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Cutting Edge: Regulatory T Cells Selectively Attenuate, Not Terminate, T Cell Signaling by Disrupting NF-κB Nuclear Accumulation in CD4 T Cells

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A key consequence of regulatory T cell (Treg) suppression of CD4 T cells is the inhibition of IL-2 production, yet how Tregs attenuate IL-2 has not been defined. Current models predict a termination of TCR signaling, by disrupting T–APC contacts, or TCR signal modification, through mechanisms such as cAMP. To directly define Treg effects on TCR signaling in CD4 T cell targets, we visualized changes in nuclear accumulation of transcription factors at time points when IL-2 was actively suppressed. Nuclear accumulation of NFAT was highly dependent on sustained TCR signaling in the targets. However, in the presence of Tregs, NFAT and AP-1 signals were sustained in the target cells. In contrast, NF-κB p65 was selectively attenuated. Thus, Tregs do not generally terminate TCR signals. Rather, Tregs selectively modulate TCR signals within hours of contact with CD4 targets, independent of APCs, resulting in the specific loss of NF-κB p65 signals. The Journal of Immunology, 2012, 188: 947–951.

Natural regulatory T cells (Tregs) counterbalance immunity by suppressing cell proliferation, survival, maturation, cytokine and/or chemokine production, and release of cytotoxic components from granules. This broad suppressive capacity is likely exerted by different mechanisms at different stages of immune activation (1, 2). Fundamental to initial T cell activation is the receipt of signals that promote cytokine production, cell proliferation, and cell survival. IL-2 is the first cytokine produced by naive T cells and is critical for successful adaptive immunity (3). Upon TCR engagement, the nuclear accumulation of NFAT, NF-κB, and AP-1, in concert, drives early IL-2 transcription (4, 5). CD28 costimulatory signaling quantitatively changes TCR signaling, enhancing NF-κB and AP-1 to promote transcription and stabilizing IL-2 mRNA (6, 7). Tregs suppress T cell activation by inhibiting cell proliferation and cytokine production, in particular early IL-2 production (8).

We mapped Treg suppression of IL-2 at the transcript and protein level to a tight kinetic window 6–10 h after initial CD4 T cell activation (9). However, the mechanism by which Tregs specifically abort IL-2 production remains unknown.

Tregs could negatively regulate T cell signals for IL-2 via CTLA-4/B7 (10) or cAMP (11) or potentially by using E3 ligases (12, 13). Alternatively, recent visualization of Treg suppressive events suggested that Tregs could terminate T cell signals by disrupting the stability or duration of CD4 T cell–APC interactions (14, 15). To define the changes in T cell signaling in target CD4 T cells activated in the presence of Tregs, we used multispectral imaging flow cytometry (Amnis ImageStream) to quantify the frequency of CD4 T cells with specific transcription factor (TF) nuclear accumulation. Tregs did not terminate T cell signaling at the time of IL-2 inhibition. Rather, signaling in targeted CD4 T cells was selectively modified by attenuation of nuclear NF-κB, but not NFAT and AP-1, through an APC-independent mechanism.

Materials and Methods

Mice and Abs

BALB/c mice (National Cancer Institute) and Thy1.1 BALB/c mice were maintained in the pathogen-free animal facility at the University of Rochester Medical Center (Rochester, NY). Abs used: mouse anti-NFAT1 IgG1 (Affinity BioReagents); mouse anti-NFAT2 IgG1, rabbit anti-p65 IgG, rabbit anti-κ Rel IgG, rabbit anti-c-Fos IgG, and rabbit anti-c-Jun IgG (Santa Cruz Biotechnology); FITC goat F(ab’)2, anti-rabbit IgG and FITC goat anti-mouse IgG1 (Southern Biotech); anti-Thy1.1 eFluor 450 (eBioscience); anti-Thy1.2 PE (BD Pharmingen).

Cell purification

CD4 T cells were isolated from spleen and lymph nodes. CD4+CD25−CD44low naive T cells were sorted by FACSAria as a source of target CD4+ T cells or control T cells (Ctrl T). Tregs were purified from a CD4-enriched population using a CD25+ MACS column (Miltenyi Biotec) (routinely >85% Foxp3+, with >85% suppression of CD4 proliferation at 1:1 target/Treg ratio). APCs were isolated from spleen by complement lysis of Thy1.2-expressing T cells. Confirmatory experiments were performed using sorted CD4+CD25+Foxp3+/GFP+ cells from Foxp3/GFP reporter mice.

Treg-suppression assay

A total of 1 × 10⁵ Thy1.1 naive target CD4+ T cells was stimulated with anti-CD3 mAb (1 µg/ml) and APCs (1 × 10⁵) in coculture with either 1 × 10⁵
Thy.1.2 Ctrl T or Thy.1.2 Tregs. Cells were harvested for functional assays at various time points. In some experiments, Thy.1 responder cells were pre-treated with 1 mM cAMP antagonist, Rp-8-Br-cAMPs (BioLog Life Science Institute), or the Src kinase inhibitor PP1 (10 μM, Axorra) was added to cultures. In some experiments, suppression was assayed following Ab-coated bead stimulation (16). M450 Dynabeads (Invitrogen) were coated with anti-CD3 (2 μg/25 μl beads) and anti-CD28 (2 μg/25 μl beads). A total of 4 × 10^6 Ab-coated beads was used to stimulate 1 × 10^5 naive target CD4^+ T cells in coculture with 1 × 10^5 Ctrl T or Tregs. At 12 h, cells were collected for p65 nuclear localization analysis (Imagestream) and IL-2 secretores by cytokine-secretion assays (Miltenyi Biotec), according to the manufacturer’s instructions. For phospho-flow, cells were fixed, permeabilized, and stained with anti-pERK mAb, according to the manufacturer’s instructions (BD Bioscience).

**Flow cytometry**

IL-2 secretores were detected by a cytokine secretion assay kit (Miltenyi Biotec), according to the manufacturer’s instructions. For phospho-flow, cells were fixed, permeabilized, and stained with anti-pERK mAb, according to the manufacturer’s instructions (BD Bioscience).

Nuclear translocation analysis on Imagestream

Cells were fixed (1% paraformaldehyde), surface stained for Thy1.1/1.2, permeabilized by 0.1% Triton X-100 (Sigma), and stained for NF-κB p65, NF-κB c-Rel, NFAT1, NFAT2, c-Fos, or c-Jun. Nuclear dye Draq5 (5 μM; Axxora) was added before analysis. Fluorescent images were visualized (>6000 events per condition) on Amnis Imagestream. A mask on the nucleus was created; within this area, colocalization of TFs and nuclear dye was measured by similarity (IDEAS software, Amnis).

DNA-binding assay

Treg suppression assay was set up as described above. At 6 h, CD4^+Thy1.1^ target cells were positively selected on Thy1.2 and lystate, and nuclear extracts were prepared (Active Motif). p65 DNA binding was quantified by TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif). Briefly, 2 μg nuclear extracts was loaded onto a 96-well plate coated with NF-κB consensus sequence, followed by anti-p65 Ab and HRP detection at 450-nm absorbance. The relative amount of p65 bound to DNA was expressed as OD.

**Results and Discussion**

*T cell signals and nuclear accumulation of TFs*

To accurately interpret Tregs effects on target T cell signaling, we first determined the relationship between continued T cell signaling and the nuclear accumulation of TFs key to IL-2 transcription. We performed single-cell analysis of TF nuclear localization using multispectral imaging flow cytometry (17). Nuclear localization was defined by a positive similarity score, representing the correlation coefficient between two fluorescent signals: the relative colocalization of the nuclear dye Draq5 and the TF NFAT2 or NF-κB p65 (Fig. 1A, 1B). Unstimulated CD4^+ T cells had a negative similarity score, corresponding to the absence of nuclear NFAT2 and NF-κB (Fig. 1A, 1B).

After 6 h of activation, most CD4^+ T cells were positive for nuclear NFAT2 (Fig. 1A, 1B) and a proportion of CD4^+ T cells exhibited nuclear localization of NF-κB (Fig. 1A, 1B). The Src kinase inhibitor PP1 ablated the nuclear localization of NFAT2 and NF-κB p65, showing the dependency on TCR/CD28 signaling (Fig. 1A). Kinetically, nuclear NFAT2 and p65 peaked at 6 h; NFAT2 was sustained through 12 h, whereas p65 declined (Fig. 1C). To understand the dynamics of nuclear NFAT and TCR signaling, we blocked Src kinase signaling at the peak of TF nuclear accumulation (6 h) and followed the frequency of cells with a nuclear pool of NFAT2 and p65 (Fig. 1D). Nuclear NFAT2 was very sensitive to termination of TCR signaling: an 80% loss in nuclear NFAT^2+ cells was noted within 1 h of PP1 addition. In contrast, p65 was relatively stable in the nucleus, with only 10.36% loss of nuclear NF-κB^+ cells within 1 h of PP1 addition, possibly reflecting non-TCR signals sustaining nuclear NF-κB p65. This single-cell assay for detection of nuclear TFs provides a sensitive platform for the detection of possible perturbations in TCR signaling mediated by Tregs.

**Sustained NFAT and AP-1 signaling in the presence of Tregs**

To determine whether Tregs broadly extinguish target T cell signaling at the time of IL-2 inhibition, we first examined TCR-dependent nuclear NFAT in the target CD4^+ T cells in coculture with Tregs or Ctrl T, non-Treg CD4 T cells (Fig. 2A, 2B). The presence of Tregs had no effect on the magnitude or timing of NFAT nuclear accumulation (Fig. 2A, 2B) at time points when IL-2 production was suppressed (Fig. 2C, 2D). Thus, IL-2 is downregulated, despite ongoing TCR-dependent signals that support NFAT nuclear accumulation. Tregs also did not change nuclear accumulation of AP-1 components c-Fos and c-Jun (Fig. 2A, 2B). The AP-1 complex is largely regulated at the protein level; therefore, we also analyzed c-Fos or c-Jun total protein in target T cells in coculture with Tregs and found no change compared with targets cultured with Ctrl T (Supplemental Fig. 1A).

The sensitivity of nuclear NFAT to perturbations in TCR signaling (Fig. 1D) and the absence of Treg-induced changes in nuclear NFAT (Fig. 2) both suggested that CD4^+ target T cell signaling remains largely intact in the presence of Tregs at the time of IL-2 regulation (Fig. 2C). To confirm ongoing upstream signaling, we used phospho-flow to measure kinase ac-

**FIGURE 1.** TCR-dependent nuclear localization of TFs. CD4^+ T cells were stimulated with anti-CD3/APCs. A. At 6 h, cells were stained for NF-κB p65 (lower panel) or NFAT2 (upper panel). Graphs gated on CD4^+ T cells and percentages of activation-induced nuclear accumulation are shown. B. Representative cell images using Imagestream. Stained with anti-NFκB or anti-NFAT mAb and the nuclear dye Draq5. Original magnification ×40. C. Kinetic analysis. Graphs show mean and SEM of the percentage of cells with similarity scores >0.5 from three independent experiments. D. CD4 cells were stimulated for 6 h, as in A, before addition of PP1. Cells were analyzed for nuclear TF and normalized to target cells in the absence of PP1; mean and SEM from three independent experiments.
tivation. Under our stimulation conditions, we were unable to detect increases in p-PLCγ or p-Zap70 in the 6–12-h time-frame. However, ERK signaling was readily detectable from 1 h after anti-CD3/APC stimulation (Fig. 2D). The presence of Tregs did not interfere with ERK signaling in the target T cells, despite concomitant inhibition of IL-2 (Fig. 2C, 2D).

**Tregs attenuate nuclear accumulation of NF-κB in CD4+ target T cells**

In contrast to NFAT and AP-1, Tregs significantly attenuated the frequency of target T cells with nuclear NF-κB p65 at 6 h after activation and frequencies had returned to unstimulated levels by 12 h (Fig. 3A, 3B). The reduction in nuclear p65 was not due to a decrease in total cellular p65 (Fig. 3C), indicating that differential generation or targeted degradation of NF-κB p65 was unlikely. An NF-κB DNA-binding assay confirmed a Treg-induced loss in nuclear NF-κB activity in CD4 targets (Fig. 3D). Interestingly, Treg attenuation of NF-κB activity was selective for p65 and was not seen for c-Rel (Supplemental Fig. 1A), suggesting that Tregs may target the p65 complexed to IκBα, rather than the c-Rel–IκBα complexes, predominant in naïve cells (18). IL-2R signaling can regulate NF-κB; thus, Tregs could modulate NF-κB by limiting IL-2 availability (19, 20). Addition of exogenous IL-2 failed to rescue the Treg-mediated change in p65 (Supplemental Fig. 1C).

**Rapid NF-κB downregulation independent of APCs**

Full effector function requires continued signaling for ≥10 h (21). To determine whether Tregs can acutely regulate T cell activation, we initiated CD4+ T cell cultures in the absence of APCs, which was not due to acute IL-2 consumption (data not shown). It is not known how the availability of individual TFs correlates with the magnitude of IL-2 production in CD4 T cells. Therefore, we dual labeled cells to examine the relationship between the loss of nuclear NF-κB and IL-2 production (Fig. 4D). IL-2 production early after activation was found in a small fraction of CD4+ T cells within the population showing an activation-induced increase in nuclear p65.
Thy1.2+ Tregs were added to the culture, and the targets were analyzed for nuclear NFAT2 (experiments.* FTregs were added to upper chamber or lower chambers. Frequency of targets cells with nuclear p65 at 12 h. Mean and SEM from three independent experiments. Representative plots from one of three independent experiments. (positive similarity score, Fig. 4D). However, there was no positive correlation between the degree of nuclear p65 (similarity score) and the amount of IL-2 produced (Fig. 4D). The data suggested that a threshold amount of nuclear NF-κB is required for IL-2 gene competency but that the degree of nuclear NF-κB does not control the magnitude of the IL-2 response. Therefore, by reducing the number of cells that reach this nuclear NF-κB threshold (Fig. 4D), Tregs appear to limit the number of IL-2–producing cells.

Mechanistically, Tregs could modulate NF-κB directly in the target T cell or indirectly via the APC. A Transwell experiment confirmed that NF-κB was only modulated when Tregs were in close proximity to targets and APCs (Fig. 4E). To test the requirement for APCs, we stimulated target T cells with anti-CD3/CD28–coated beads (16) with or without Tregs, using conditions in which Tregs successfully inhibited IL-2 (Supplemental Fig. 2D, 2E). In the absence of APCs, Tregs retained the ability to inhibit nuclear NF-κB in target T cells (Fig. 4F). Tregs also retained the ability to attenuate NF-κB in cultures with fixed APCs (data not shown). Thus, Tregs rapidly and selectively attenuated NF-κB T cell activation signals in CD4 targets, independent of the APCs.

Our results suggested that models of Treg action whereby Tregs modulate the frequency or duration of T–APC conjugation (14, 15) cannot fully account for the early inhibition of IL-2. Rather, qualitative changes in T cell signaling, with a decrease in available nuclear NF-κB, appear to underlie early suppressive events. Interestingly, an in vivo study using an NF-κB luciferase reporter also showed decreased pathogen-induced NF-κB activation with Tregs present (22). The targeting of such a fundamental signaling pathway by Tregs suggested that attenuated NF-κB may account for the ability of Tregs to modulate the activities of many cell types, from mast cells to B cells (1, 23, 24). Lymphocytes may be particularly sensitive to Treg downregulation of NF-κB p65 if costimulation or inflammatory cytokines are limited (self-Ag) but would override suppression when costimulatory signals, particularly CD28/NF-κB or TNF-α, are upregulated.

The downstream consequence of reduced NF-κB in CD4 T cells has not been established. We do not know whether there is a direct linkage between Treg-attenuated NF-κB and loss of IL-2. Once a threshold level of nuclear NF-κB is achieved, the amount of nuclear NF-κB p65 does not impact the magnitude of the IL-2 response. Therefore, Tregs appear to limit the number of T cells reaching that nuclear NF-κB threshold. Loss of NF-κB could modify the expression of a number of antiapoptotic molecules, such as Bcl-2, and cell cycle-promoting molecules, like CDK (25), leading to the indirect loss of early IL-2 producers as a result of a failure to support their survival. Alternatively, reduced NF-κB could lead to an alteration in the balance of TFs, with a predominant NFAT signal. NFAT signals trigger an anergy-related gene profile, with the upregulation of E3 ligase-associate genes (26). Interestingly, we showed that Tregs induced a unique transcriptional program in CD4 targets that showed the most overlap with ionomycin-induced anergy (27).

We have shown that Tregs do not terminate T cell–activation signals but instead induce a unique signaling program in CD4+ target T cells with sustained NFAT/AP-1 but significantly reduced nuclear NF-κB p65. This early NF-κB modulation is independent of the APCs. We propose that Treg suppression occurs in distinct mechanistic phases, with early modulation that can occur in an APC–independent fashion, qualitatively altering T cell signaling, and a later phase during which modulation of the APCs may terminate T–APC interactions. Thus, the context in which CD4+ T cells encounter Tregs may make them differentially sensitive to these two phases and, hence, account for the heterogeneity and controversy in the timing and proposed mechanisms of Treg suppression.
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Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure 1. Specificity of Tregs regulation of TFs and lack of a role for IL-2 consumption or cAMP. Naïve Thy1.1+ CD4+ target T cells were co-cultured with Thy1.2+ Ctrl T cells or Tregs. After anti-CD3/APC stimulation, cells were collected at various time points and transcription factors NFAT1, NFAT2, c-Fos, c-Jun and c-Rel, were analyzed on Imagestream.

A) Total amount of transcription factors were determined by measuring total intensity of transcription factors in Thy1.1+ target cells. Mean and SEM from 3 independent experiments were shown.

B) No change in c-Rel activation in target T cells in the presence of Tregs. Percentage of nuclear c-Rel (left) and total intensity of c-Rel (right) in Thy1.1+ target cells were shown.

C) Treg suppression of p65 was IL-2 independent. Treg suppression assay was set-up as above in the presence or absence of exogenous IL-2. Data are expressed as % of control CD4+ target T cell cultures with nuclear NFκB p65 at 12h. Tregs and Tregs +IL-2 not significantly different. Mean and SEM from 3 independent experiments were shown. Not significantly different (ns), 2-tailed student t.

D-F) Thy1.1+ CD4+ target T cells were pre-treated with cAMP antagonist and then cultured with Thy1.2+ control T cells or Tregs. 6hr and 12hr after anti-CD3 mAb stimulation, cells were collected and analyzed for NFκB and IL-2 secretion respectively (Miltenyi cytokine secretion assay). D) Representative plots of nuclear NFκB at 6hr gated on Thy1.1+ target T cells, one of two independent experiments. E) Representative dot plots of IL-2 secretors at 12 h gated on Thy1.1+ target T cells. F) Percentage of IL-2 suppression. Mean and SEM from 4 independent experiments. Not significantly different (ns), 2-tailed student t.
Supplementary Figure 2. CD28 co-stimulatory signals and the role of APC in Treg suppression of p65 and IL-2. Naïve Thy1.1+ CD4+ target T cells were co-cultured with Thy1.2+ Ctrl T cells or Tregs. Cells were either stimulated with anti-CD3/APC or anti-CD3 plus anti-CD28/APC. Anti-CD28 was given at a high concentration (1 μg/ml) to provide significant co-stimulatory signals with increased p65. Thy1.1+ target T cells were then analyzed by p65 nuclear localization assay on Imagestream at 6h (A and B) or IL-2 cytokine secretion assay at 12h (C). A) Representative data from one of 3 experiments. B) and C) Mean and SEM from 3 independent experiments were shown. * p<0.05 by paired 2-tailed student t test.

D-E) Naïve Thy1.1+ CD4+ target T cells (CFSE labeled) were co-cultured with Thy1.2+ Ctrl T cells or Tregs and stimulated by anti-CD3/CD28 coated beads at various bead per well concentrations. D) Proliferation was measured by CFSE-dilution at 72h of culture. B) IL-2 secretion at 12h of culture. One of 2 independent experiments was shown.