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Regulatory T cells suppress CD4$^+$ T cells through NFAT-dependent transcriptional mechanisms

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Abstract

Regulatory T cells (Tregs) control autoreactive T cells by inhibiting activation-induced proliferation and cytokine expression. The molecular mechanisms responsible for the inactivation of effector T cells by Tregs remain yet to be fully characterized. We report that T-helper cells stimulated in the presence of Tregs quickly activate NFAT1 and have increased NFAT1-dependent expression of the transcription repressor Ikaros. NFAT1 deficiency or dominant-negative Ikaros compromises Treg-mediated inhibition of T-helper cells *in vitro* and *in vivo*. Thus, our results place NFAT-dependent mechanisms as general regulators of T-cell tolerance and show that Treg-mediated suppression of T-helper cells results from the activation of NFAT-regulated gene expression.

Keywords Ikaros; NFAT; regulatory T cell
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Introduction

A major mechanism of peripheral tolerance is suppression by regulatory T cells (Tregs) [1]. Several subtypes of Tregs have been defined based on the specific developmental processes involved in their generation. tTregs develop in the thymus and constitute approximately 5–10% of the total peripheral CD4$^+$ T-cell population [1]. In contrast to tTregs, peripheral pTregs develop in peripheral lymphoid tissues from naïve CD4$^+$ progenitors [2]. Several models have been proposed to explain how Tregs mediate suppression of T-cell activation [3], including secretion of immunomodulatory cytokines [4,5] and cell-to-cell contacts mediated by proteins such as CTLA-4 [6] or LAG-3 [7]. Regardless of which mechanisms are in play in any given context, the general outcome is an inhibition of proliferation and cytokine secretion. The molecular events that occur within the suppressed T cell that are ultimately responsible for inhibiting T-cell activation remain yet to be fully characterized.

The family of NFAT transcription factors is composed of four calcium-/calcineurin-regulated members, NFAT1-4, and a more distantly related NFAT5 [8]. In T cells, engagement of the TCR causes an elevation of intracellular calcium and the activation of calcineurin, which in turn dephosphorylates NFAT proteins leading to their nuclear translocation. NFAT proteins play major roles not only during T-cell activation but also in the induction of T-cell tolerance [9,10].

Here, we show that NFAT1 is required for effective Treg-mediated suppression of T-helper (Th) cell activation. When T cells are activated in the presence of Tregs, they upregulate the NFAT1-dependent expression of Ikaros, which is necessary to achieve efficient suppression. Our results support a central role for NFAT1 in the regulation of Treg-mediated suppression of Th cells.

Results and Discussion

NFAT1-deficient T-helper cells show resistance to Treg-mediated suppression

Previous studies have shown that NFAT1 directs the expression of a specific set of genes responsible for the inhibition of Th1 cell effector functions in response to tolerizing stimuli [9–11]. To determine whether NFAT1 could regulate Treg-mediated suppression of effector Th-cell activation, we assessed the ability of Tregs to suppress NFAT1-deficient Th1 cells. *In vitro* differentiated Th1 cells from wild-type (WT) or *Nfat1*$^{-/-}$ DO11.10 mice were stimulated for 48 h with ovalbumin$_{323–339}$ peptide-loaded antigen-presenting cells (APC-OVA) in the presence or absence of WT CD4$^+$CD25$^+$Foxp3$^+$ Tregs isolated from BALB/c mice. Whereas WT T cells were efficiently suppressed by Tregs, T cells from *Nfat1*$^{-/-}$ mice were resistant to Treg-mediated suppression (Fig 1A). Similar experiments using OT-II mice and Tregs from B6 Foxp3-RFP mice confirmed a significant decrease (~ 3-fold) in the susceptibility to Treg-mediated

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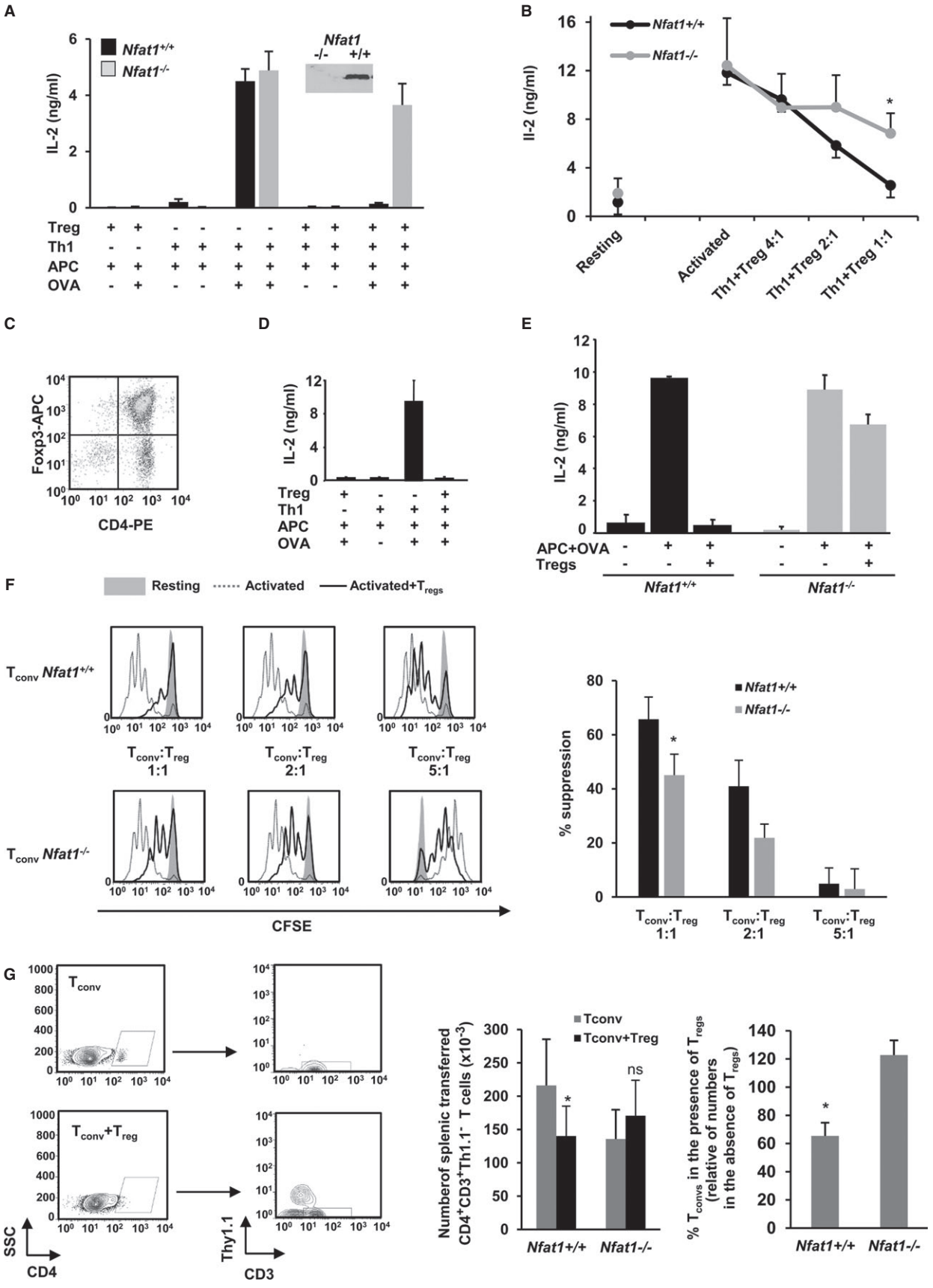


Figure 1. NFAT1-deficient T cells are more resistant to suppression by Tregs.

A, B DO11.10 (A) or OTII (B) WT or *Nfat1*$^{-/-}$ Th1 cells were activated with APC-OVA for 48 h with or without WT CD4$^+$CD25$^+$FoxP3$^+$ Tregs (mean ± s.e.m. of 5 (A) or 3 (B) experiments, *t*-test, *$P < 0.05$). Inset: immunoblot for NFAT1.

C FACS of Tregs from BALB/c mice expanded *in vitro* for 1 week.

D, E IL-2 production in DO11.10 WT (D, E) or *Nfat1*$^{-/-}$ (E) Th1 cells stimulated with APC-OVA for 48 h with or without expanded Tregs (ratio 1:1) (mean + s.e.m. of 2 and 3 independent experiments performed in triplicate, respectively).

F C57BL6 WT or *Nfat1*$^{-/-}$ naïve CD4$^+$ T cells (T_{conv}) labeled with CFSE were stimulated with 0.5 μg/ml anti-CD3 antibody and APCs for 72 h with or without Tregs at indicated ratios. Graph shows % of inhibition of CFSE dilution from 3 experiments (mean + s.e.m., *t*-test, *$P < 0.05$).

G *Rag1*$^{-/-}$ mice were adoptively transferred with 2.5 × 10^6 naïve WT or *Nfat1*$^{-/-}$ CD4$^+$Thy1.2$^+$ T cells (T_{conv}) with or without 5 × 10^5 Foxp3$^+$RFP$^+$Thy1.1$^+$ Tregs. Seven days later, CD4$^+$CD3$^+$Thy1.1$^-$ T_{conv} in spleens was measured by FACS. Graphs show numbers of cells and relative percentage of suppression of T_{conv} expansion (mean + s.e.m. from 8 experiments, Wilcoxon, *$P < 0.05$).

Source data are available online for this figure.

suppression in Th1 cells lacking NFAT1, ensuring that this was not a strain-specific effect (Fig 1B).

Tregs activate and expand when encountering their cognate antigen *in vivo*, and CD28 signaling appears crucial to maintain efficient Treg-suppressive activity [12,13]. CD4$^+$CD25$^+$ T cells expanded for 1 week (Fig 1C) remained suppressive (Fig 1D), but still failed to effectively inhibit *Nfat1*$^{-/-}$ T cells (Fig 1E). As we had seen in anergic cells [10,14], effector NFAT1-deficient Th1 cells were less susceptible to Treg-mediated suppression. Further analyses using naïve CD4$^+$ T cells confirmed that the requirement for NFAT1 also extended to this T-cell population (Fig 1F). To confirm the NFAT1 requirement *in vivo*, we measured the ability of Tregs to suppress the homeostatic proliferation of CD4$^+$ T cells transferred into lymphopenic hosts. One week after adoptively transferring CD4$^+$CD25$^-$ T cells into *Rag1*$^{-/-}$ hosts, the number of donor T cells in the spleen was reduced by more than 40% when Tregs were co-transferred. However, *Nfat1*$^{-/-}$ T-cell expansion was not similarly affected (Fig 1G).

To confirm the role of NFAT1 in the regulation of the susceptibility of T cells to Tregs, *Nfat1*$^{-/-}$ DO11.10 Th1 cells were transduced with retrovirus expressing NFAT1. Positively infected T cells were re-stimulated with APC-OVA in the presence of Tregs. *Nfat1*$^{-/-}$ Th1 cells regained susceptibility to Tregs upon restoration of NFAT1 expression (Fig 2A). Similar results were obtained when cells were transiently transfected with a plasmid expressing NFAT1 (Fig 2B). Altogether, these results indicated that NFAT1 activity in Th cells is required to effectively respond to Tregs. It is possible, however, that other NFAT proteins might also cooperate with NFAT1 in this program. NFAT4 has been shown to exert a negative control on T-cell responses, and NFAT1/NFAT4-deficient mice present a more severe lymphoproliferative phenotype than NFAT1-deficient mice [15,16]. Furthermore, cells lacking these two NFAT proteins have also been shown to be resistant to Treg-mediated suppression [17]. Our data show, however, that T cells that lack just NFAT1 show no major alterations in their responses to TCR engagement, while rendering them less susceptible to Treg-mediated suppression.

Suppressed CD4$^+$ T cells upregulate the expression of *Ikaros*

We and others have previously identified genes that inhibit T-cell activation that are also expressed in an NFAT-dependent manner in tolerized T cells [18]. We showed that the expression of one such gene, *Ikaros*, was also upregulated (~ 15-fold) in suppressed Th1 cells (Fig 2C). The NFAT1 dependence of Ikaros was further confirmed by the inability of *Nfat1*$^{-/-}$ T cells to upregulate its expression when activated in the presence of Tregs (Fig. 2D). Expression of genes that are known to maintain T cells in an unresponsive state suggested the possibility that Tregs might stably suppress effector T cells. In fact, it has been previously reported that Tregs can anergize naïve CD4$^+$ T cells [19] and Tregs have been shown to induce anergy in arthritogenic self-reactive T cells *in vivo* [20]. To test this hypothesis, DO11.10 Th1 cells were stimulated with APC-OVA in the presence of Tregs for 24 h. Th1 cells were then purified and rested for 4 h before being re-stimulated. Previously suppressed T cells regained responsiveness when re-stimulated in the absence of Tregs (Fig 2E), indicating that while Treg-induced NFAT-dependent expression of specific genes inhibited T-cell activation, it was not sufficient to induce a long-lasting unresponsive state.

Ikaros mediates silencing of *Il2* expression in anergic T cells by inducing epigenetic modifications that maintain the *Il2* promoter locus in a closed state [21–24]. We assessed whether those modifications were also induced by Tregs. Our data revealed that the presence of Tregs resulted in a slight deacetylation of the *Il2* locus, a much smaller effect than reported in anergic cells [23] (Fig 2F). It is likely, thus, that additional signals to those transduced by the presence of Tregs might be required to induce a long-lasting, stable state of unresponsiveness in responder T cells. Accordingly, we could not detect upregulation of several other anergy-associated genes (data not shown), suggesting that specific differences in the programs of gene expression activated in anergic and suppressed T cells might determine divergences between these two forms, passive versus dominant, of T-cell tolerance [25].

Naïve T cells can also be differentiated into Tregs in the periphery when activated in the presence of specific signals and cytokine environments [26]. To assess whether iTregs also rely on NFAT1-mediated mechanisms, WT and *Nfat1*$^{-/-}$ Th1 cells were co-cultured with *in vitro* generated iTregs (Fig 2G) and stimulated with APC-OVA. Contrary to what we had seen with tTregs, iTregs efficiently suppressed both WT and *Nfat1*$^{-/-}$ T cells (Fig 2H). Interestingly, iTregs could not induce upregulation of *Ikaros* (Fig 2I). The mechanisms involved in iTreg- and tTreg-mediated suppression appear, thus, to be different, and the requirement for NFAT1 is likely specific for tTregs.

Early nuclear NFAT1 translocation occurs in T cells stimulated in the presence of Tregs

Expression of anergy-inducing genes occurs in response to the activation of NFAT1 in the absence of its major transcriptional partner in activated T cells, AP-1 [10]. Indeed, specific activation of NFAT using the calcium ionophore, ionomycin, induced a marked upregulation of *Ikaros* expression, which was prevented when costimulatory pathways were also activated with PMA (Fig 3A). We, thus, determined

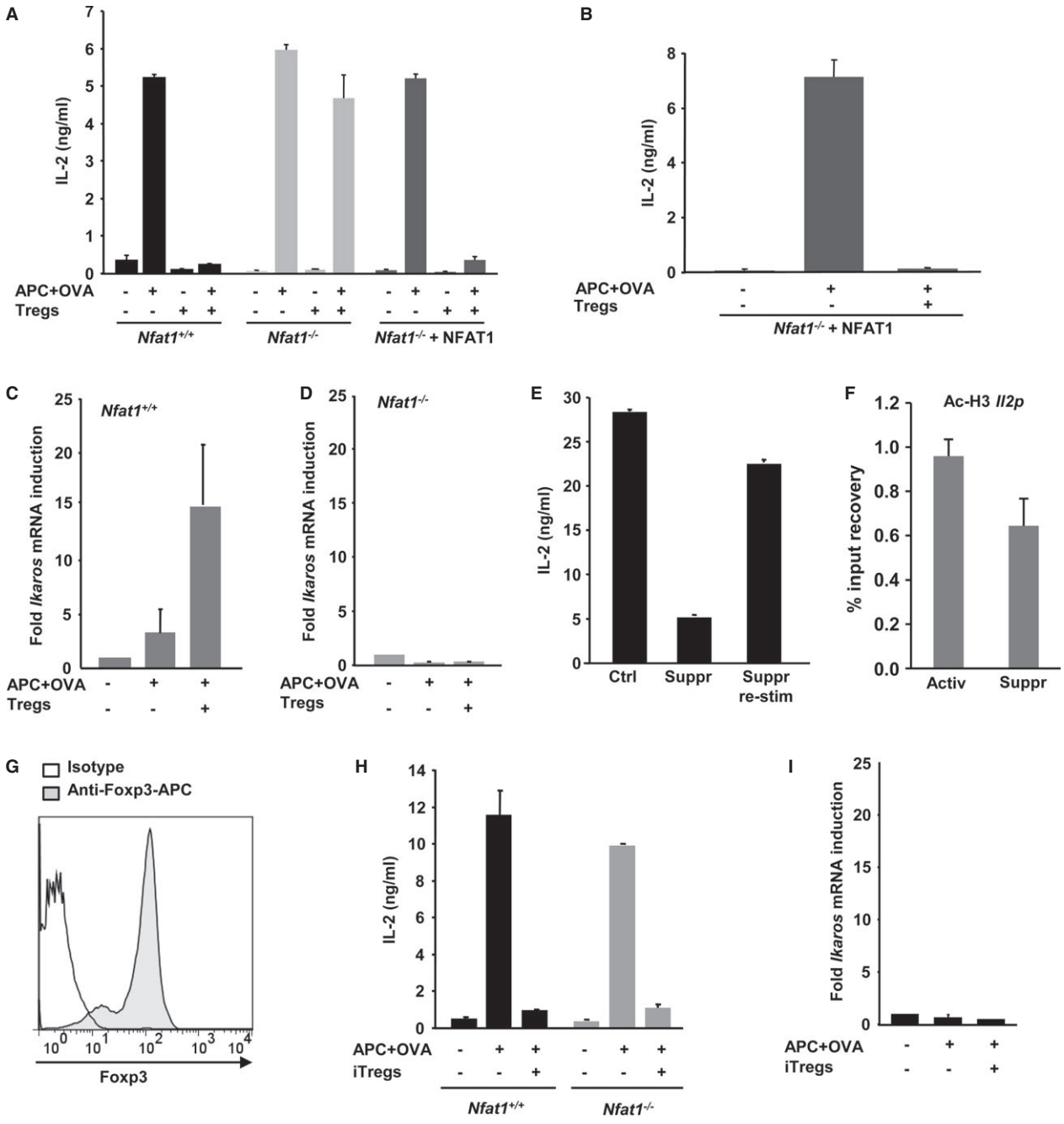


Figure 2. Suppressed CD4$^+$ T cells upregulate the NFAT1-dependent expression of *Ikaros*.

A Th1 cells from WT or *Nfat1*$^{-/-}$ DO11.10 mice transfected with a control (WT and *Nfat1*$^{-/-}$ cells) or NFAT1-expressing (*Nfat1*$^{-/-}$ cells) retrovirus were stimulated with APC-OVA for 48 h with or without WT Tregs (1:1). IL-2 production determined by ELISA (mean + s.e.m. of three experiments).

B *Nfat1*$^{-/-}$ DO11.10 Th1 cells transfected with a plasmid expressing NFAT1 were activated as described in (A) (mean + s.e.m. of three experiments).

C, D WT (C) and *Nfat1*$^{-/-}$ (D) DO11.10 Th1 cells were stimulated with APC-OVA with or without Tregs (1:1) for 12 h. Th1 cells were reisolated and RNA obtained. Results (mean + s.e.m. of three experiments) show *Ikaros* mRNA fold induction relative to resting cells.

E IL-2 expression in DO11.10 Th1 cells activated as described in (A). Th1 cells were reisolated, rested for 4 h, and restimulated (mean + s.e.m. of three experiments).

F WT and *Nfat1*$^{-/-}$ Th1 cells were stimulated with APC-OVA in the presence or absence of Tregs (1:1) for 24 h. Th1 cells were reisolated and Histone H3 acetylation at the *Il2* promoter measured by ChIP (mean + s.e.m. of three experiments).

G FACS analysis of Foxp3 expression in iTregs.

H WT and *Nfat1*$^{-/-}$ DO11.10 Th1 cells were stimulated with APC-OVA in the presence or absence of iTregs (1:1) for 48 h. IL-2 values are mean + s.e.m. from three experiments.

I Th1 cells were activated for 12 h as in (H). Th1 cells were reisolated and RNA obtained. Results (mean + s.e.m. of three experiments) show *Ikaros* mRNA fold induction relative to resting cells.

Source data are available online for this figure.

Figure 3. Early activation of NFAT1 in suppressed T cells.

A Th1 cells were activated in with 1 μM ionomycin or 20 nM PMA + 500 nM ionomycin for 6 h. Expression of *Ikaros* was assessed by qPCR. Values (fold induction relative to resting cells) are mean + s.e.m. from three experiments (*t*-test. $*P < 0.05$).

B Nuclear extracts from DO11.10 Th1 cells stimulated with APC-OVA in the presence or absence of Tregs (1:1). DNA–protein complexes containing NFAT proteins were analyzed by EMSA.

C NFAT1 subcellular localization and c-Jun phosphorylation were determined by immunofluorescence in resting OT-II Th1 (Thy1.2)$^+$ cells or cells treated with ionomycin or stimulated with APC-OVA in the presence or absence of Thy1.1$^+$ CD4$^+$CD25$^+$ Tregs for 4–6 h. DAPI was used to stain nuclei.

D Splenic DCs were loaded with OVA and cocultured for 24 h with OT-II Th1 cells in the presence or absence of Foxp3-RFP$^+$ Tregs. Expression of CD80 and CD86 was determined in CD11c$^+$CD4$^-$ DC populations.

E Th1 cells were activated with plate-bound anti-CD3 with or without soluble anti-CD28 in the presence of increasing numbers of Tregs. Results (mean ± s.e.m. from three experiments) show relative expression of IL-2 compared with the levels detected in the absence of Tregs.

F, G IL-2 production in DO11.10 Th1 cells stimulated with APC-OVA in the presence or absence of Tregs (E) or iTregs (F) (1:1) for 48 h. Where indicated, blocking antibodies for CTLA-4 were also added. Graph shows mean + s.e.m. of three experiments (one-way ANOVA, $*P < 0.05$).

Source data are available online for this figure.

whether NFAT1 would be activated in T cells stimulated in the presence of Tregs. Nuclear extracts prepared from DO11.10 Th1 cells activated for 4 h with APC-OVA in the presence of Tregs were analyzed by EMSA, which showed that nuclear NFAT was present in T cells during Treg-mediated suppression (Fig 3B). To confirm these results in B6 mice, OTII-Thy1.2$^+$ Th1 cells were stimulated in the presence or absence of Thy1.1$^+$ Tregs. After 4 h, cells were analyzed by immunofluorescence. NFAT1 remained cytosolic in resting cells but translocated into the nucleus in Th1 cells that had been activated either in the presence or absence of Tregs (Fig 3C). As mentioned above, it is NFAT1 in the absence of AP-1 activation that induces the expression of tolerance-associated genes, and immunofluorescence analysis revealed that Tregs also prevented c-Jun phosphorylation in T cells (Fig 3C). Although Tregs have been shown to inhibit signaling events downstream of the TCR, including the calcium/NFAT pathway [27], our data show that early after stimulation in the presence of Tregs, T cells can still activate NFAT but show reduced cJun activation. The presence of an active NFAT protein without concomitant activation of AP-1 should allow suppressed T cells to upregulate the NFAT-dependent AP-1-independent expression of Ikaros [10].

It has been shown that Tregs can prevent the expression of B7 proteins on dendritic cells (DCs) to generate costimulation-deficient cells through mechanisms that involve CTLA-4 [6,28]. To determine whether this mechanism could explain our results, we analyzed the expression of CD80 and CD86 in DCs in suppression reactions and saw that Tregs induced a clear downregulation of CD80 and CD86 (Fig 3D). Providing direct costimulation using anti-CD28 antibodies resulted in reduced efficacy of Treg-mediated suppression (Fig 3E). Furthermore, as previously reported [29,30], suppression assays in the presence of a blocking anti-CTLA-4 antibody confirmed that blockade of CTLA-4 prevented Treg-mediated suppression (Fig 3F); the blocking antibodies had however no effect on the activity of iTregs (Fig 3G). Our data do not exclude that signals directly transmitted by Tregs to effector T cells may also mediate the activation of regulatory gene expression. Supporting this possibility, upregulation of *Grail* was described when naïve T cells were activated just with anti-CD3 antibodies in the presence of Tregs [19]. Also, direct engagement of B7 proteins on effector T cells by CTLA-4 expressed on Tregs has been proposed as a mechanism of suppression [31].

Ikaros activity is required for optimal Treg-mediated suppression

Ikaros has been shown to inhibit IL-2 expression in CD4$^+$ T cells [21,22]. Thus, we assessed Ikaros' role in the Treg-mediated suppression of IL-2 expression. Our results showed that Th1 cells transfected with a plasmid expressing a dominant-negative form of Ikaros (IK-DN) mimicked NFAT1-deficient T cells and became more refractory to Tregs (Fig 4A). Similar results were obtained using CD4$^+$CD25$^-$ T cells isolated from transgenic mice expressing IK-DN (Fig 4B). Furthermore, WT Tregs could not suppress wasting induced by the transfer of naïve IK-DN T cells into *Rag1*$^{-/-}$ hosts as efficiently as the one caused by transfer of WT T cells (Fig 4C). These results confirmed that the activity of Ikaros in CD4$^+$ T cells is required for efficient Treg-mediated suppression. While necessary, this, however, may not be the only mechanism that represses *Il2* transcription in suppressed T cells. Treg-mediated activation of ICER/CREM complexes in effector T cells has also been shown to participate in the inhibition of IL-2 production [32]. The possibility remains that these complexes may also regulate Treg-mediated suppression by directly modulating NFAT activity in suppressed T cells [33].

Overall, our results show that NFAT1-regulated gene expression is required for efficient Treg-mediated suppression of CD4$^+$ T cells. These experiments duly correlate NFAT1 activation in suppressed T cells with the expression of T-cell inactivating genes and identify Ikaros as a regulator of suppression that is required for optimal inhibition of T-cell effector function, thereby providing a basis for how Tregs maintain Th cells in a suppressed state.

Materials and Methods

Mice

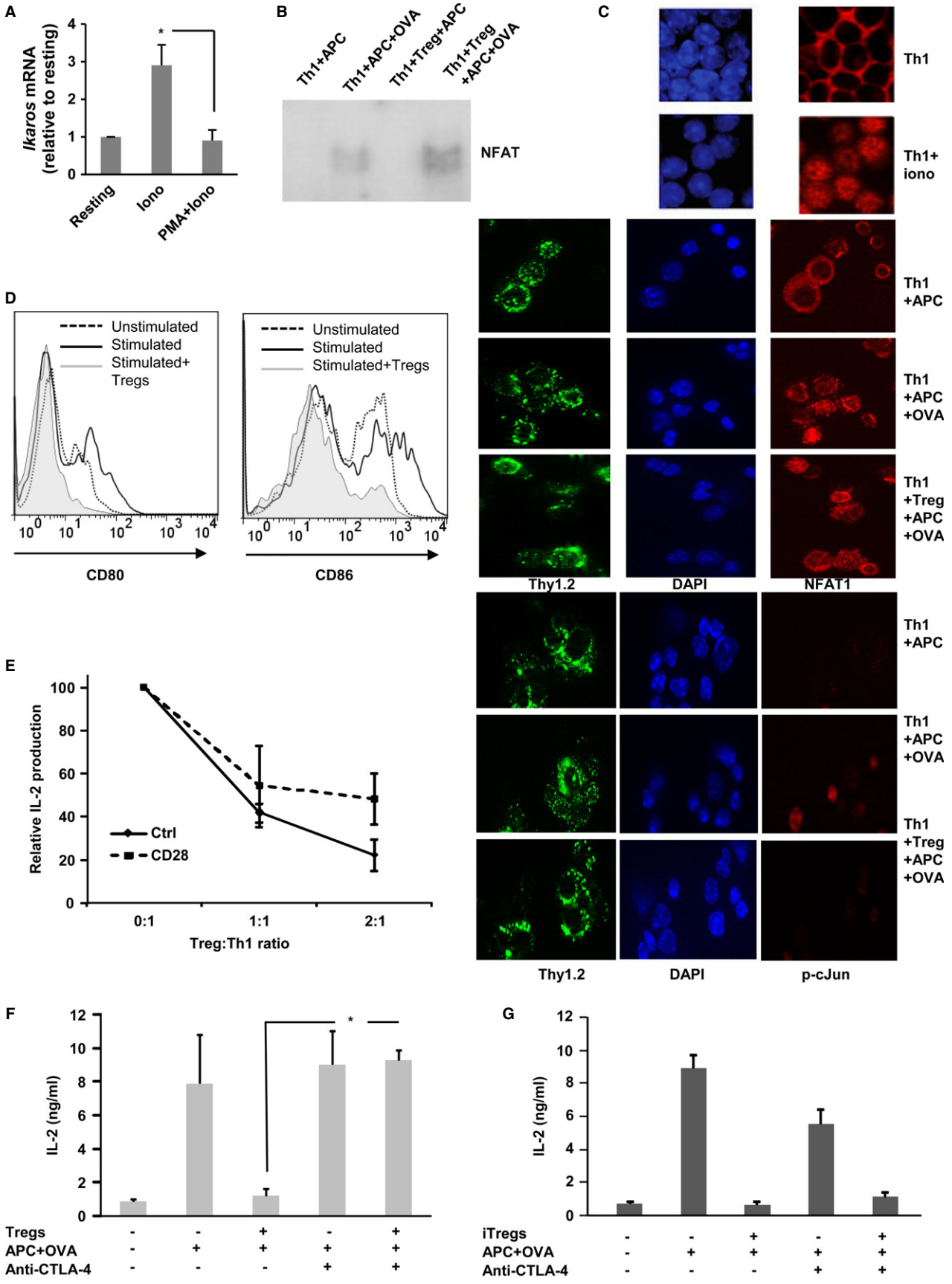
Strains description is available in Supplementary Methods.

T-cell isolation

CD4$^+$ and CD4$^+$CD25$^+$ cells were isolated from the spleen and lymph nodes of mice using magnetic beads coupled to anti-CD4 or anti-CD25 antibodies (Life Technologies). Alternatively, Foxp3$^+$ Tregs were isolated from *Foxp3-RFP* mice by sorting for CD4 and RFP expression.

In vitro Th1 differentiation

CD4$^+$ T cells were differentiated *in vitro* as previously described [22]. Details in Supplementary Methods.



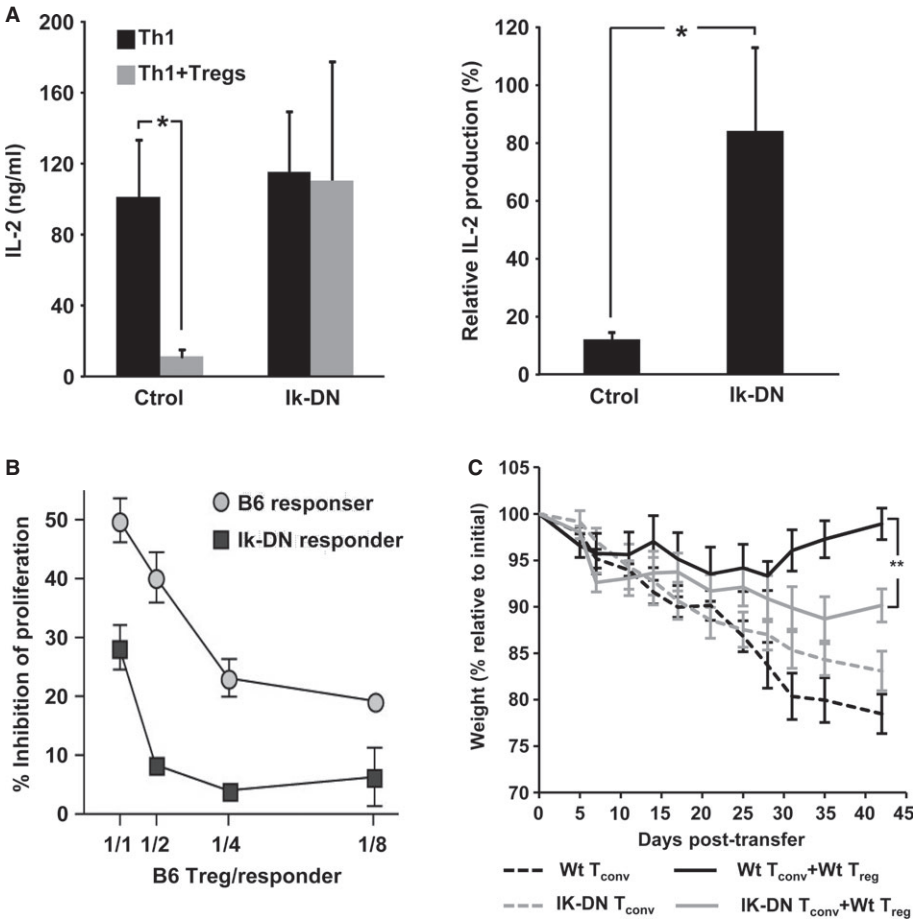


Figure 4. Treg-mediated suppression requires Ikaros activity.

A Th1 cells were transfected with a plasmid expressing IK-DN or a control vector and activated in the presence of Tregs (1:1). Percent suppression of IL-2 expression was calculated (mean + s.e.m. from four experiments, *t*-test, $*P < 0.05$).

B B6 or B6-IkDN/+ $CD4^+CD25^-$ T cells (Thy1.2$^+$) were labeled with CFSE and activated with APC and soluble anti-CD3 in the presence of increasing amounts of $CD4^+CD25^+$ Treg from B6-Thy1.1$^+$ mice. After 3 days, suppression of responder cell proliferation was determined by FACS, assessing the degree of inhibition of CFSE dilution (mean ± s.e.m.) from two experiments.

C $CD25^-CD4^+$ T_{conv} cells from WT or IK-DN B6 mice were transferred into B6-*Rag1*$^{-/-}$ mice. Twenty days later, mice received PBS or $CD25^+$ $CD4^+$ Treg purified from WT donors. Recipients were weighed (mean ± s.e.m.) and observed for symptoms of diarrhea approximately every 2 days (5 mice per group. one-way ANOVA $^{**}P < 0.01$).

Source data are available online for this figure.

Treg expansion

Tregs were stimulated with 0.25 μg/ml immobilized anti-CD3 and anti-CD28 for 24 h and cultured for 1 week in the presence of 200 U/ml murine IL-2 (Cell Signaling).

iTreg generation

Naive $CD4^+$ T cells were stimulated with 0.5 μg/ml immobilized anti-CD3 and anti-CD28 in the presence of IL-2 (50 U/ml) and TGFβ-1 (3 ng/ml) (eBioscience) for 1 week.

In vitro suppression assays

DO11.10 or OTII effector T cells were stimulated for 24–72 h using T-cell-depleted splenocytes (APCs) loaded with 0.1–1 μM OVA in the presence of pre-activated Tregs isolated from BALB/c, C57BL6, or *Foxp3-RFP* mice (anti-$CD3^+$ anti-CD28 for 24 h before the assay). Where indicated, 10 μg/ml anti-CTLA-4 antibody (UC10-4B9. Biolegend) was added. Alternately, CFSE-labeled $CD4^+CD25^-$ T cells were activated with APCs and 0.5–4 μg/ml soluble anti-CD3 in the presence of different amounts of purified Tregs from C57BL/6 Thy1.1 or *Foxp3-RFP* mice, for 72 h. Responder cells were identified as $CD4^+Thy1.1^-$ or $CD4^+RFP^-$, and suppression of proliferation was determined by assessing inhibition of CFSE dilution.

In vivo suppression assays

2.5×10^6 $CD4^+CD25^-$ T cells from Thy1.2 WT or *Nfat1*$^{-/-}$ mice were adoptively transferred into Thy1.2 *Rag1*$^{-/-}$ B6 mice i.p. with or without 5×10^5 Thy1.1 $Foxp3^+RFP^+$ Tregs. Seven days post-transfer, splenic T cells were identified as $CD3^+CD4^+Thy1.1^-$.

Alternatively, to induce experimental colitis, 1×10^6 $CD4^+CD25^-$ T cells from C57Bl/6 or B6-Ik-DN mice were adoptively transferred i.p. into *Rag1*$^{-/-}$ B6 recipients. Twenty days later, groups of six mice received PBS or 0.25×10^6, i.p. $CD25^+CD4^+$ WT Tregs. Recipients were weighed and observed for symptoms of diarrhea approximately every 2 days.

ELISA and immunofluorescence

See Supplementary Methods for detailed description.

Transfection and retroviral infection of T cells

T cells were transfected by electroporation using a nucleofector electroporator (Amaxa). Retrovirus expressing NFAT1 and conferring G418 resistance (from an IRES) were generated using the Phoenix ecotropic packaging cell line (A gift from Dr G. Nolan, Stanford University, CA). Viral supernatants were used to infect T cells by spin infection at a minimal ratio of five virions/cell. Cells were grown for 1 week in 500 μg/ml G418 to allow selection of positively infected T cells.

Semiquantitative real-time PCR (qPCR)

qPCRs were performed as previously described [22]. Detailed description and primer sequences are provided in Supplementary Methods.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from resting, stimulated, or suppressed cells. NFAT-specific oligonucleotides (5′-AGCTAGCT-AGGAATATTCCTGGATGATC) were labeled with [^{32}P]ATP and incubated with nuclear extracts (3–5 μg) and 100 ng/μl poly(dI-dC) for 20 min [14]. DNA–protein complexes were resolved in polyacrylamide gel electrophoresis on a 4% non-denaturing gel.

Chromatin immunoprecipitation (ChIP) assays

ChIp assays suppressed and activated Th1 cells were performed as previously described [22]. See Supplementary Methods for detailed description and primer sequences.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Differences between multiple groups were analyzed by one-way ANOVA with a Tukey's *post hoc* test. Comparisons between data pairs were analyzed using a paired *t*-test (two-tail distribution) or a Mann–Whitney test as indicated in each figure.

Supplementary information for this article is available online: http://embor.embopress.org

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

DSS, AJ, SB, RMT, SB, and EFZ performed the experiments. ADW and FM designed the studies. DSS and FM wrote the paper. All authors contributed to the analysis of the data and approved the manuscript.

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