Protein kinase C-\(\eta\) controls CTLA-4–mediated regulatory T cell function

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Regulatory T (T\(\text{reg}\)) cells, which maintain immune homeostasis and self-tolerance, form an immunological synapse (IS) with antigen-presenting cells (APCs). However, signaling events at the T\(\text{reg}\) cell IS remain unknown. Here we show that the kinase PKC-\(\eta\) associated with CTLA-4 and was recruited to the T\(\text{reg}\) cell IS. PKC-\(\eta\)-deficient T\(\text{reg}\) cells displayed defective suppressive activity, including suppression of tumor immunity but not of autoimmune colitis. Phosphoproteomic and biochemical analysis revealed an association between CTLA-4–PKC-\(\eta\) and the GIT2-\(\alpha\)PIK-PAK complex, an IS-localized focal adhesion complex. Defective activation of this complex in PKC-\(\eta\)-deficient T\(\text{reg}\) cells was associated with reduced depletion of CD86 from APCs by T\(\text{reg}\) cells. These results reveal a CTLA-4–PKC-\(\eta\) signaling axis required for contact-dependent suppression and implicate this pathway as a potential cancer immunotherapy target.

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Here we show that, by analogy with the PKC-θ–CD28 interaction in T eff cells, which promotes their activation and function\(^9\), the CD28-related receptor CTLA-4, which is highly expressed on T reg cells and is required for their suppressive function\(^{14,15}\), physically recruits another member of the novel PKC (nPKC) subfamily, PKC-η, which localizes at the T reg cell IS after stimulation. This association required phosphorylated serine residues in PKC-η and a conserved, membrane-proximal motif in the cytoplasmic tail of CTLA-4, respectively. Although development of T reg cells and expression of typical T reg cell markers were normal in PKC-η-deficient (Prkch\(^−/−\)) T reg cells, these cells displayed a grossly impaired contact-dependent suppressive activity in vitro and in vivo, which was associated with a grossly defective activation of the transcription factors NFAT and NF-κB. Lastly, we show that defective activation of a focal adhesion complex consisting of the kinase PAK and two additional proteins, PAK-interacting exchange factor (PIX) and G protein–coupled receptor kinase-interacting protein 2 (GIT2), which was physically associated with CTLA-4 and PKC-η, was associated with reduced ability of Prkch\(^−/−\) T reg cells to serially engage APCs; this provides a potential mechanistic basis for the impaired suppressive activity of these cells. Therefore, strategies that interfere with this signaling pathway may be beneficial for inhibiting T reg cell function in cancer and, hence, boosting the immune response against tumors.

**RESULTS**

**Phospho-PKCN-η interacts with CTLA-4 in the T reg cell IS**

By analogy to the interaction of PKC-θ with the CTLA-4–related costimulatory receptor CD28, which is critical for the activation of T eff cells\(^9\), we first tested whether any PKC isoform could physically interact with CTLA-4. Using T hybridoma cells, we found that CTLA-4 coimmunoprecipitated with a higher than normal molecular weight species of PKC-η (Fig. 1a), but not with any other T cell–expressed PKC isoforms (Supplementary Fig. 1a). We reasoned that the shift in molecular weight of PKC-η might be due to phosphorylation. Indeed, treatment with alkaline phosphatase partially reversed the shift of the higher-molecular-weight species of PKC-η and resulted in the appearance of a lower-molecular-weight species (Fig. 1b), which indicated that CTLA-4 interacts predominantly with phosphorylated PKC-η.

We found phosphorylated PKC-η in unstimulated Foxp3\(^+\) T reg cells but not in naive T cells (Fig. 1c), despite the fact that expression of mRNA encoding PKC-η and PKC-η protein was not substantially different between T eff cells and T reg cells (Supplementary Fig. 1b). Using a T reg cell–APC conjugation system and confocal microscopy, we observed that, relative to PKC-θ, PKC-η preferentially localized in the T reg cell IS, where it partially colocalized with the TCR (Fig. 1d).

We analyzed the relative intensity of the fluorescence signals of enhanced green fluorescent protein (eGFP)-tagged PKC-η or PKC-θ fusion proteins in the T reg cell IS by calculating the intensity ratio in the IS versus the opposite T cell pole. About 15% (3/20) of imaged cells displayed preferential localization of PKC-θ in the IS (ratio ≥5), consistent with the observation that a similar percentage of cells in this population were Foxp3\(^+\) (by intracellular staining), representing contaminating activated T eff cells in the induced (i)T reg cell culture. Among the remaining cells, the intensity ratios for PKC-η and PKC-θ were 2.84 ± 0.22 and 1.51 ± 0.18 (average ± s.e.m.; \(P < 0.0001\), two-sided Student’s t-test), respectively (data not shown). Taken together, these results indicate that phosphorylated PKC-η associates with CTLA-4 in T reg cells and, furthermore, that PKC-η preferentially colocalizes with CTLA-4 in the IS.

**Development of Foxp3\(^+\) T reg cells is independent of PKC-η**

Given the critical role of CTLA-4 in contact-dependent T reg cell suppressive function, which involves depletion of CD80 and CD86 from APCs\(^{14,15}\), we next examined the CD4\(^+\)Foxp3\(^+\) T reg cell population in the lymphoid organs of Prkch\(^−/−\) mice by intracellular Foxp3 staining. The number and frequency of CD4\(^+\)Foxp3\(^+\) T reg cells was not significantly altered in the thymi and spleens of these mice (Fig. 2a,b and Supplementary Fig. 2a), which suggested that PKC-η is dispensable for in vivo development of CD4\(^+\)Foxp3\(^+\) T cells. Cells that have been ‘licensed’ to become Foxp3\(^+\) expressed similar levels of typical T reg cell markers, including Foxp3, TCRβ, CTLA-4, CD25, immunomodulatory receptor GITR and CD44 (Fig. 2c–j). Thus, PKC-η is dispensable for natural (n)T reg cell development in vivo, and for iT reg cell differentiation in vitro as assessed by expression of typical T reg cell markers (data not shown).

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**Figure 1** IS recruitment and CTLA-4 interaction of PKC-η in T reg cells. (a) Immunoblot analysis of T hybridoma cells left unstimulated (−) or stimulated (+) with anti-CD3 (α-CD3) plus CD86-Fc (B7) for 5 min. CTLA-4 IPs (+ and −) or whole cell lysates (WCL) were immunoblotted with Abs to detect the indicated proteins. Arrowheads mark the two PKC-η species, p-PKCN-η indicated phosphorylated PKC-η. (b) Immunoblot analysis of immunoprecipitates (IPs) or WCL from T hybridoma cells stimulated with anti-CD3 plus CD86-Fc for 5 min. CTLA-4 IPs were left untreated or treated with alkaline phosphatase (AP) before immunoblotting. (c) Immunoblot analysis of cytosol or nuclear fractions from sorted CD4\(^+\)CD8\(^−\)Foxp3\(^+\) (naive) and CD4\(^+\)Foxp3\(^+\) (T reg) cells (2 \(×\) 10\(^6\) each) from Prkch\(^−/−\) Fig mice. Long and short refer to long (10 min) or short (1 min) exposure times. (d) Confocal imaging of PKC-η (top) or PKC-θ (bottom) and TCRβ (TCR) localization in in vitro differentiated iT reg cells from AND TCR-Tg Rag2\(^−/−\) mice, which were retrovirally transduced with eGFP-tagged mouse PKC-θ or PKC-η 1 d after anti-CD3 and anti-CD28 stimulation. Sorted GFP\(^+\) T cells (~90% Foxp3\(^+\) by intracellular staining; data not shown) obtained on day 4 and stimulated for 5–10 min by conjugation with mouse cytochrome C–pulsed lipopolysaccharide-stimulated B cells were fixed and analyzed. eGFP-tagged PKC, TCR and nuclear DAPI staining are shown in green, red and blue, respectively. DIC, differential interference contrast. Data are representative of at least four experiments (a–c) and at least 100 cells collected from three independent experiments (d).
PKC-θ is required for Foxp3+ Treg cell suppressive function

We next explored the possibility that PKC-θ might be required for the suppressive function of Foxp3+ Treg cells. To enable definitive identification of Treg cells, we crossed Prkch−/− mice with mice coexpressing Foxp3 and enhanced GFP under the control of the endogenous Foxp3 promoter (Foxp3-IRES-eGFP, where IRES is the internal ribosome entry site; hereafter called FIG) to generate Prkch−/− FIG mice. We sorted CD4+GFP+ Treg cells from Prkch+/+ and Prkch−/− FIG mice, and found that, upon stimulation, they produced similar amount of interleukin 10 (IL-10; Fig. 2k), a cytokine that can mediate the suppressive activity of Treg cells.

We next considered the possibility that, despite the presence of an apparently normal Treg cell population in Prkch−/− mice, the suppressive activity of these cells may be defective. We first used a conventional in vitro Treg cell suppression assay, which assessed the proliferation of naive T cells cocultured with Treg cells and stimulated with anti-CD3 plus splenic dendritic cells (DCs) as an APC source. The percentage of dividing effector T (Teff) cells was consistently greater in cultures with Prkch−/− Treg cells compared to Teff cells cocultured with Prkch+/+ Treg cells (Fig. 3a). The Prkch−/− Treg cells were substantially impaired in their ability to suppress the proliferation of Teff cells even at high Treg/Teff cell ratios, which indicated that PKC-θ expression by Treg cells is important for their function.

To determine the importance of PKC-θ in Treg cell function in vivo, we used two distinct experimental models, namely, homeostatic T cell population expansion and tumor growth. Transfer of naive CD4+ CD62L+ T cells into immunodeficient mice leads to their massive proliferation, a process generally referred to as homeostatic expansion, although it is likely that some of this proliferation is antigen-driven. Treg cells control the population expansion of Teff cells in a lymphopenic environment. We adoptively transferred purified naive CD45.1+ CD4+ T cells, either alone or in the presence of sorted Prkch+/+ or Prkch−/− CD45.2+ CD4+GFP+ Treg cells, into Rag1−/− mice, and determined T cell numbers 1 week after transfer. In the presence of Prkch+/+ Treg cells, CD45.1+ T eff cell numbers were significantly reduced in all secondary lymphoid organs we examined; in contrast, we observed minimal or no reduction in T eff cell population expansion in the presence of Prkch−/− Treg cells (Fig. 3b–d). Similar numbers of Prkch+/+ and Prkch−/− Treg cells were present in these tissues (Supplementary Fig. 2b).

We next investigated the ability of Prkch−/− Treg cells to inhibit the immune response against a growing tumor. We adoptively transferred splenocytes, depleted of CD25+ T cells, into Rag1−/− mice as a source of Teff cells in the absence or presence of Treg cells 1 day before inoculation of B16-F10 melanoma cells. Transfer of CD25+ T cell–depleted splenocytes alone resulted in relatively small skin tumors, whereas mice receiving Teff cells together with Prkch+/+ Treg cells developed significantly larger tumors than mice receiving Treg cells alone, indicating that the absence of PKC-θ expression on Treg cells impaired the ability of Treg cells to control tumor growth.

**Figure 2** Development of Foxp3+ Treg cells is independent of PKC-θ. (a–d) Cell counts (log10) of CD4+Foxp3+ cells from thymi (a), spleens (b), peripheral lymph nodes (pLN; c) and mesenteric lymph nodes (mLN; d) of 8–12-week-old Prkch−/− or Prkch+/+ mice determined by intracellular Foxp3 staining. Each data point represents a single mouse. Horizontal lines represent mean values. (e–j) Geometric mean fluorescence intensity (geoMFI) of Foxp3 (e), TCRβ (f), CTLA-4 (g), CD25 (h), GITR (i) and CD44 (j) expression determined on gated CD4+Foxp3+ Treg cells from Prkch+/+ or Prkch−/− mice. (k) IL-10 production (measured by enzyme-linked immunosorbent assay (ELISA)) by CD4+GFP+ Treg cells from Prkch+/+ and Prkch−/− FIG mice, which were stimulated with plate-bound anti-CD3 mAb and CD86-Fc in the presence of IL-2 overnight. Shown are mean ± s.e.m. (n = 3). ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001. Data are representative of three independent experiments.

**Figure 3** Contact-dependent suppression by Treg cells depends on PKC-θ. (a) In vitro suppression assay measuring the proliferation of CellTrace Violet–labeled naive B6 CD4+CD25− T eff cells cocultured in the absence (1:0) or presence of Foxp3+ Treg cells from Prkch+/+ or Prkch−/− FIG mice at the indicated T eff/Treg cell ratios and stimulated with anti-CD3 mAb and splenic DCs for 3 d. Percentages of CellTrace Violet-diluting T eff cells were calculated (left). Flow cytometry dot plots of dye-diluting T eff cells cultured with Treg cells at a 1:1 ratio are shown (right). (b–d) In vivo homeostatic proliferation assay showing the number (log10) of B6.SJL CD45.1+ naïve T cells recovered from spleens (b), peripheral lymph nodes (pLN; c) and mesenteric lymph nodes (mLN; d) of recipient Rag1−/− mice 7 d after intravenous transfer of naive T cells (2 × 106 cells) in the absence (none) or presence of CD4+GFP+ Treg cells (0.5 × 106 cells) sorted by flow cytometry from Prkch+/+ or Prkch−/− FIG mice. Each data point represents a single mouse. Horizontal lines represent mean values. (e) Sequential measurements of B16-F10 melanoma growth in groups of naive CD4+ CD62L+ T cells transferred into Rag1−/− mice 7 d after intravenous transfer of naive CD4+CD25− T eff cells (n = 6) or with 0.5 × 106 CD4+GFP+ Treg Cells from Prkch+/+ (n = 8) or Prkch−/− (n = 6) FIG mice, and were inoculated 1 d later with tumor cells (2 × 105 cells). Tumor diameters along two perpendicular axes were measured 2–3 times per week. Shown are mean ± s.e.m. analyzed by analysis of variance (ANOVA) with post hoc Bonferroni’s corrections. *P < 0.05; **P < 0.01; ***P < 0.001. Data are representative of at least five (a) and at least three (b–e) independent experiments.
massive B16 tumors, which reflected inhibition of the T eff cell antitumor response by the cotransferred T reg cells (Fig. 3e). Cotransfer of Prkch−/− T reg cells resulted in substantially reduced tumor growth, similar to that seen in mice receiving only T eff cells (Fig. 3e). Taken together, these results indicate that in the absence of PKC-η, the in vivo suppressive function of T reg cells is attenuated, which leads to enhanced homoeostatic proliferation and antitumor immunity.

Consistent with the importance of PKC-η in T reg cell suppressive functions, Prkch−/− mice exhibit lymphadenopathy, reflected by increased numbers of T cells and B cells, as well as a higher proportion of CD44hi T cells, characteristic of an activated phenotype23. Indeed, Prkch−/− CD44hi T cells secreted significantly elevated amounts of effector cytokines, including IL-2, IFN-γ, IL-4 and IL-17A (Fig. 4a–d), upon in vitro stimulation with anti-CD3 plus anti-CD28 monoclonal antibodies (mAbs). Consistent with this hyperactive phenotype, Prkch−/− mice displayed elevated serum titers of IgE (Fig. 4e) and autoantibodies against double-stranded DNA and histone (Fig. 4f) at 8–12 weeks of age, which implies that the immune system is deregulated and hyperactive in the absence of PKC-η.

We also assessed the ability of Prkch+/+ and Prkch−/− T reg cells to inhibit the development of autoimmune colitis in an established T cell transfer model. Although transfer of naive T cells alone into Rag1−/− recipient mice induced weight loss, indicative of the development of chronic inflammatory bowel disease, cotransfer of either Prkch+/+ or Prkch−/− GFP+ T reg cells protected the recipients against weight loss (Supplementary Fig. 3a) and inhibited the population expansion of T eff cells (Supplementary Fig. 3b). Thus, despite the in vitro (Fig. 3a) and in vivo (Fig. 3b–e) severe defects in their suppressive function, Prkch+/− T reg cells were still able to protect, albeit incompletely, recipient mice against the development of colitis. This finding suggests that in this particular disease model, Prkch+/− T reg cells use an alternative, PKC-η-independent suppressive mechanism(s), e.g., IL-10-mediated suppression (Fig. 2k). Furthermore, increased proliferation (or localization) of Prkch−/− T reg cells in the inflammatory bowel environment may compensate for their defective intrinsic suppressive function. Indeed, we found greater numbers of cotransferred Prkch−/− T reg cells relative to Prkch+/+ T reg cells in the secondary lymphoid organs of the recipient mice, and this effect tended to be more pronounced in mesenteric lymph nodes, which drain the site of inflammation (Supplementary Fig. 3c). Together, these findings suggest that PKC-η is not globally required for all forms of T reg cell–induced suppression, and that it is dispensable for T reg cell–mediated inhibition of colitis.

Mapping and importance of CTLA-4–PKC-η interaction

The predominant association of phosphorylated PKC-η with CTLA-4 in T reg cells suggested that phosphorylated residues mediate this interaction. To pinpoint potential phosphorylation site(s) in PKC-η required for its interaction with CTLA-4, we created mutant vectors encoding PKC-η with substitutions in six predicted phosphorylation sites, transfected these mutant vectors into JTAg cells, a Jurkat cell derivative that does not express CD28, along with a vector encoding full-length CTLA-4, and immunoprecipitated the transfected CTLA-4 proteins. Upon costimulation with anti-CD3 mAb and anti-CD86-Fc recombinant protein, we found that mutations of amino acids Ser28 and Ser32 in the C2 domain, or Ser317 in the V3 domain of PKC-η abolished the interaction with CTLA-4; as a control, substitution of three other phosphorylation sites (Ser327, Thr656 or Ser675) did not affect this interaction (Fig. 5a). Thus, phosphorylation of Ser28 and Ser32 or Ser317 in PKC-η is critical for the interaction with CTLA-4.

To determine whether the association between PKC-η and CTLA-4 is required for the suppressive function of T reg cells, we generated bone marrow (BM) chimeric mice by retrovirally reconstituting Prkch−/− FIG BM cells with wild-type PKC-η or a mutant that does not interact with CTLA-4, PKC-η (S28A,S32A). The retroviral vector used coexpressed a nonsignaling rat CD2 (rCD2) to allow isolation of transduced T cells. We sorted transduced T reg cells (rCD2*GFP+CD45.2+) from chimeric mice and assayed them for their ability to suppress the in vivo homoeostatic proliferation of cotransferred naive CD45.1+ T cells. Unlike T reg cells expressing wild-type PKC-η, T reg cells reconstituted with PKC-η(S28A,S32A) were incapable of suppressing naive T cell proliferation in the spleens and lymph nodes of adoptively transferred recipients (Fig. 5b–d).

To map the critical motif in the cytoplasmic tail of CTLA-4 that is required for the interaction with PKC-η, we substituted four conserved tail residues, i.e., a membrane–proximal positively charged motif (K188XXKKR193), where X is any amino acid other than K or R), a proline-rich motif (Pro205,Pro206,Pro209) and two tyrosine residues (Tyr201 or Tyr218). We found that mutation of the positively charged K188XXKKR193 motif as well as complete deletion of the CTLA-4 cytoplasmic tail (deletion of amino acids 183–223; Δ183–223) greatly reduced the association with PKC-η; in contrast, substitution of the conserved tyrosine residues or the proline-rich motif did not affect the interaction (Fig. 5e). This membrane–proximal motif is highly conserved throughout evolution from fish to primates (Supplementary Fig. 4a). Partial truncation of the cytoplasmic tail of CTLA-4 (Δ192–223), which...
left intact nine membrane-proximal residues, including Lys188 and Lys191, resulted in a residual association with PKC-ζ, which, however, was weaker than that displayed by full-length CTLA-4 (Supplementary Fig. 4b). T<sub>Treg</sub> cells expressing CTLA-4 with a similar incomplete truncation of the cytoplasmic tail have been reported to retain suppressive activity<sup>23,24</sup>. We also found that the interaction between CTLA-4 and PKC-ζ was not affected by PP2, an inhibitor of Src-family kinases (data not shown), consistent with the lack of effect of Tyr201 or Tyr218 substitutions in CTLA-4 tail on this interaction. Taken together, these results reveal that the CTLA-4–PKC-ζ interaction is necessary for the suppressive function of T<sub>Treg</sub> cells, thereby implicating PKC-ζ in a signaling axis linking CTLA-4 to T<sub>Treg</sub> cell–mediated suppression.

**Impaired CD86 depletion by Prkch<sup>−/−</sup> T<sub>Treg</sub> cells**

In comparison to T<sub>eff</sub> cells, T<sub>Treg</sub> cells preferentially form aggregates with APCs<sup>1,25</sup>, and this is due in part to the higher expression of adhesion molecules such as LFA-1 (ref. 26) and neuropilin-1 (ref. 27) on T<sub>Treg</sub> cells. Such T<sub>Treg</sub> cell–APC engagement has been implicated as a potential suppression mechanism, as it allows T<sub>Treg</sub> cells to effectively compete with T<sub>eff</sub> cells in engaging APCs and, thus, inhibit T<sub>eff</sub> cell activation<sup>25</sup>. However, activation of LFA-1 and its conversion to a high-affinity state, as measured by adhesion to its ligand, ICAM-1, was intact in Prkch<sup>−/−</sup> T<sub>Treg</sub> cells (Supplementary Fig. 5). Prkch<sup>−/+</sup> and Prkch<sup>−/−</sup> T<sub>Treg</sub> cells expressed similar amounts of neuropilin-1, CD39 and CD73 (data not shown); the latter two are cell-surface molecules that have been implicated in T<sub>Treg</sub> cell–mediated suppression through an adenosine-dependent action<sup>28</sup>.

To elucidate the signaling mechanism potentially responsible for the PKC-ζ–mediated suppression, we performed a phosphoproteomic analysis of Prkch<sup>−/+</sup> versus Prkch<sup>−/−</sup> T<sub>Treg</sub> cells and found that PAK2 and GIT2, two components of a focal adhesion complex that promotes focal adhesion disassembly and, hence, cellular motility<sup>29,30</sup>, were substantially hypophosphorylated in Prkch<sup>−/−</sup> T<sub>Treg</sub> cells (Supplementary Fig. 6a). A complex of these two proteins together with the guanine nucleotide exchange factors PIX and BPIX has been found to translocate to the T cell IS and to be required for optimal T<sub>eff</sub> cell activation<sup>31</sup>. Moreover, CTLA-4 immunoprecipitates from anti-CD3– plus anti-CD8–costimulated Prkch<sup>−/−</sup> T<sub>Treg</sub> cells contained not only PKC-ζ but also GIT2, αPIX and PAK (Fig. 6a). These proteins were also present in PKC-ζ immunoprecipitates (data not shown). Of note, recruitment of this complex was unique to CTLA-4 costimulation, because the association of the GIT2-αPIX-PAK complex with CTLA-4 was barely above background level when the cells were costimulated with anti-CD3 plus anti-CD28 mAbs (Supplementary Fig. 6b). Thus, this particular signaling event is not shared between CTLA-4 and CD28. Furthermore, the activating phosphorylation of PAK kinases was severely reduced in Prkch<sup>−/−</sup> T<sub>Treg</sub> cells (Fig. 6b), which indicated impaired activation of this complex.

Given the impaired activation of PAK, and because PAK was found to be required for TCR-induced transcriptional activation of NFAT and the CD28 response element (which includes an NF-κB binding site) in Jurkat T cells<sup>31,32</sup>, we also examined whether Prkch<sup>−/−</sup> T<sub>Treg</sub> cells displayed impaired activation of these transcription factors after costimulation with anti-CD3ε plus anti–CTLA-4 mAbs. In these conditions, we observed a severe defect in NFATc1 and NF-κB activation in the Prkch<sup>−/−</sup> T<sub>Treg</sub> cells (Fig. 6c).

Because the GIT-PIX-PAK complex promotes cellular motility through focal adhesion disassembly<sup>29,30</sup>, we investigated whether the defective activation of the GIT2-αPIX-PAK complex in Prkch<sup>−/−</sup> T<sub>Treg</sub> cells might result in a more stable conjugation of Prkch<sup>−/−</sup> T<sub>Treg</sub> cells with APCs. We noticed that the efficiency of conjugation between Prkch<sup>−/−</sup> T<sub>Treg</sub> cells and APCs was significantly higher by comparison to APC conjugates of Prkch<sup>−/+</sup> T<sub>Treg</sub> cells (Fig. 6d), suggesting that in Prkch<sup>−/−</sup>
Treg cells impaired activation of the GIT2-αPix-Pak complex leads to defective breaking of Treg cell–APC contacts.

If Prkch−/− Treg cells display impaired dissociation from engaged APCs because of defective activation of the GIT2-αPix-Pak complex, this defect may be expected to translate into reduced ability of Prkch−/− Treg cells to serially engage new APCs, which, in turn, could result in reduced suppressive activity. This prediction is based on findings that Treg cells can capture CD80 and CD86 from APCs, a process that depletes the ligands required for CD28 costimulation of Treg Cells15 and is thus a potential mechanism of contact-dependent Treg cell–mediated suppression. To address this possibility, we tested the ability of Prkch−/+ versus Prkch−/− Treg cells to deplete CD86 from cocultured CD45.2+CD11c+ APCs as an indirect measure of APC engagement by the Treg cells. Prkch−/+ and Prkch−/− Treg cells were equally capable of depleting CD86 from these APCs (Fig. 6e). However, upon subsequent addition of a second pool of APCs, distinguished from the first APC population by their CD45.1 expression, Prkch−/− Treg cells had a significant delay in their ability to deplete CD86 from these newly introduced APCs, as indicated by the fact that it took them about fourfold longer (~16 h versus ~4 h) to execute the same level of CD86 depletion as that accomplished by Prkch−/+ Treg cells (Fig. 6e). This observation supports the notion that the relative inefficiency of Prkch−/− Treg cells in serially engaging new APCs and, hence, in effectively depleting APC-expressed CD86, could account, at least in part, for their reduced suppressive activity. Our findings imply that a Treg cell–intrinsic signaling mechanism dependent on CTLA-4–PKC-η is required in order to manifest the cell-extrinsic suppressive function of CTLA-4 toward Treg cells.

DISCUSSION

We report an interaction of CTLA-4 with PKC-η and preferential IS localization of PKC-η in Treg cells upon their activation. Moreover, PKC-η and its CTLA-4 association were required for the contact-dependent suppressive function of Treg cells, albeit not for their development. Although Prkch−/− Treg cells displayed defective suppressive activity in a conventional in vitro suppression assay, and in in vivo homeostatic proliferation and antitumor immunity models, they could still inhibit the development of autoimmune colitis. Our findings provide two potential explanations for these apparently discrepant observations. First, enhanced expansion of Prkch−/− Treg cells in the inflammatory bowel environment, which was particularly noticeable in mesenteric lymph nodes of recipient mice, could compensate for the reduced intrinsic suppressive function of these cells. Second, Treg cells use various mechanisms of suppression, and the relative importance of each mechanism depends on the environment and the context of the immune response. For example, similar to Prkch−/+ Treg cells, Ctla4−/− Treg cells suppress the development of colitis through intact compensatory expression of IL-10 (ref. 33), and we found that Prkch−/− Treg cells expressed normal amounts of IL-10. Thus, the CTLA-4–associated PKC-η–dependent suppressive pathway that we describe here may be less relevant in Treg cell suppressive mechanisms that do not depend on Treg cell–APC contact but, rather, on soluble mediators. If our observations of a dispensable role of PKC-η in Treg cell protection against colitis can be extended to other autoimmune disease models, this would suggest that strategies that selectively inhibit PKC-η or its association with CTLA-4 may disable Treg cells from inhibiting antitumor immunity without affecting the ability to control autoimmune disease.
of these T$_{reg}$ cells to protect against autoimmune diseases, a notion with important potential translational implications.

The T$_{reg}$ cell defect revealed by our study may explain the increased cytokine production and higher amounts of serum IgE and autoantibodies in Prkch$^{-/-}$ mice, although intrinsic hyperactivity of the T$_{eff}$ cells in these mice could also contribute to this increase. Nevertheless, it is interesting to note that mice with a T$_{reg}$ cell–specific deletion of Ctla4 display similar increases in cytokine, IgE and autoantibody amounts$^{14}$, which reinforces the notion of a functionally relevant link between CTLA-4 and PKC-$\eta$ at the level of T$_{reg}$ cell–mediated suppression.

The recruitment of a GIT2-$\alpha$PIX-PAK complex to CTLA-4 (and PKC-$\eta$) upon TCR plus CTLA-4 (but not CD28) costimulation of T$_{reg}$ cells and its impaired activation in Prkch$^{-/-}$ T$_{reg}$ cells suggest a potential mechanism underlying their impaired suppressive activity, based on previous findings that this macromolecular complex is required for the disassembly of focal adhesions in neurons and epithelial cells$^{29,30}$. Given the reported localization of this complex to the T cell IS and its importance for activation of T cells$^{31}$, it is possible that the TCR-activated GIT2-$\alpha$PIX-PAK complex destabilizes T cell–APC contacts, thereby promoting conversion of the mature, concentric IS into an unstable IS (i.e., kinapse), which is important for the motility of T cells and their ability to serially engage new APCs$^{34}$. In the context of contact-dependent, T$_{reg}$ cell–mediated suppression, increased stability of T$_{reg}$ cell–APC conjugates and the concomitant reduction in their ability to serially engage new APCs would be translated to impaired overall suppressive activity. Consistent with this notion, we found that Prkch$^{-/-}$ T$_{reg}$ cells form more efficient contacts with APCs in comparison with their Prkch$^{+/-}$ counterparts and, furthermore, these Prkch$^{-/-}$ T$_{reg}$ cells displayed a reduced ability to deplete CD86 from a second set of added APCs, likely a reflection of impairment in their disengagement from the first set of APCs. When considered at the population level and the relatively long time during which T$_{reg}$ cells have the opportunity to engage APCs, this effect could translate into an overall reduction in depletion of CD86 and, consequently, reduced suppression of T$_{reg}$ cells. Hence, our findings identify a T$_{reg}$ cell–intrinsic signaling mechanism consisting of a CTLA-4–bound PKC-$\eta$–GIT2-$\alpha$PIX-PAK complex, which has a role in promoting the cell-extrinsic suppressive function of T$_{reg}$ cells–expressed CTLA-4 on T$_{eff}$ cells.

The biology of CTLA-4 is complex and its mechanisms of action in T cells are still incompletely understood. This complexity reflects, to a large extent, the fact that CTLA-4 is expressed both in T$_{eff}$ cells where it functions in a cell-autonomous manner in cis to inhibit their activation, and in T$_{reg}$ cells where it operates in a cell-nonautonomous manner in trans to dampen responses of T$_{eff}$ cells$^{17,18,35}$. Earlier studies did not distinguish between these functions, but with the conditional deletion of Ctla4 in T$_{reg}$ cells only, it became evident that expression of CTLA-4 by T$_{eff}$ cells is in most (but not all) cases important for their suppressive function through depletion of CD80 and CD86 from APCs$^{14,15,36}$. Ctla4$^{-/-}$ mice display fatal lymphoproliferative disorder characterized by the systemic infiltration of pathogenic self-reactive T cells$^{37,38}$. Although Prkch$^{-/-}$ mice displayed moderate lymphadenopathy, increase in memory-activated T cells and cytokine expression, and elevated IgE and autoantibodies at a relatively young age, they lived into adulthood (~1 year) with no gross signs of pathology (data not shown). Several functional disparities could account for these different phenotypes. First, CTLA-4 inhibits thymocyte negative selection$^{39}$, and, as a result, Ctla4$^{-/-}$ mice harbor autoreactive T cells that cause tissue damage$^{40}$. However, thymic selection is largely intact in the absence of PKC-\(\eta\)$32, which suggests that the lack of overt autoreactivity and lymphoproliferation in Prkch$^{-/-}$ mice might limit the self-destructive nature of the hyperactive Prkch$^{-/-}$ T$_{eff}$ cells. Second, the inhibitory effect of CTLA-4 is mediated by a combination of non–mutually exclusive mechanisms consisting of T$_{reg}$ cell–intrinsic as well as T$_{reg}$ cell–intrinsic mechanisms, and both mechanisms can cooperate to dampen T$_{reg}$ cell responses. Third, our findings strongly suggest that the intrinsic ability Prkch$^{-/-}$ T$_{reg}$ cells to deplete CD86 from APCs by transendocytosis (which depends on the CTLA-4 extracellular domain) is intact, and that only the newly described signaling function of CTLA-4, i.e., the dissociation of T$_{reg}$ cells from APCs mediated by the PKC-$\eta$–GIT2-$\alpha$PIX-PAK complex (which depends on the CTLA-4 cytoplasmic domain) is impaired. In contrast, T$_{reg}$ cells from germ-line Ctla4$^{-/-}$ mice or Foxp3-Cre conditional Ctla4$^{-/-}$ mice lack both of these CTLA-4 functions.

Our findings of altered APC engagement and presumed reduced motility of Prkch$^{-/-}$ T$_{reg}$ cells may be related to the reported ability of CTLA-4 to reverse the TCR stop signal and promote the motility of T$_{eff}$ cells, possibly via LFA-1 activation$^{41,42}$. In contrast, T$_{reg}$ cells have been found to be resistant to the CTLA-4–induced reversal of the TCR stop signal, which results in longer contact times with APCs$^{43}$, a scenario that would lead to lower overall T$_{reg}$ cell motility and less frequent serial encounters with new APCs. However, we did not observe a defect in LFA-1 expression or activation in Prkch$^{-/-}$ T$_{reg}$ cells; furthermore, although LFA-1 promotes the formation of a stable IS, our findings point to a defect at a later stage, i.e., disassembly of the IS in the Prkch$^{-/-}$ T$_{reg}$ cells.

Clinical use of anti–CTLA-4 heralded a new era of cancer immunotherapy by targeting immunosuppression$^{44}$. In many tumor tissues, infiltrating T$_{reg}$ cells restrict the function of T$_{eff}$ cells and, therefore, inhibiting critical T$_{reg}$ cell signaling molecules that are important for their function could lead to enhanced antitumor responses$^{45}$. Here we showed that phosphorylated PKC-$\eta$ is recruited to the T$_{reg}$ cell IS and is associated with the cytoplasmic tail of CTLA-4, and demonstrated the critical importance of this association for the contact-dependent suppressive activity of T$_{reg}$ cells. Hence, the CTLA-4–PKC-$\eta$ axis could represent a key therapeutic target for T$_{reg}$ cell–dependent suppression in controlling cancer.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

K.-F.K. and G.F. designed experiments, collected data, performed analyses and wrote the paper; J.C., T.Y. and T.S. performed microscopy experiment; A.J.C.-B. was involved in experiments and data collection; S.B. performed and assisted in melanoma studies; Y.Z. and J.R.Y. did the phosphoproteomic experiments and analyses; G.K. and M.K. provided critical reagents and were involved in study design; N.R.I.G. and A.A. designed the study, analyzed data and wrote the paper.
COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Antibodies and reagents. mAbs specific for mouse CD3 (clone 145-2C11), CD28 (clone 37.51) or CTLA-4 (clone UC10-18B) were purchased from Biologend, as were fluorophore-conjugated anti-CD4 (clone GK1.5), anti-CD8 (53-6.7), anti-Foxp3 (clone FJK-16s), anti-CD25 (clone PC61), anti-CD44 (clone IM7) and anti-GITR (clone DTA-1) mAbs. Anti-human CD3 mAb (OKT3) was purified in-house. Polyclonal anti-PKC-θ (sc-212), anti-PI3K-α (C-15 and sc-215), anti-PAK (N-20 and sc-882), anti-NFATC1 (7A6), anti-lamin B (M-20) and anti–α-tubulin (TU-02) Abs were obtained from Santa Cruz Biotechnology. Anti-p65 (NF-kB), anti-GIT2 and anti-αPIX Abs were obtained from Cell Signaling Technology. Anti-Foxp3 Abs (clone 150D/E4 for immunoblotting and clone FJK-16s for flow cytometry) were purchased from eBiosciences. Alexa Fluor 647–conjugated anti-mouse Ig and Alexa Fluor 555–conjugated anti-rabbit Ig were obtained from Molecular Probes. Digitonin was obtained from EMD Chemicals. Calf intestinal alkaline phosphatase was purchased from New England BioLabs. Recombinant CD86-Fc was previously described46.

Plasmids. Plasmids of full-length human Prkch and mouse Cta4 were generated via PCR amplification and cloned into the pEP4/HisC expression vector or pMIG retroviral vector, respectively. Prkch and Cta4 point mutations were generated using Quikchange II Site-directed Mutagenesis Kit (Stratagene). Cta4 mutants encoding variants with cysteine tail deletions (amino acids 183–223 and 192–223) were generated via PCR amplification.

Mice and primary cell cultures. C57BL/6 (B6; CD45.2+), B6.SJL (CD45.1+) and Prkch−/− (CD45.2)22 mice were housed and maintained under specific pathogen–free conditions, and manipulated according to guidelines approved by the LIAI Animal Care Committee and the Animal Care and Use Committee of The Scripps Research Institute. The Prkch−/− mice are now available from the Jackson Laboratories (B6.Cg-Prkch-tm1.1Gasc/J). Foxp3-ires-eGFP (FIG) mice were obtained from the Jackson Laboratories. Prkch−/− × Foxp3-IRES-eGFP (Prkch−/− FIG) mice were generated by crossing FIG mice with Prkch−/− mice22, 8–12-week-old mice with no preference for genders were used. CD4+ T cells were isolated by anti-CD4 (BD Biosciences) positive selection, and were cultured in RPMI-1640 medium (Mediatech, Inc.) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 1 mM sodium pyruvate, 1 mM MEM nonessential amino acids, and 100 U/mL each of penicillin G and streptomycin (Life Technologies).

Immunoprecipitation and immunoblotting. Simian virus 40 large T antigen-transfected human embryonic Jurkat T cells (JTag) and MCC-specific hybridoma T cells have been described previously44; these cell lines have not been recently short tandem repeat–profiled but tested negative for mycoplasma contamination. JTag cells in logarithmic growth phase were transfected with plasmid DNAs by electroporation and incubated for 48 h. Transfected cells were stimulated with an anti-human CD3 (OKT3) mAb plus recombinant CD86-Fc and a cross-linking Ab (JTag) or anti-CTLA-4 mAb (T Hybridoma) for 5 min. Cell lysis in 1% NP-40 lysis buffer (50 mM Tris-HCl, 50 mM NaCl and 5 mM EDTA), immunoprecipitation and immunoblotting were carried out as previously described43.

Enzyme-linked immunosorbent assay. Serum IgE was quantified using capture and biotinylated mAbs from CALTAG Laboratories, as previously described47. Autoantibodies specific for double-stranded DNA (dsDNA) and histone were determined using plates coated with salmon sperm DNA (Life Technologies) or calf thymus histone (Roche), respectively. Detection was carried out using biotinylated anti-mouse IgG, streptavidin conjugated HRP and ABTS substrates (Bio-Rad). Relative IgG serum titers were calculated by dividing the absorbance values of experimental samples by the negative control values.

Isolation of mRNA, cDNA synthesis and real-time PCR. Total RNA was extracted from sorted CD4+GFP+ and CD4+GFP− cells of FIG mice using the RNeasy kit (Qiagen). RNA was used to synthesize cDNA by the SuperScript III FirstStrand cDNA synthesis kit (Life Technologies). Gene expression was determined using real-time PCR with iTaq SYBR Green (Bio-Rad) in the presence of the following primer sets: mouse Cta4 forward: 5′-ATCTAGATCCCACGGCCTAG-3′; reverse: 5′-GGGCGATGGTCTTGATCAA-3′; Prkch (forward: 5′-CAAGCATTTCAGCAGGAA-3′; reverse: 5′-TGTTCCCAATACCTCCAG-3′) and the housekeeping β-actin (Actb). Relative gene expression levels were determined in triplicates and calculated using the 2−ΔΔCt and normalized to the abundance of Actb.

Microscopy. CD4+ T cells were purified from moth cytoschrome C (MCC)–specific mice expressing the transgenic AND TCR crossed with Rag2−/− (AND-Tg × Rag2−/−) mice and stimulated by immobilized anti-CD3e (14-251; 10 μg/ml) and anti-CD28 (PV-1; 1 μg/ml) in the presence of mouse recombinant IL-2 (10 ng/ml) and human recombinant TGFβ (5 ng/ml) for 3 d. The cells were retrovirally transduced with vectors encoding eGFP-tagged mouse PKC-θ or PKC-η for 24 h 1 d after the initial stimulation. On day 4 or later, the cells were sorted on a FACSAria (BD) to obtain purified (≥90%) GFP+ cells, which were maintained in culture. B cells purified from B10.BR mice and stimulated by LPS (Difco; 10 μg/ml) plus MCC (5 μg/ml) were cocultured with CD4+GFP+ Treg cells (0.125 × 106 to 4 × 106) sorted from Prkch−/− or Prkch−/− FIG mice in the presence of an anti-mouse CD3 mAb.

In vitro suppression assay. Flow cytometry–sorted naïve CD4+CD26L2 Treg cells were labeled with 5 μM of CellTrace Violet according to the manufacturer’s protocol (Life Technologies). CD11c+ splenic DCs were purified according to the manufacturer’s instructions (Miltenyi Biotech). Labeled Treg cells (2 × 106) and splenic DCs (5 × 105) were cocultured for 3 d with CD4+GFP+ Treg cells (0.125 × 106 to 4 × 106) sorted from Prkch−/− or Prkch−/− FIG mice in the presence of an anti-mouse CD3 mAb.

Homeostatic expansion model. Naïve CD4+CD26L2+ cells from congenic B6.SJL (CD45.1+) and CD4+GFP+ Treg (CD45.2+) cells from Prkch−/− and Prkch−/− FIG mice were sorted using ARIA Cell Sorter (BD Biosciences). Two × 106 naïve T cells were transferred intravenously alone or together with 0.5 × 106 CD4+GFP+ Treg cells into Rag1−/− mice; this was done in a blinded fashion and assigned randomized with no pre-established inclusion or exclusion criteria. Mice were killed 7–10 d after transfer. Spleens, peripheral lymph nodes and mesenteric lymph nodes were harvested, and each population was independently analyzed by flow cytometry. To achieve reasonable power, at least 5 mice/group (15 mice/experiment) were used. Additional mice were added to experiments as appropriate.

B16 melanoma model. Splenocytes from normal B6 mice were depleted of CD25+ cells using biotinylated anti-CD25 mAb (clone PC61, eBiosciences) and streptavidin–conjugated beads (BD Biosciences). CD4+GFP+ Treg cells were sorted using flow cytometry from Prkch−/− or Prkch−/− FIG mice. 15 × 106 CD25-depleted splenocytes were adoptively transferred alone or together with 0.5 × 106 Prkch−/− or Prkch−/− CD4+GFP+ Treg cells into recipient Rag1−/− mice; this was done in a blinded fashion and assigned in a randomized fashion with no pre-established inclusion or exclusion criteria. 2 × 106 B16-F10 melanoma cells were inoculated intradermally on the right shaved flank the next day. Tumor size was measured using an electronic dial caliper 2–3 times/week44. To achieve reasonable power, at least 5 mice/group (15 mice/experiment) were used. Additional mice were added to experiments as appropriate.

BM chimeras. cDNAs encoding full-length human wild-type or S28A,S32A substituted Prkch were subcloned into a modified pMIG retroviral vector containing IRES and non-signaling rat CD2 gene (lacking the cytoplasmic tail). BM chimeras were produced in irradiated B6 mice as previously described49. Briefly, BM cells were flushed from the femurs and tibias of Prkch−/− FIG mice that have been pretreated with 5-fluorouracil to enrich for stem cells. BM cells were cultured in DMEM (Mediatech, Inc.) containing 10% FBS, IL-3 (20 ng/ml), IL-6 (25 ng/ml) and stem cell factor (SCF; 100 ng/ml). Retroviral infections were carried out for two consecutive days. 1 × 106 infected BM cells were intravenously injected into irradiated B6 mice. Analyses were performed
double-positive Treg cells were sorted on an ARIA Cell Sorter, and their immunofluorescence analysis.

Stable isotope labeling by amino acids in cell culture and phospho- medium supplemented with 13C and 15N-labeled lysine and arginine for stable isotope labeling by amino acids in cell culture (SILAC) labeling. GFP+ Treg cells plus anti–CTLA-4 mAbs for 5 min. sorted by flow cytometry were left unstimulated or stimulated with anti-CD3 and immediately assayed on an LSRII flow cytometer to determine double-positive Treg cells (5 × 10⁴) cultured with CD45.2+CD11c+ splenic DCs. For the CD86 depletion experiment, Treg cells (5 × 10⁴) were cultured with CD45.2+CD11c+ splenic DCs (2.5 × 10⁴) for 9 h, followed by addition of CD45.1+CD11c+ splenic DCs (2.5 × 10⁴) from B6.SJL mice for another 9 h. Cells were collected at different time points and stained with fluorophore-conjugated Abs specific for CD11c, Annexin V, CD4, CD86, I-Ab, CD45.1 or CD45.2.

Stable isotope labeling by amino acids in cell culture and phosphoproteomic analysis. Prkch+/+and Prkch−/− FIG naive CD4+ T cells were differentiated into iTreg cells as described above in regular RPMI-1640 medium or medium supplemented with 13C and 15N-labeled lysine and arginine for stable isotope labeling by amino acids in cell culture (SILAC) labeling. GFP+ Treg cells sorted by flow cytometry were left unstimulated or stimulated with anti-CD3 plus anti–CTLA-4 Abs for 5 min. Prkch+/+ and Prkch−/− FIG cell lysates were mixed at a 1:1 ratio, and 300 µg of the protein mixture was precipitated with 5x volume of cold acetone. After centrifugation at 14,000g (10 min at 4 °C), protein pellets were solubilized and reduced with 100 mM Tris-HCl, pH 9. Enriched phosphopeptides were analyzed by the MudPIT LC-MS/MS method. MS analysis was performed using an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher). A cycle of one full-scan mass spectrum (300–1,800 m/z) at a resolution of 60,000 followed by 20 data-dependent MS/MS spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation.

MS data were analyzed by the Integrated Proteomics Pipeline IP2 (Integrated Proteomics Applications; http://www.integratedproteomics.com/). The tandem mass spectra were searched against the European Bioinformatics Institute's International Protein Index mouse target-decoy protein database. Protein false discovery rates were controlled below 1% for each sample. In ProLuCID database search, the cysteine carboxamidomethylation was set as a stable modification, and phosphorylation on serine, threonine or tyrosine was configured as differential modification. Peptide quantification was performed by Census software, in which the isotopic distributions for both the unlabeled and labeled peptides were calculated and this information was then used to determine the appropriate mass-to-charge ratio (m/z) range from which to extract ion intensities. Phosphopeptides were further evaluated with IP2 phospho analysis module, which computes Ascore and Debunker score.

Statistical analysis. Statistical analyses were performed using, unless otherwise stated, analysis of variance (ANOVA) with post hoc Bonferroni’s corrections. Unless otherwise indicated, data represent the mean ± s.e.m., with P < 0.05 considered statistically significant.

10–12 weeks after reconstitution to determine cells coexpressing GFP (for Foxp3 expression) and rat CD2 (for transgene expression) using anti-rCD2 mAb (clone OX-34, Biolegend). Cells were pooled from spleens and peripheral LN of 4–5 chimeric mice, and enriched for CD4+ cells. GFP+CD2+ double-positive Treg cells were sorted on an ARIA Cell Sorter, and their in vivo suppressive function was analyzed in a homeostatic expansion assay as described above.

Treg cell–APC coculture. GFP+ Treg cells (5 × 10⁴) from Prkch+/+ or Prkch−/− FIG mice were cultured with CellTrace Violet-labeled splenic CD11c+ APCs (5 × 10⁴) for the indicated times. Cells were carefully collected with cut tips and immediately assayed on an LSRII flow cytometer to determine double-positive (GFP+Violet+) conjugates. For the CD86 depletion experiment, Treg cells (5 × 10⁴) were cultured with CD45.2+CD11c+ splenic DCs for 5 min, followed by addition of CD45.1+CD11c+ splenic DCs (2.5 × 10⁴) from B6.SJL mice for another 9 h. Cells were collected at different time points and stained with fluorophore-conjugated Abs specific for CD11c, Annexin V, CD4, CD86, I-Ab, CD45.1 or CD45.2.

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Statistical analysis. Statistical analyses were performed using, unless otherwise stated, analysis of variance (ANOVA) with post hoc Bonferroni’s corrections. Unless otherwise indicated, data represent the mean ± s.e.m., with P < 0.05 considered statistically significant.
Erratum: Protein kinase C-η controls CTLA-4–mediated regulatory T cell function

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In the version of this article initially published, the second affiliation for Nicholas R.J. Gascoigne was incorrect. The correct affiliation is The Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore. The error has been corrected in the HTML and PDF versions of the article.