

in the evolution of global cellular regulatory networks composed of both transcriptional and signaling elements (24). Further work will be needed to compare in quantitative terms the contributions of gene duplication and recombination to the evolutionary process.

The strategy used here of targeted domain recombination between proteins that belong to a specific signaling network could facilitate the engineering of other protein networks of interest, a fundamental goal of synthetic biology. Genes known to belong to a target pathway could be deconstructed into domains and used to build small libraries of domain recombinations that are subsequently screened for the desired function.

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27. We thank A. Chau for help with Matlab, N. Helman and J. Walter for help with fluorescence microscopy, and members of the Lim laboratory for critical reading of this manuscript and assistance. This work was supported by grants from the Howard Hughes Medical Institute, the Packard Foundation, the NIH, and the NIH Nanomedicine Development Centers (W.A.L.). S.G.P. was supported by a Long-Term Postdoctoral Fellowship from the Human Frontier Science Program. P.W. was supported by the Li Foundation. S.G.P. and W.A.L. conceived the project. S.G.P. and J.G. performed the experiments. P.W. assisted with initial experiments. S.G.P., J.G., and W.A.L. analyzed the results. S.G.P. and W.A.L. wrote the manuscript. The authors declare no competing financial interests.

Supporting Online Material

www.sciencemag.org/cgi/content/full/328/5976/368/DC1
Materials and Methods

Figs. S1 to S9

Table S1

References

23 September 2009; accepted 9 March 2010
10.1126/science.1182376

Protein Kinase C- θ Mediates Negative Feedback on Regulatory T Cell Function

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T cell receptor (TCR)-dependent regulatory T cell (T_{reg}) activity controls effector T cell (T_{eff}) function and is inhibited by the inflammatory cytokine tumor necrosis factor- α (TNF- α). Protein kinase C- θ (PKC- θ) recruitment to the immunological synapse is required for full T_{eff} activation. In contrast, PKC- θ was sequestered away from the T_{reg} immunological synapse. Furthermore, PKC- θ blockade enhanced T_{reg} function, demonstrating PKC- θ inhibits T_{reg} -mediated suppression. Inhibition of PKC- θ protected T_{reg} from inactivation by TNF- α , restored activity of defective T_{reg} from rheumatoid arthritis patients, and enhanced protection of mice from inflammatory colitis. T_{reg} freed of PKC- θ -mediated inhibition can function in the presence of inflammatory cytokines and thus have therapeutic potential in control of inflammatory diseases.

CD4⁺ CD25⁺ regulatory T cells (T_{reg}) suppress the function of CD4⁺ and CD8⁺ effector T cells (T_{eff}) through a T cell receptor (TCR) engagement and cell contact-dependent mechanism (1–3). Inflammatory signals delivered by cytokines like tumor necrosis factor- α

(TNF- α) decrease T_{reg} activity (4, 5), perhaps as a mechanism to reduce interference by T_{reg} cells in immune responses to pathogens. In rheumatoid arthritis, T_{reg} cells circulate in normal numbers, but they have decreased activity *ex vivo* (5, 6). Besides the negative signals initiated by TNF- α , T_{reg} also receive inhibitory signals via the TCR. Akt activation by the TCR can reduce T_{reg} function and thus appears to be tightly regulated (7). This suggests TCR signaling in T_{reg} can negatively feed back to inhibit T_{reg} -mediated suppression. Moreover, TCR signaling leads to formation of the immunological synapse within seconds of T cell activation. Thus, the differences in TCR signaling in T_{reg} may emerge at the level of the immunological synapse (IS), a structured interface between T cells and antigen-presenting cells

(APCs) where TCR signalosomes are assembled (8). Whereas T_{reg} can form stable contacts with APCs with functional consequences both *in vitro* and *in vivo* (9–11), signaling events in the T_{reg} IS have not been investigated.

To study signaling in the human T_{reg} IS, we developed a model system on supported planar bilayers containing the mobile fluorescently labeled intercellular adhesion molecule-1 (ICAM-1) and antigen surrogate anti-CD3 (the signaling subunit of the TCR) antibodies and CD4⁺ CD25⁺ T_{eff} or CD4⁺ CD25⁺ T_{reg} freshly isolated from peripheral blood (fig. S1, A to C). T_{eff} and T_{reg} both formed IS, defined by a symmetric pattern consisting of a central cluster of anti-CD3 surrounded by a ring of ICAM-1 (12, 13) (Fig. 1A). T_{reg} ISs were more stable than T_{eff} ISs (fig. S2, A and B), which displayed symmetry breaking within 20 min as previously described (14). *Ex vivo* expanded, human umbilical cord blood T_{reg} (15) displayed similar behavior to adult peripheral blood T_{reg} (fig. S2, C to E). We measured recruitment of TCR proximal signaling molecules to IS by staining with phospho-Src kinase activation loop and Zap70 kinase interdomain A tyrosine 319 antibodies and imaging with total internal reflection fluorescence microscopy (TIRFM) (16). Signals were quantified on the basis of unbiased measurement of IS proximal fluorescence intensity. T_{eff} IS displayed significantly higher amounts of phospho-Src than T_{reg} (fig. S3A); however, we observed a similar intensity of phosphorylation of the downstream kinase Zap70 (fig. S3B). We next explored the protein kinase C- θ (PKC- θ) pathway, which is downstream of Src family kinases (17) and mediates IS breaking (14), because Zap70 phosphorylation appeared normal in T_{reg} .

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PKC- θ recruitment to the IS leads to recruitment of Carma-1, a MAGUK (membrane-associated guanylate kinase) protein, which enables the assembly of a Carma-1-Bcl10-Malt1 complex necessary for transcription factor NF- κ B activation and subsequent T_{eff} activation (18). We quantified PKC- θ and Carma-1 recruitment in ISs on planar bilayers by TIRFM. T_{eff} ISs recruited PKC- θ in a broad pattern overlapping with early TCR signaling, as previously reported (14) (Fig. 1B). T_{reg} displayed sixfold lower PKC- θ recruitment to IS than that of T_{eff} , and this recruitment was focused in a limited area defined by a small number of bright puncta (Fig. 1B). The same difference in the ability to recruit PKC- θ was found with ex vivo expanded, human umbilical cord blood T_{reg} and expanded $CD4^+ CD25^- T_{eff}$ cells (fig. S4). CD28 co-stimulation plays an important role in PKC- θ recruitment to IS (13, 17). Thus, we compared the ability of T_{reg} and T_{eff} to recruit PKC- θ in the presence of a CD28 ligand, CD80, in the bilayer. The co-stimulatory signal increased the amounts of PKC- θ recruited to ISs in both T_{reg} and T_{eff} (fig. S5). Even in the presence of CD80 in the bilayer, the T_{reg} still have significantly less PKC- θ recruited to the IS than T_{eff} ($P < 0.001$). Interestingly, total PKC- θ expression was even higher in T_{reg} compared with T_{eff} (fig. S6). Moreover, the confocal imaging revealed that PKC- θ was sequestered away from the T_{reg} IS, whereas PKC- θ in T_{eff} accessed the IS and formed cytoplasmic puncta (Fig. 1C and fig. S7). T_{reg} IS also displayed significantly less Carma-1 recruitment than T_{eff} IS (Fig. 1D). Thus, recruitment of PKC- θ and its downstream target to the IS are reduced in T_{reg} .

We further dissected the TCR and LFA-1 integrin-dependent components of PKC- θ recruitment by using bilayers containing either anti-CD3 or ICAM-1, respectively. TCR engagement in T_{eff} was necessary and sufficient for increased PKC- θ recruitment on the basis of a twofold increase in PKC- θ fluorescence intensity on anti-CD3-only bilayers compared with fluorescence on ICAM-1-alone bilayers (Fig. 1, E and F). TCR engagement alone in T_{reg} recruited 11-fold less PKC- θ than in T_{eff} , whereas LFA-1 engagement alone recruited 3.5-fold more PKC- θ than in T_{eff} (Fig. 1, E and F). Thus, TCR triggering in T_{reg} actually down-regulates PKC- θ recruitment to the IS by 7.7-fold compared with basal recruitment by LFA-1 engagement alone (Fig. 1F).

We hypothesized that PKC- θ activation may be part of a negative feedback loop controlling T_{reg} function because TCR signals are essential for T_{reg} function but suppress PKC- θ recruitment. We investigated whether inhibition of PKC- θ with compound 20 (C20) (19) may affect the suppressive function of human $CD4^+ CD25^+ T_{reg}$ cells. T_{eff} function was measured as cytokine secretion and cell proliferation. C20 treatment of only the T_{reg} cells significantly up-regulated their suppressive ability, even in the presence of CD28-mediated costimulation, but did not induce suppressive activity in treated T_{eff} (Fig. 2, A

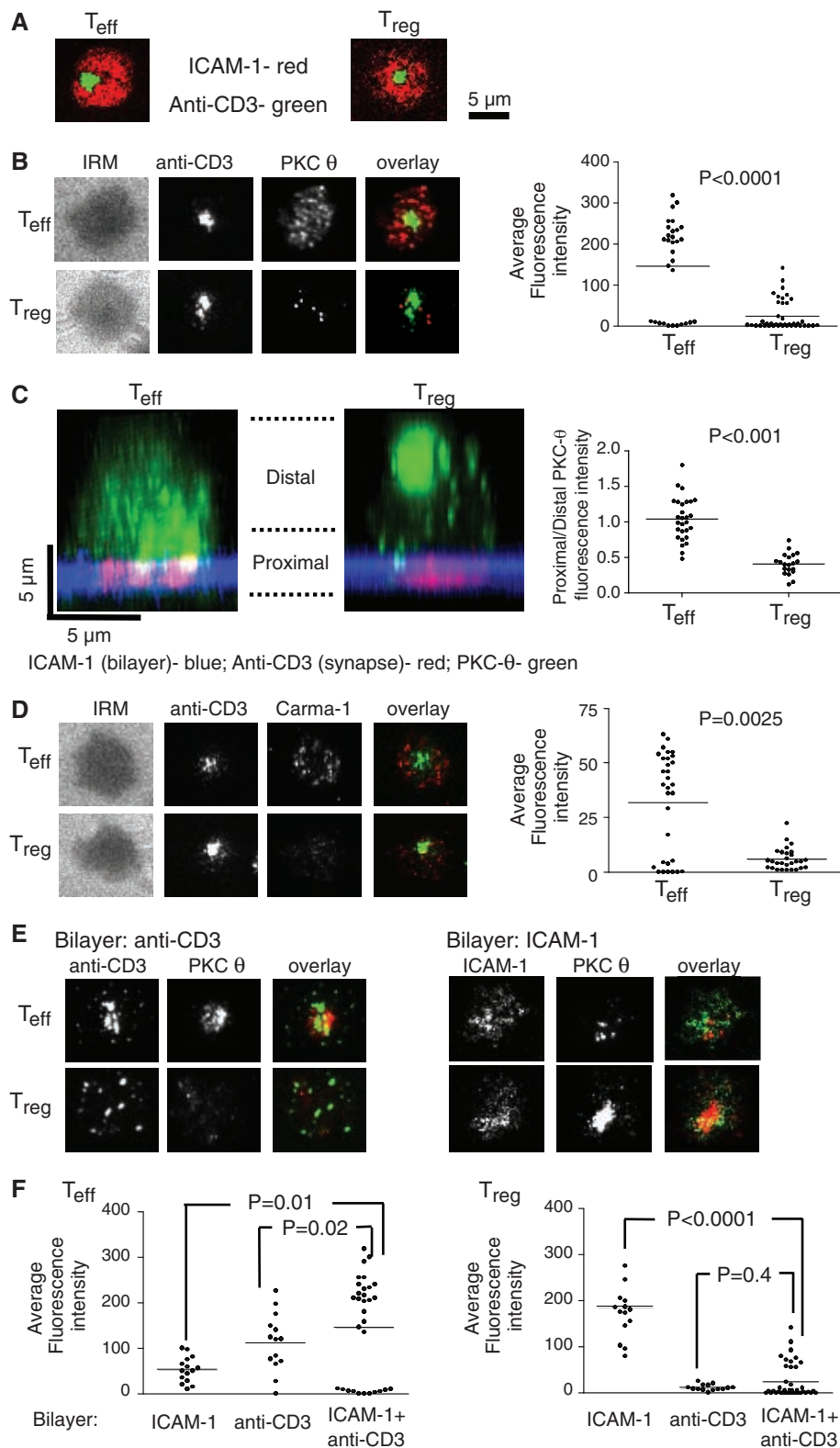


Fig. 1. Human T_{reg} form altered ISs. Human T_{eff} and T_{reg} were introduced into bilayers containing anti-CD3 (5 μ g/ml) and ICAM-1 at 250 molecules/ mm^2 (A to D); fixed at 6 min; and permeabilized, stained, and imaged by TIRFM [(A), (B), (D), and (E)] or by confocal microscopy (C). (A) Localization of ICAM-1 (red) and anti-CD3 (green) in the IS. (B and D) Staining and average fluorescence intensity of PKC- θ (B) and Carma-1 (D). (C) Distribution of endogenous PKC- θ in cells. Histogram shows average of (bottom) planes "proximal"/upper planes "distal" ratio of anti-PKC θ intensity per cell. The images show representative images. (E and F) PKC- θ staining (E) and average fluorescence intensity (F) in T_{eff} or T_{reg} on bilayers containing only anti-CD3 (left) or ICAM-1 (right). Data are representative of three [(A) to (D)] or five [(E) and (F)] different experiments. P values were calculated by Mann-Whitney test.

and B, and fig. S8A). Consistent with imaging data showing that co-stimulation up-regulates the recruitment of PKC- θ to ISs in T_{reg} (fig. S5), untreated T_{reg} demonstrated reduced ability to suppress T_{eff} function in the presence of CD28 antibodies [40% and 25%, respectively (fig. S8A)], and this difference was abrogated by treatment with C20. Inhibition of PKC- θ seemed to increase T_{reg} suppressive function in general, without preference for specific helper T cell type (fig. S8B). C20 also significantly increased T_{reg} function in an APC-dependent assay (fig. S8C).

It has been reported that the $CD4^+ CD25^{high}$ T_{reg} cells are the most potent suppressors in vitro (20); we therefore sorted $CD4^+$ T cells according to their CD25 expression (fig. S1D), pretreated the sorted T_{reg} with C20, and cocultured the $CD4^+ CD25^{int}$ or $CD4^+ CD25^{high}$ T cells with target $CD4^+ CD25^-$ T cells. Although the $CD4^+ CD25^{high}$ T cells manifested a greater suppressive activity, pretreatment with C20 significantly enhanced their suppression of interferon- γ (IFN- γ) secretion (fig. S8D). Lastly, the effect of C20 on

T_{reg} function was time-dependent; we observed the peak of suppressive function after 30 min of treatment (fig. S8E).

TCR-induced DNA binding of NF- κ B p65 and p50 subunits, which indicates NF- κ B activation, and the ability to proliferate were greatly reduced by C20 in both T_{reg} and T_{eff} (Fig. 2C and fig. S9). To test whether activation of NF- κ B was a critical PKC- θ target in control of T_{reg} activity, we inhibited NF- κ B activation by using two different types of inhibitors, and both significantly increased T_{reg} activity (Fig. 2D). Moreover, treatment of T_{reg} with analogs of C20 with different median inhibitory concentration (IC₅₀) values demonstrated that, of the analogs tested, only PKC- θ inhibitors with IC₅₀ \leq 1 nM significantly up-regulated T_{reg} suppressive function (fig. S10). Lastly, neutralizing antibodies against transforming growth factor- β (TGF- β) receptor II completely blocked the suppressive function of T_{reg} induced by inhibition of PKC- θ either in APC-free or in presence of APC in the coculture (Fig. 2E and fig. S8C), suggesting the possible in-

volvement of TGF- β presented by T_{reg} as a suppressive mechanism. To confirm the conclusion that inhibition of PKC- θ up-regulates the suppressive activity of human T_{reg} , we specifically silenced PKC- θ gene expression by using RNA interference (siRNA). The specific PKC- θ small interfering RNA (siRNA) reduced PKC- θ expression by 80% (Fig. 2F). Moreover, silencing of PKC- θ significantly increased T_{reg} -mediated suppression of IFN- γ secretion by T_{eff} (Fig. 2F). PKC- θ silencing in T_{eff} resulted in the expected down-regulation of IFN- γ secretion and cell proliferation (fig. S11). We concluded that PKC- θ activity induced by TCR signaling mediates a negative feedback loop that reduces the activity of human $CD4^+ CD25^{high}$ T_{reg} to suppress cytokine secretion and proliferation of T_{eff} in vitro.

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that results in the destruction of joint architecture (22). Recent studies in RA patients demonstrated that the function of $CD4^+ CD25^{high}$ T_{reg} is impaired (5, 6). We used T_{reg} purified from peripheral blood of 25 RA patients

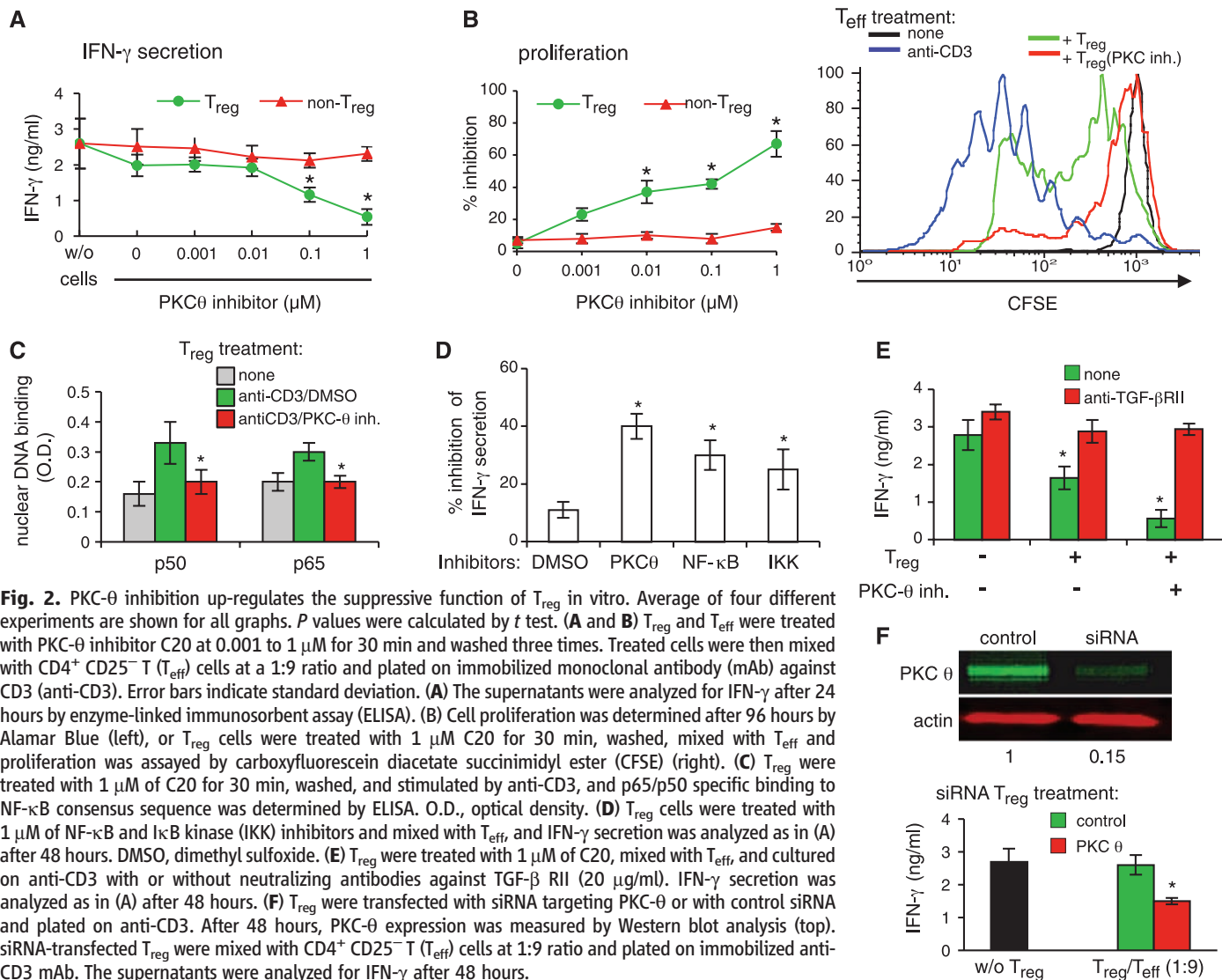


Fig. 3. Inhibition of PKC- θ rescues and protects T_{reg} function. **(A and B)** Freshly purified T_{reg} from healthy donors and RA patients were treated or not with PKC- θ inhibitor C20 for 30 min at 1 μ M, washed three times, mixed with $CD4^+CD25^-$ T cells at ratio 1:3, and plated on immobilized anti-CD3. The supernatants were analyzed for IFN- γ after 24 to 48 hours by ELISA. **(C to E)** T_{reg} cells from healthy donors were treated with PKC- θ inhibitor C20 [(C) and (E)] or with PKC- θ siRNA (D) as described above and cocultured with $CD4^+CD25^-$ T cells with or without TNF- α (50 ng/ml). IFN- γ secretion was determined after 48 hours by ELISA [(C) and (D)], and proliferation was determined after 96 hours (E). Error bars indicate standard deviation. **(F)** Untreated or TNF- α -treated (50 ng/ml, for 24 hours) T_{reg} were introduced to bilayers with anti-CD3 and ICAM-1, fixed, and imaged by confocal microscopy. Representative images are shown with ICAM-1 (blue), anti-CD3 (red), and PKC- θ (green). Plots show the distribution of endogenous PKC- θ in cells. Each bar shows average of (bottom planes "synapse proximal"/upper planes "synapse distal") ratio of anti-PKC θ intensity per cell. Data points from three independent experiments are included in the analysis.

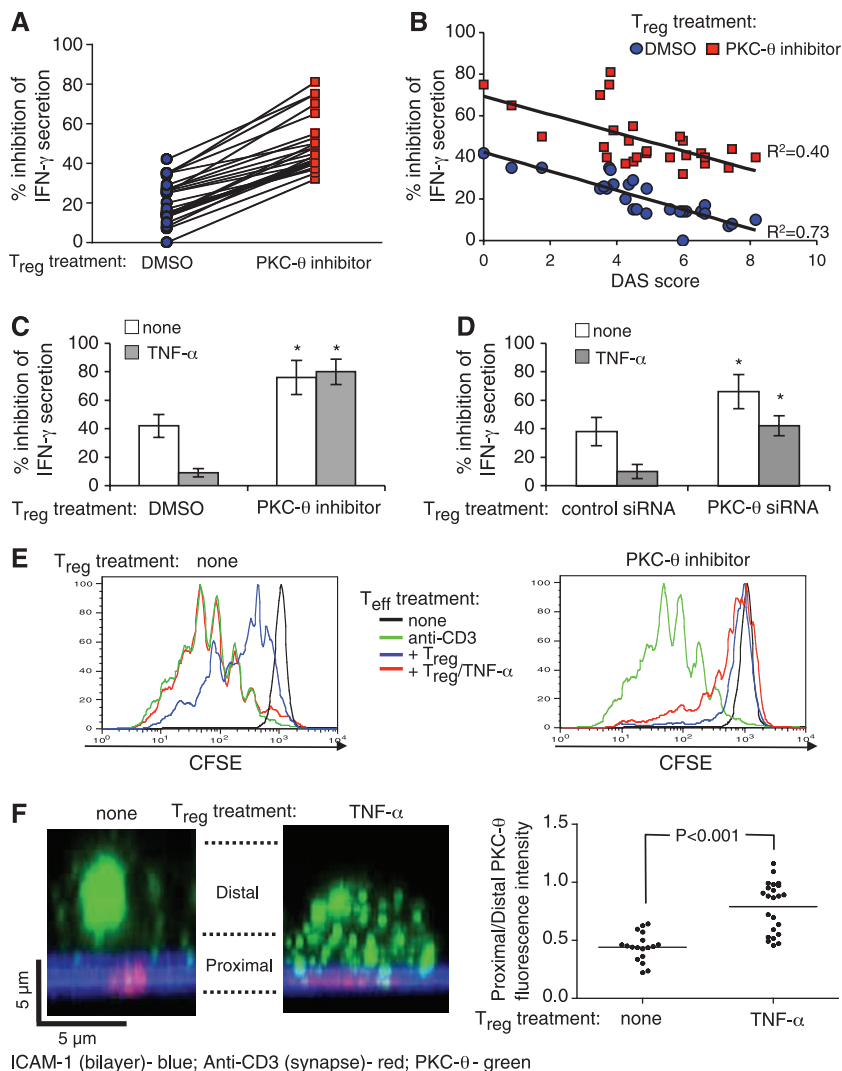
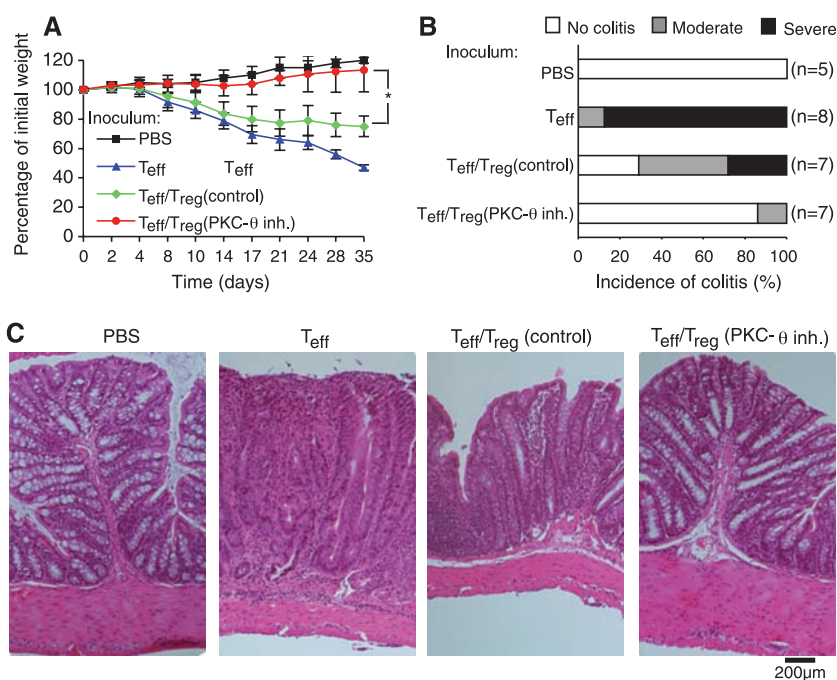


Fig. 4. Treatment with PKC- θ inhibitor up-regulates T_{reg} function in vivo. Colitis was induced in C57BL/10.PL $TCR\alpha^{-/-}$ $\beta^{-/-}$ mice (31). Disease progression was monitored by **(A)** body weight loss, **(B)** colitis score, and **(C)** histology. Numbers in parentheses indicate number of mice. Combined data of three independent experiments are presented. P values were calculated by t test. PBS, phosphate-buffered saline.



with different severities of disease (fig. S12) and found, despite anergic state and comparable T_{reg} numbers to healthy donors (fig. S13A), that RA T_{reg} demonstrated significantly reduced suppression of IFN- γ from autologous $CD4^+ CD25^- T_{eff}$ (fig. S13B). The loss of function was due to defective intrinsic function of T_{reg} from RA patients and not due to increased resistance of T_{eff} (fig. S13C), consistent with previously published results (5). Treatment with C20 significantly increased the suppressive function of T_{reg} purified from all 25 RA patients (Fig. 3A) to levels comparable to those of healthy donor-derived T_{reg} (30 to 50% inhibition at a T_{reg}/T_{eff} of 1:3, fig. S13B). Moreover, the defective T_{reg} function in RA patients was inversely correlated with the disease active score (DAS score), and the shift in IFN- γ secretion was similar across the disease score spectrum (Fig. 3B). Thus, inhibition of PKC- θ boosts the suppressive function of T_{reg} isolated from RA patients independent of the severity of disease.

Treatment of T_{reg} cells with TNF- α inhibits their activity and down-regulates expression of the T_{reg} master regulator transcription factor Foxp3 (5). We investigated the possibility that elimination of the PKC- θ -mediated negative feedback on T_{reg} function may render T_{reg} resistant to inhibition by TNF- α . As expected, in the presence of TNF- α , T_{reg} displayed significantly reduced inhibition of IFN- γ secretion and proliferation in T_{eff} , and this effect was largely reversed by C20 or PKC- θ -specific siRNA (Fig. 3, C to E, and fig. S14A). Moreover, C20 prevented TNF- α -induced down-regulation of Foxp3 in T_{reg} (fig. S14B). Strikingly, TNF- α treatment induced increased PKC- θ recruitment to IS in T_{reg} by decreasing sequestration at the distal pole (Fig. 3F), consistent with the idea that TCR-activated PKC- θ mediates negative feedback on T_{reg} function that is further enhanced by TNF- α .

Lastly, we determined the ability of C20 to increase T_{reg} function in vivo by using a colitis model in $TCR\alpha^{-/-} \beta^{-/-}$ mice induced by transfer of the $CD4^+ CD25^- CD45RB^{high} T_{eff}$ cells (23, 24). Treatment of murine T_{reg} with C20 up-regulated their suppressive function in vitro (fig. S15, A and B). Moreover, C20-treated $CD4^+ CD25^+ T_{reg}$ cells provided significant protection from colitis, as demonstrated by normal weight gain and normal histology of the distal colon in seven of eight mice (Fig. 4, A to C). C20 treatment increased the number of T_{reg} recovered from mesenteric lymph nodes and the spleen (fig. S15C). This protection was significantly greater than that afforded by T_{reg}

left untreated (Fig. 4, A to C). Treatment of $CD4^+ CD25^- T_{eff}$ with C20 before transfer with untreated T_{eff} did not protect mice from colitis (fig. S16). Thus, PKC- θ inhibition significantly increased the suppressive effect of T_{reg} in vivo.

T_{reg} function is crucial to prevent autoimmunity in mice and humans. Although T_{reg} numbers in patients suffering from autoimmune diseases are similar to healthy controls, T_{reg} function is defective, probably caused by negative regulation of T_{reg} by the inflammatory milieu (20). In the present study, we demonstrate that PKC- θ is sequestered in the distal pole of T_{reg} cells in a manner that reduces its recruitment to the IS. Moreover, inhibition of PKC- θ protects both mouse and human T_{reg} against negative effects of TNF- α , which appears to act in T_{reg} by unleashing PKC- θ from the distal pole. In T_{eff} , PKC- θ is part of a strong positive circuit with free access to TCR signalosomes in the IS, NF- κ B activation, increased expression of IL-2, proliferation, and survival (17, 25, 26). PKC- θ is not sequestered in the distal pole of T_{eff} , although the negative regulators like Csk binding protein are (27). In contrast, we noted that T_{reg} display increased IS stability, consistent with decreased PKC- θ activity in the IS and attenuated NF- κ B activation. The proposed role of NF- κ B in inhibition of T_{reg} function is consistent with the system-wide role of this family of transcription factors in promoting inflammation (28). IS stabilization may enhance T_{reg} function on the basis of recent evidence for an important role of IS in T_{reg} effects mediated through dendritic cells (1, 29).

In conclusion, formation of IS induces altered signaling pathways in T_{reg} , which is characterized by reduced recruitment of Src kinases, PKC- θ , and Carma-1 to the IS. Moreover, in T_{reg} , PKC- θ acts as a proinflammatory mediator, and this effect is enhanced by TNF- α . Indeed, T_{reg} treated with C20 displayed enhanced ability to prevent autoimmune colitis and to restore function of T_{reg} from RA patients. Thus, targeting the PKC- θ -mediated negative feedback loop enhances the activity of T_{reg} and makes them resistant to cytokines associated with the inflammatory milieu found in some autoimmune diseases. Thus, inhibition of PKC- θ in T_{reg} may be a valuable component in T_{reg} adoptive immunotherapy to treat autoimmunity and graft versus host disease (30).

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31. Materials and methods are available as supporting material on Science Online.
32. This work was funded by NIH through the NIH Roadmap for Medical Research PN2 EY016586 and R01 AI43542 to M.L.D.; a Leukemia and Lymphoma Translational Research grant 6098-09 to B.B. S.K. was supported by the Osaka University Immunology Frontier Research Center. B. Blazar is an ad hoc advisor to Becton Dickinson. Becton Dickinson has purchased licensing rights for the use of T_{reg} cells to prevent alloresponses and autoimmunity. An MTA agreement is required for use of compound 20. M.D. and A.Z.-Z. have applied for patents for the use of compound 20 and RNA interference against PKC θ in adoptive immunotherapy (patent application nos. 61173237 and 61286871).

Supporting Online Material

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Materials and Methods
Figs. S1 to S16
References

16 December 2009; accepted 4 March 2010
Published online 25 March 2010;
10.1126/science.1186068
Include this information when citing this paper.