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Defective Activation of Protein Kinase C and Ras-ERK Pathways Limits IL-2 Production and Proliferation by CD4⁺CD25⁺ Regulatory T Cells

Somnia P. Hickman,* Jaeseok Yang,* Rajan M. Thomas,† Andrew D. Wells,† and Laurence A. Turka²*†

Naturally occurring CD4⁺CD25⁺ regulatory T cells (Tregs), which play an important role in the maintenance of self-tolerance, proliferate poorly and fail to produce IL-2 following stimulation in vitro with peptide-pulsed or anti-CD3-treated APCs. When TCR proximal and distal signaling events were examined in Tregs, we observed impairments in the amplitude and duration of tyrosine phosphorylation when compared with the response of CD4⁺CD25⁻ T cells. Defects were also seen in the activity of phospholipase C-γ and in signals downstream of this enzyme including calcium mobilization, NFAT, NF-κB, and Ras-ERK-AP-1 activation. Enhanced stimulation of diacylglycerol-dependent pathways by inhibition of diacylglycerol metabolism could overcome the “anergic state” and support the ability of Tregs to up-regulate CD69, produce IL-2, and proliferate. Our results demonstrate that Tregs maintain their hyporesponsive state by suppressing the induction and propagation of TCR-initiated signals to control the accumulation of second messengers necessary for IL-2 production and proliferation. The Journal of Immunology, 2006, 177: 2186–2194.

The maintenance of tolerance in the periphery is an active process that involves the cooperative integration of many pathways, including signals mediated through the engagement of inhibitory receptors, anti-inflammatory cytokines, and immunoregulatory cells (1–5). Thymus-derived CD4⁺CD25⁺ regulatory T cells (Tregs)² are perhaps the best-characterized population of immunoregulatory T cells (6). In addition to CD25, Tregs constitutively express CTLA-4 (7), glucocorticoid-induced TNFR (8, 9), and the forkhead-winged transcription factor Foxp3 (10). Tregs constitute 5–10% of the total CD4⁺ T cells in the periphery and are critical not only for the control of autoimmunity, but also have been implicated in modulating responses to infectious diseases and promoting allograft acceptance (11, 12).

Tregs have been termed anergic, based on their relative lack of proliferation and IL-2 production in vitro compared with nonregulatory CD4⁺CD25⁻ T cells (13), however, the mechanism underlying this anergic phenotype is not completely understood. Calcium mobilization was shown to be severely impaired in Tregs upon TCR engagement with peptide-pulsed, and to a lesser extent with anti-CD3 treated, APCs in vitro (14). Defective JNK activation has also been observed following treatment with PMA/ionicmycin (15). Finally, a recent analysis of human Treg cell lines generated from cord blood has uncovered defects in Ras, MEK1/2, and ERK1/2 activation (16). Interestingly, T cells rendered anergic by TCR stimulation in the absence of costimulation exhibit similar biochemical defects in TCR signaling.

In this study, we sought to gain a better understanding of the molecular mechanisms behind the failure of Tregs to synthesize IL-2 through the examination of several signaling pathways involved in T cell activation and IL-2 production. The prevailing theme we observed was that Tregs exhibited diminished responses of all pathways examined, both in the absolute amplitude and temporal duration, when compared with the responses of CD4⁺CD25⁻ T cells. Interestingly, enhancing activation of the protein kinase C (PKC) and Ras pathways by either inhibition of diacylglycerol (DAG) metabolism to support activation of DAG-dependent pathways, or treatment with the phorbol ester PMA, enabled Tregs to both produce IL-2 and undergo extensive proliferation. Collectively, these data demonstrate that Tregs are not globally unresponsive to stimuli that normally induce IL-2, but have systems in place that quench signal initiation and propagation resulting in limited accumulation of second messengers and reduced activation of pathways that are essential for IL-2 transcription.

Materials and Methods

Mice

BALB/c mice were purchased from The Jackson Laboratory and maintained under specific pathogen-free conditions in the animal facilities of the University of Pennsylvania.

Media, reagents, Abs, and flow cytometry

All cells were grown in RPMI 1640 (Mediatech Cellgro) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEFES (all obtained from Invitrogen Life Technologies), 50 μM 2-ME (Sigma-Aldrich), and 10% heat-inactivated FCS (HyClone). Biotin anti-CD25 (7D4), anti-B220, anti-CD11b, anti-CD8, anti-MHC class II, anti-CD4 (RM4-5), anti-CD69-PE, streptavidin-PE, streptavidin-allophycocyanin, purified and FITC anti-CD3 (2C11) were purchased from BD
P. Driscoll, R. Lo, A. D. Hyper, R. A. Burns, and K. L. Ley.

Results

Treg proliferation

Tregs fail to sustain phosphorylation of tyrosine substrates upon activation

Preparation of cytoplasmic and nuclear extracts

T Cell}

Preparation of Ab-coated beads

Flow cytometry was used to measure intracellular calcium levels in T cells labeled with anti-CD4 and anti-CD25 and loaded with the calcium-binding dye Indo-1 (Molecular Probes) by reading the emission at 530 (blue) and 405 (violet) nm at different time points and calculating the violet/blue emission ratio using FlowJo. Baseline calcium fluorescence was acquired in biotinylated-2C11 (10 μg/ml) was added after 1 min, streptavidin (0.25 μg/ml) was added for 3 min, and ionomycin (1 μM) was added after 7 min.

Quantitative IL-2 bead array

For quantification of IL-2 production, supernatants were collected after 18 h. One hundred microliters of supernatant was incubated with an equivalent volume of anti-IL-2-coated beads (105/condition) in a 96-well plate for 2 h. Supematant (the cytoplasmic fraction) was collected and stored at −80°C. 25 μl of lysis buffer was added to the nuclear pellet remaining in the tube. The pellet was subjected to constant agitation for 30 min at 4°C followed by centrifugation for 10 min at 14,000 × g. The nuclear fraction (supernatant) was collected and stored at −80°C until ready to use. A total of 1 × 106 and 0.5 × 106 cell equivalents was used for Western blotting of nuclear and cytoplasmic fractions, respectively.

Results

Tregs fail to sustain phosphorylation of tyrosine substrates upon activation

TCR engagement activates numerous protein tyrosine kinases leading to the phosphorylation of specific substrates which participate in several second messenger cascades necessary for T cell activation and IL-2 production (18). When TCR proximal signaling at the level of tyrosine phosphorylation was examined to identify possible defects in Tregs, we made two primary observations. First, we found that while the overall pattern of tyrosine phosphorylation remained similar between Tregs and CD4+ CD25+ T cells (Fig. 1A), the degree of maximal induction of phosphorylated substrates was lower in Tregs than on TCR engagement. To exclude the possibility that decreased tyrosine phosphorylation was due to enhanced tyrosine phosphatase activity, we treated cells with the broad spectrum tyrosine phosphatase inhibitor sodium pervanadate (250 μM), which supports PKC activation (20). Given reports describing the possibility that decreased tyrosine phosphorylation was due to an increased tyrosine phosphatase activity in Tregs, we treated cells with the broad spectrum tyrosine phosphatase inhibitor sodium pervanadate (250 μM). However, cell stimulation with beads (Fig. 1B), the degree of maximal induction of phosphorylated substrates was lower in Tregs than on TCR engagement. To exclude the possibility that decreased tyrosine phosphorylation was due to an enhanced tyrosine phosphatase activity, we treated cells with the broad spectrum tyrosine phosphatase inhibitor sodium pervanadate (250 μM). However, cell stimulation with beads (Fig. 1B), the degree of maximal induction of phosphorylated substrates was lower in Tregs than on TCR engagement.

Decreased phospholipase C (PLC)-γ1 activity in Tregs

TCR-dependent tyrosine phosphorylation leads to the formation of a molecular scaffold of adapter molecules, including LAT, SLP-76, and Gads. PLC-γ1 is also recruited to this complex where it serves to activate distinct signaling pathways that support the nuclear recruitment of NFAT, AP-1, and NF-kB and regulate transcription of the IL-2 gene. Phosphorylated PLC-γ1 catalyzes phosphatidylinositol 4,5-bisphosphate hydrolysis to generate inositol 1,4,5-trisphosphate, which increases cytosolic-free calcium, and DAG, which supports PKC activation (20). Given reports describing decreased activity in PLC-γ1 and calcium flux in anergic T cells (21) and Tregs (14), we next assessed TCR-induced PLC-γ1 tyrosine phosphorylation. As shown in Fig. 2A, CD3-induced phosphorylation of PLC-γ1 was only slightly lower in Tregs than in CD4+ CD25+ T cells. A more marked difference was seen following CD3/CD28 stimulation. Specifically, CD3/CD28 costimulation of Tregs resulted in a 1.5-fold increase in phosphorylated (p) PLC-γ1 activity compared to TCR stimulation alone.
We next examined signaling events downstream of PLC-Ca2+ mobilization and its effects on NFAT in Tregs and CD4+CD25− T cells stimulated with anti-CD3-coated beads in the presence or absence of CD28 co-stimulation for 5 min (A) or up to 60 min (B), or with sodium pervanadate (100 μM) for 1 min (C). Whole cell lysates were resolved by SDS-PAGE and blotted with anti-phosphotyrosine mAb 4G10 to determine protein expression. Data from one representative experiment of two are shown.

Calcium mobilization and its effects

We next examined signaling events downstream of PLC-γ1 activation, focusing first on calcium mobilization which was assessed by flow cytometry using the calcium-sensitive dye Indo-1. Consistent with previous data (14), the amplitude of calcium increase was attenuated in Tregs following CD3 stimulation when compared with CD4+CD25− T cells and could not be restored with coligation of CD3 with anti-CD4 mAb (Fig. 2A and data not shown). Treatment of cells with the calcium ionophore, ionomycin, or the drug thapsigargin, an inhibitor of the sarco(endo)plasmic reticulum Ca2+-ATPase pump, induced comparable elevations of intracellular Ca2+ response indicating that the function of calcium release-activated calcium channels in Tregs, which is directly responsible for Ca2+ influx, was normal (Fig. 2B and data not shown). Thus, these data confirm that impaired calcium mobilization in Tregs is caused by a more proximal signaling defect.

Calcium mobilization is essential for NFAT activation as it activates the calcium-dependent phosphatase, calcineurin which then dephosphorylates NFAT on specific amino acids residues revealing its nuclear localization sequence (22). We next assessed the affect reduced calcium mobilization had on the nuclear accumulation of NFAT in Tregs and CD4+CD25− T cells. We found that detectable accumulation of NFATc2 and NFATc1 occurred at 1 and 5 h, respectively, upon anti-CD3 stimulation of CD4+CD25− T cells. In Tregs, however, the appearance of these molecules was both delayed and diminished except in the case of PMA/ionomycin treatment where NFATc2 in particular was strongly induced (Fig. 2C).

Impaired activation of the Ras-ERK pathway in Tregs

As noted above, DAG is the other major second messenger generated upon hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC-γ1. It allosterically activates the cysteine-rich (C1) domain-containing proteins Ras guanyl nucleotide-releasing protein and PKC-θ (23–25) resulting in the activation of the Ras-ERK-AP-1 and NF-κB pathways, respectively. In addition, expression of dominant-negative Ras in the Jurkat T cell line inhibited the induction of CD69, thus emphasizing the critical role Ras plays in the TCR/CD3-mediated expression of this early T cell activation marker (26). Hence, we used CD69 and ERK as surrogates for the activation of the Ras pathway in Tregs. As shown in Fig. 3A, CD3 stimulation alone induces CD69 in nearly all CD4+CD25− T cells. Although less than half of Tregs express CD69 following stimulation with anti-CD3 or anti-CD3/CD28, cotreatment with CD3 plus PMA up-regulates CD69 in virtually all Tregs (CD3 alone, 37%; CD3/CD28, 42%; vs CD3/PMA, 95%). Consistent with the observed differences in CD69 induction, levels of ERK phosphorylated in response to CD3 or CD3/CD28 costimulation were also reduced in Tregs when compared with CD4+CD25− T cells (Fig. 3B). The addition of PMA to CD3 stimulation enabled prolonged expression of p-ERK in Tregs, and also augmented the early induction of p-ERK in CD4+CD25− T cells. Thus, these data confirm that TCR/CD3-mediated triggering of the Ras-ERK pathway is compromised in Tregs and suggest that PMA enhances activation of this signaling pathway in Tregs, as it does in CD4+CD25− T cells.

Impaired signal transduction in Tregs is not due to PLC-γ1 or Ras degradation

A report by Heissmeyer et al. (27) investigating the signaling defects in ionomycin-induced anergic T cells found that PLCγ1 was specifically targeted for degradation upon TCR engagement. To determine whether this was occurring in Tregs, culture lysates were analyzed for PLC-γ1 and Ras expression. As Fig. 4 shows, PLC-γ1 and Ras were equivalently expressed in Tregs and CD4+CD25− T cells following CD3 or CD3/CD28 stimulation at all time points analyzed with both populations displaying slightly reduced levels at the later time point. In a preliminary experiment,
we were also unable to detect any differences in PKC-θ levels between the two populations (data not shown). Thus, these data suggest that Tregs, unlike ionomycin-anergized T cells, are using mechanisms other than the targeted degradation of PLC-γ1, Ras, or PKC-θ to dampen TCR-induced signals.

PMA can reverse the hyporesponsive state of Tregs

The data generated thus far showed a modest defect in calcium mobilization and suggested a more pronounced defect in DAG-dependent pathways. Moreover, we and others find that PMA/ionomycin (1 h) can induce the production of IL-2 by Tregs (Fig. 5 and Ref. 16), thus indicating that Tregs are not globally defective in their ability to produce this cytokine. Importantly, PMA but not ionomycin synergizes with TCR signals to mediate IL-2 production from Tregs indicating that defective DAG-dependent responses, and not calcium-dependent signals, are primarily responsible for the failure of TCR/CD3 stimulation to induce IL-2 in Tregs (Fig. 5).

Inhibition of DAG metabolism enables Tregs to proliferate and to produce IL-2

The requirement for PMA to promote IL-2 production in TCR/CD3-stimulated Tregs suggested that neither CD3 nor CD3/CD28 costimulation induced adequate activation of PKC or Ras perhaps due to the limited induction of p-PLC-γ1 with consequent reduction in DAG. To more directly test this hypothesis, we used a pharmacologic inhibitor of DAG metabolism. In vivo, DAG is metabolized by DAG lipase or DAG kinase (DGK) to yield glycerol and free fatty acids or phosphatidic acid, respectively (28). Stimulation of Tregs with CD3 in the presence of the DGK inhibitor R59022 resulted in increased levels of CD69, which was even more pronounced when CD3/CD28 costimulation was provided (Fig. 6A). Although CD3/R59022 or CD3/CD28 treatments alone were unable to support the production of IL-2 above the levels of detection, the combined treatments (CD3/CD28/R59022) synergized to strongly up-regulate IL-2 at both the mRNA and protein level (Fig. 6B and data not shown). The hypoproliferative response of Tregs was also reversed as Tregs stimulated with CD3/CD28 in the presence of APCs and R59022 proliferated to a similar extent as when stimulated with anti-CD3 and given exogenous IL-2 (Fig. 6C). CD4+CD25+ T cells, and likewise also demonstrated enhanced IL-2 production and proliferation when DAG metabolism was inhibited (Fig. 6, B and C, right panels).

Stimulation of DAG-dependent pathways correlates with enhanced accumulation of c-Rel and c-Jun

Having shown that strengthening DAG-dependent signaling pathways was effective in eliciting IL-2 from Tregs, we were interested in assessing the influence of these treatments upon the induction of c-Rel, c-Jun, and c-Fos, given their specific involvement in IL-2
transcription. All three transcription factors were maximally induced in both T cell populations following PMA/ionomycin treatment (Fig. 7). When Tregs were activated through the TCR, PMA costimulation functioned best to support the nuclear accumulation of c-Rel and c-Jun followed next by CD3/CD28/R59022 treatment. A similar finding was observed for CD4+/CD25+/H11002 T cells except that c-Jun was equivalently expressed at high levels in response to either PMA or CD28 costimulation and the addition of R59022 to the latter yielded no obvious enhancement in expression. Expression levels of c-Fos, the limiting component of the Jun/Fos heterodimeric transcription factor AP-1 are notable as TCR-stimulated expression was significantly lower in comparison to PMA/ionomycin induced-expression for both Tregs and CD4+/CD25+/H11002 T cells. Thus, these data show that the induction levels of NF-kB and AP-1 family members correlates with IL-2 production in Tregs and expressed as a ratio.

Discussion

CD4+/CD25+ Tregs are characteristically anergic to TCR stimuli mediated by splenocytes and fail to expand or produce IL-2 in vitro, while paradoxically, the same stimuli induces the robust proliferation of CD4+/CD25− T cells. In this study, we provide evidence for defects in pathways downstream of PLC-γ1 and particularly implicate inefficient activation of PKC and Ras as a causal factor in the failure of Tregs to produced IL-2 in response to TCR stimulation. Furthermore, we find that Tregs like CD4+/CD25− T cells retain the ability to produce IL-2 under select circumstances involving TCR-initiated signals and, in agreement with other studies, in response to pharmacologic stimuli (16, 29, 30).

A region located 300-bp immediately upstream of the transcriptional start site of the IL-2 gene must undergo chromatin remodeling before transcription initiation (31, 32). Recently, this region in Tregs was shown to exist in a closed chromatin configuration following PMA/ionomycin; a fact which was suggested to be the mechanism governing the failure of this treatment to elicit IL-2 (15). Our experiments were conducted for the same duration and IL-2 was quantified by the same means as the aforementioned study but yielded substantially different results. Markedly, we could readily detect IL-2 at the protein (Fig. 5) and mRNA level (data not shown) in CD4+/CD25+/H11001 Tregs upon PMA/ionomycin stimulation. The possibility that contamination of Tregs with CD4+/CD25+/H11002 T cells contributed to the results we obtained can also be excluded because expression of IL-2 mRNA and protein remained significantly lower in Treg cultures following CD3 and CD3/PMA treatments, respectively (Fig. 5).

TCR-dependent signaling activity can be detected for as long as 10 h after T cell-APC conjugate formation and is a requirement if a naive T cell is to reach its full effector potential (33). However, analysis of tyrosine-phosphorylated substrates surprisingly revealed that Tregs, unlike CD4+/CD25− T cells, were unable to sustain the activation of many proteins likely to be involved in transducing or amplifying the TCR signal. Ligand-induced down-regulation of the TCR is also dependent upon protein tyrosine phosphorylation and is directly proportional to the magnitude of

FIGURE 3. Weak induction of CD69 and ERK activation in Tregs. A, Tregs (left panel) or CD4+/CD25− T cells (center panel) were stimulated for 18 h with CD3-coated beads alone (shaded histogram), or in the presence of CD28 (dotted line), PMA (thin line), or were left untreated (thin line). CD69 expression (right panel) was determined by flow cytometric analysis. B, Cells were stimulated with Ab-coated beads in the presence of PMA for the time indicated as previously described. Whole cell lysates were resolved by SDS-PAGE and immunoblotted with anti-phospho-ERK (upper panel). Blots were stripped and reprobed with anti-ERK-specific Abs (lower panel). Data from one representative experiment of three are shown. Quantification of p-ERK levels were normalized to the relative quantity of total ERK and expressed as a ratio.
the signal transduction cascade induced upon TCR triggering (34). Tregs and CD4+CD25- T cells expressed equivalent levels of TCR/CD3 and stimulation led to a comparable dose-dependent down-regulation of this complex from the surface (data not shown). Moreover, we found that maximum TCR down-regulation could be detected as early as 5-h poststimulation in both populations and was sustained for 24 h provided the stimulus remained throughout the culture period, consistent with a previous report (35). Thus, serial engagement and down-regulation of the TCR occurs normally in Tregs and the failure to do so cannot account for the diminutive phosphorylation response.

The ability of Tregs to moderate signal strength thus altering the outcome of stimulation has been described previously. Using the Tet on/off system to modulate the expression of the selecting peptide in the thymus, the Mathis and Benoist group (36) has demonstrated that CD4+CD25+ Treg cells are considerably more resistant to clonal deletion following negative selection than conventional CD4+CD25+ T cells. Regulating the expression and activation of key signaling molecules such as PLC-γ1 may be a built-in component of this program. The diminished activity of PLC-γ1 in Tregs is likely to contribute not only to the delay and reduced appearance of NFAT in the nucleus, but also to attenuated activation of the PKC and Ras-ERK directed pathways as well. This phenotype has been documented previously in cells rendered anergic by sustained ionomycin treatment where it was first found that NFAT1 in the absence of AP1 induces T cell anergy (37). Characterization of this model further has revealed that PLC-γ1 and PKC-θ are degraded by an E3 ligase-dependent mechanism upon activation (27). In contrast, we did not observe evidence of PLC-γ1, Ras, or PKC-θ degradation as Tregs stimulated for up to 60 min expressed these proteins at levels that were equivalent to that detected in CD4+CD25- T cells (Fig. 4 and data not shown). In sum, these data indicate that Tregs are using different signals to modulate T cell activation, CD45 is of particular interest. Changes in the synaptic localization and lipid raft partitioning of CD45 during T cell activation have been described by several investigators (38–40). Additionally, Zhang et al. (41) have shown that raft-excluded CD45 results in sustained ERK activation, enhanced synaptic raft clustering, and increased IL-2 production by T cells following activation. That the positioned exclusion of CD45 or other phosphatases away from the immunological synapse during T cell activation may be altered in Tregs is an intriguing possibility which should be closely examined in light of a recent report demonstrating that the cross-linking of CD45 on Tregs with specific mAbs could abrogate their suppressive action (42).

Costimulation is critical for optimal activation of AP-1 and NF-κB as the combined treatment on nonregulatory T cells augments the nuclear levels of these proteins more strongly than TCR stimulation alone (43, 44). The NF-κB family member c-Rel is of particular importance as it serves dually to participate in chromatin remodeling of the IL-2 promoter and transcription of the IL-2 gene (45). In Tregs, the induction of c-Rel and c-Jun by TCR stimulation was almost equivalent to that seen by PMA/ionomycin. In contrast, c-Fos expression was clearly different as levels induced upon TCR triggering were dramatically lower than levels resulting from PMA/ionomycin treatment. Because c-Fos must be transcriptionally induced upon T cell activation and given also that IL-2 transcription is dependent on the cooperative association of multiple transcription factors acting in concert, our data would suggest

FIGURE 4. Equivalent expression of Ras and PLC-γ1 in Tregs and CD4+CD25- T cells. Whole cell lysates from sort-purified cells following CD3 or CD3/CD28 stimulation were resolved by SDS-PAGE and blotted with anti-PLC-γ1 (upper panel), anti-Ras (middle panel), and anti-actin (lower panel). Quantitative analysis of Western results in A for actin-normalized levels of PLC-γ1 and Ras in cells stimulated for the time indicated.

FIGURE 5. IL-2 production by Tregs following CD3/PMA stimulation. Tregs or CD4+CD25- T cells were stimulated with anti-CD3-coated beads alone or in the presence of PMA or ionomycin. Supernatants were collected 24 h later and analyzed for IL-2. Data from one representative experiment of two are shown.
that induction of c-Fos may be the rate-limiting molecule for IL-2 production in our system.

Despite the fact that DGK inhibition in the presence of CD3/CD28 costimulation was permissive to support the up-regulation of CD69 and production of IL-2 by Tregs, generally, responses resulting from CD3/PMA treatment were more robust. The efficacy of PMA treatment over DGK inhibition to support the proliferation of Tregs, for example, can be explained by the fact that PMA, unlike DAG, is not readily metabolized. Hence, the activity of the downstream pathways stimulated by PMA leading to IL-2 production should be prolonged. Second, DAG production is dependent on the activity of PLC-γ1 and we show here that Tregs, unlike CD4+CD25+ T cells, are only slightly amenable to enhancement in PLC-γ activity when provision of signal 1 (CD3/TCR cross-linking) and signal 2 (CD28 costimulation) is given (Fig. 2A). Thus, even when DGK is inhibited, DAG production is subject to limitations in PLC-γ1 activation.

If IL-2 production and proliferation are a measure of the degree of T cell activation, no stimulatory conditions used in this study or in others have demonstrated the ability to fully reverse the hyporesponsive phenotype of Tregs and enable these cells to function completely as CD4+CD25+ T cells (Fig. 6). Notably, several reports have indicated that Foxp3, which is preferentially expressed in CD4+CD25+ Tregs, is a critical element in their regulatory program (10). In a recent report, Bettelli et al. (46) demonstrated that Foxp3 can physically associate with NFAT and NF-κB negatively affect their transcriptional but not DNA-binding activities. Furthermore, ectopic expression of Foxp3 in scurfy-derived (Foxp3-deficient) T cells significantly lowered the high level of NFAT and NF-κB transcriptional activity normally observed in these cells following stimulation. Hence, even under the most permissive conditions where PKC- and Ras-dependent pathways are fully active in Tregs, the action of Foxp3 upon the transcriptional activities of NFAT and NF-κB may provide an additional level of regulation to limit IL-2 synthesis.

It is clear from this work that Tregs have the capacity to produce IL-2 but the signaling pathways operative within these cells serve to actively inhibit signal transduction and prevent full activation.
The authors have no financial conflict of interest.

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Strengthening of DAG-directed pathways using pharmacological correlates with enhanced induction of c-Rel and c-Jun. Nuclear extracts of CD3/PMA stimulation two are shown.

FIGURE 7. IL-2 production by Tregs following CD3/PMA stimulation correlates with enhanced induction of c-Rel and c-Jun. Nuclear extracts (10⁶ cell equivalents) were prepared from Tregs (A) or CD4⁺ CD25⁺ T cells (B) following a 4-h stimulation with CD3 or CD3/CD28-coated beads in the presence or absence of the DGK inhibitor R59022 (10 μM) and were resolved by SDS-PAGE and immunoblotted with anti-c-Rel, anti-c-Jun, anti-c-Fos, or anti-histone 1. Data from one representative experiment of two are shown.

Strengthening of DAG-directed pathways using pharmacological agents is a novel approach by which these cells can be expanded in vitro for potential use in immunotherapy.

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Disclosures

The authors have no financial conflict of interest.

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