAid M-MuLV reverse transcriptase following the recommendations of the supplier (MBI Fermentas). Real-time PCR was performed using the following oligonucleotides: FoxP3 forward: CTTATCCGATGGGCCATCCTGGAAG, FoxP3 reverse: TTCCAG-GTGGCGGGGTTTCTG; IL-2 forward: CCTGAGCAGGATGGAGA-ATTACAGG, IL-2 reverse: GCACTCAAATGTGTTGTGAGAGCCC; and elongation factor-1 α (EF1α) forward: GATTACAGGGACATCT-CAGGCTG, EF1α reverse: TATCTCTTCTGGCTGTAGGGTGG. Oligonucleotides were chosen to span at least one intron at the level of genomic DNA. Real-time PCR analyses to quantify the expression of IL-2, FoxP3, and EF1α mRNAs were performed in triplicates on an iCycler (Bio-Rad Laboratories) using the IQ SYBR Green Supermix (Bio-Rad Laboratories). After normalization of the data according to the expression of EF1α mRNA, the relative expression levels of IL-2 and FoxP3 mRNAs were calculated.

**Online supplemental material.** Fig. S1 shows that the production of IL-2 by DKO CD4<sup>+</sup> CD25<sup>-</sup> T cells is sensitive to TGF-β. Fig. S1 is available at <http://www.jem.org/cgi/content/full/jem.20041538/DC1>.

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## REFERENCES

- Shevach, E.M. 2002. CD4<sup>+</sup> CD25<sup>+</sup> suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2:389–400.
- Piccirillo, C.A., and A.M. Thornton. 2004. Cornerstone of peripheral tolerance: naturally occurring CD4(+)CD25(+) regulatory T cells. *Trends Immunol.* 25:374–380.
- Serfling, E., F. Berberich-Siebelt, S. Chuvpilo, E. Jankevics, S. Klein-Hessling, T. Twardzik, and A. Avots. 2000. The role of NF-AT transcription factors in T cell activation and differentiation. *Biochim. Biophys. Acta.* 1498:1–18.
- Crabtree, G.R., and E.N. Olson. 2002. NFAT signaling: choreographing the social lives of cells. *Cell.* 109:S67–S79.
- Hogan, P.G., L. Chen, J. Nardone, and A. Rao. 2003. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* 17:2205–2232.
- Xanthoudakis, S., J.P. Viola, K.T. Shaw, C. Luo, J.D. Wallace, P.T. Bozza, D.C. Luk, T. Curran, and A. Rao. 1996. An enhanced immune response in mice lacking the transcription factor NFAT1. *Science.* 272:892–895.
- Schuh, K., B. Kneitz, J. Heyer, U. Bommhardt, E. Jankevics, F. Berberich-Siebelt, K. Pfeffer, H.K. Muller-Hermelink, A. Schimpl, and E. Serfling. 1998. Retarded thymic involution and massive germinal center formation in NF-ATp-deficient mice. *Eur. J. Immunol.* 28:2456–2466.
- Hodge, M.R., A.M. Ranger, F.C. de la Brousse, T. Hoey, M.J. Grusby, and L.H. Glimcher. 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity.* 4:397–405.
- Oukka, M., I.C. Ho, F.C. de la Brousse, T. Hoey, M.J. Grusby, and L.H. Glimcher. 1998. The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity.* 9:295–304.
- Ranger, A.M., M. Oukka, J. Rengarajan, and L.H. Glimcher. 1998. Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. *Immunity.* 9:627–635.
- Rengarajan, J., B. Tang, and L.H. Glimcher. 2002. NFATc2 and NFATc3 regulate T(H)2 differentiation and modulate TCR-responsiveness of naive T(H) cells. *Nat. Immunol.* 3:48–54.
- Chuvpilo, S., E. Jankevics, D. Tyrsin, A. Akimzhanov, D. Moroz, M.K. Jha, J. Schulze-Luehrmann, B. Santner-Nanan, E. Feoktistova, T. Konig, et al. 2002. Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. *Immunity.* 16:881–895.
- Thornton, A.M., E.E. Donovan, C.A. Piccirillo, and E.M. Shevach. 2004. Cutting edge: IL-2 is critically required for the in vitro activation of CD4<sup>+</sup>CD25<sup>+</sup> T cell suppressor function. *J. Immunol.* 172:6519–6523.
- Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of CD25<sup>+</sup>CD4<sup>+</sup> naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J. Immunol.* 162:5317–5326.
- Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor FOXP3. *Science.* 299:1057–1061.
- Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. *Nat. Immunol.* 4:330–336.
- Khattari, R., T. Cox, S.A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells. *Nat. Immunol.* 4:337–342.
- Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2002. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 3:135–142.
- McHugh, R.S., M.J. Whitters, C.A. Piccirillo, D.A. Young, E.M. Shevach, M. Collins, and M.C. Byrne. 2002. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity.* 16:311–323.
- Thornton, A.M., and E.M. Shevach. 1998. CD4<sup>+</sup> CD25<sup>+</sup> immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188:287–296.
- Baecher-Allan, C., V. Viglietta, and D.A. Hafler. 2002. Inhibition of human CD4(+)CD25(+high) regulatory T cell function. *J. Immunol.* 169:6210–6217.
- George, T.C., J. Billsborough, J.L. Viney, and A.M. Norment. 2003. High antigen dose and activated dendritic cells enable Th cells to es-

- cape regulatory T cell-mediated suppression in vitro. *Eur. J. Immunol.* 33:502–511.
23. Brunkow, M.E., E.W. Jeffery, K.A. Hjerrild, B. Paepfer, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27:68–73.
24. Wildin, R.S., F. Ramsdell, J. Peake, F. Faravelli, J.L. Casanova, N. Buist, E. Levy-Lahad, M. Mazzella, O. Goulet, L. Perroni, et al. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* 27:18–20.
25. Bennett, C.L., J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, and H.D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27:20–21.