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Comment on "Constitutive Nuclear Localization of NFAT in Foxp3 $^{+}$ Regulatory T Cells Independent of Calcineurin Activity"

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Letters to the Editor

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Comment on "Constitutive Nuclear Localization of NFAT in Foxp3$^+$ Regulatory T Cells Independent of Calcineurin Activity"

Recently, Li et al. (1) reported that a fraction of NFATc2 and NFATc1 proteins is constitutively localized in the nuclei of primary regulatory T (Treg) cells. In contrast to our own experience, the authors claimed that this NFAT translocation in resting Treg cells is independent of calcineurin activity. Therefore, we studied the proportion of phosphorylated and dephosphorylated NFATc2 in whole cell lysates of sorted primary conventional T (Tcon) cells and Treg cells.

Resting murine, but not human, Treg cells indeed possessed a small portion of dephosphorylated NFATc2 (Fig. 1A and 1B, *lane 1*). However, all CsA-treated samples, unstimulated Tcon and Treg cells, as well as stimulated Tcon cells serving as controls, showed almost no dephosphorylated NFATc2 both in murine (Fig. 1A, *lanes 2*, *6*, *4*) and in human primary cell lysates (Fig. 1B, *lanes 3*, *6*, *4*, *8*). Accordingly, CsA-treated unstimulated Treg cells showed no dephosphorylated NFATc2 in nuclear extracts of murine cells and slightly lower MFI of NFATc2 in nuclei of human cells.

Altogether, the main message that constitutive nuclear localization of NFATc2 is independent of calcineurin activity in Treg cells (1) could not be confirmed by our data.

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Response to Comment on "Constitutive Nuclear Localization of NFAT in Foxp3$^+$ Regulatory T Cells Independent of Calcineurin Activity"

Our finding of constitutive nuclear localization of NFAT in regulatory T cells (Tregs) was based on results from Western blots, confocal microscopy, chromatin-immunoprecipitation, and functional assays in cell culture (1). Western blots detected NFATc1 and NFATc2 in nuclear preparations isolated from resting or cyclosporin (CsA)-treated Tregs, and nuclear localization was directly visible by confocal microscopy. Chromatin immunoprecipitation using anti-NFATc1 and -NFATc2 Abs demonstrated nuclear NFAT in Tregs selectively bound to Foxp3 target genes, whereas nuclear NFAT in activated non-Tregs was bound to a different a set of genes. Consistent with these results, CsA preferentially

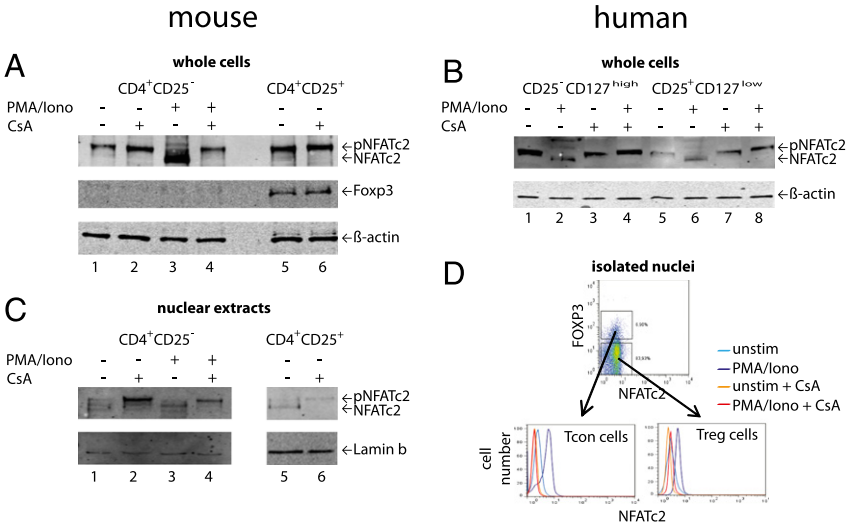

FIGURE 1. Sorted murine CD4$^+$CD25$^-$ (Tcon) and CD4$^+$CD25$^+$ (Treg) or human CD25$^-$CD127^{high} (Tcon) and CD25$^+$CD127^{low} (Treg) cells were preincubated with 100 nM CsA or DMSO (control) for 30 min, and stimulated with PMA/ionomycin for 30 min or left unstimulated. (**A**–**C**) Immunoblot analysis. (**D**) Flow cytometric measurement of isolated nuclei (method described in Ref. 2). Representative data out of three experiments each.

blocked anti-CD3/CD28–induced activation/proliferation of non-Tregs, but not Tregs, in the presence of exogenous IL-2. Although the mechanisms responsible for constitutive NFAT nuclear translocation remain to be elucidated, several possibilities were proposed, including NFAT dephosphorylation by phosphatases other than calcineurin, attenuated NFAT phosphorylation due to decreased phosphokinase activity, or stable binding to Foxp3, which retains NFAT in the Treg nucleus regardless of phosphorylation status.

Scheel et al. present results that they believe contradict our findings. In Fig. 1A, the authors show a small fraction of dephosphorylated NFATc2 in the whole cell lysate of resting Tregs, which disappears following CsA treatment. In CsA-treated human Tregs, no dephosphorylated NFATc2 is identified (Fig. 1B). In nuclear extracts of resting Tregs, both dephosphorylated and phosphorylated NFATc2 are detected, but in the nuclear extract of CsA-treated Tregs, only phosphorylated NFATc2 is detected (Fig. 1C). The disappearance/absence of dephosphorylated NFATc2 in the CsA-treated Tregs led the authors to conclude that there is no calcineurin-independent constitutive nuclear NFAT in Tregs.

This conclusion discounted the presence of phosphorylated nuclear NFATc2 identified in the CsA-treated Tregs as shown in their Fig. 1C (*lane 6*), which stands as direct evidence of calcineurin-independent nuclear NFATc2. Apparently, this evidence was overlooked because the authors were focused on dephosphorylated NFATc2 as evidence for nuclear NFAT. Therefore, the results of Scheel et al. are in fact supportive of rather than contradictory to our findings. As indicated in our article (1), the phosphorylation status of the constitutive nuclear NFAT in Tregs is yet to be determined. If the constitutive nuclear NFAT in Tregs is indeed phosphorylated, it would provide a mechanistic explanation for its CsA-resistant nuclear translocation in Tregs. The nature of the CsA-sensitive dephosphorylated NFATc2 shown in Fig. 1A and 1C is unclear. There is a possibility that these NFATc2 species represent calcineurin-dephosphorylated NFATc2 due to inadvertent activation of the Tregs. This possibility is supported by the presence of nuclear NFAT in the "unstimulated" non-Treg cell shown in Fig. 1C (*lanes 1* and *5*). These unstimulated cells appear to be activated because in truly unstimulated/resting non-Treg cells, NFATc2 should reside in the cytoplasm (2).

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