Phosphatase PP2A is requisite for the function of regulatory T cells

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Homeostasis of the immune system depends on the proper function of regulatory T cells (T<sub>reg</sub> cells). Compromised suppressive activity of T<sub>reg</sub> cells leads to autoimmune disease and graft rejection and promotes anti-tumor immunity. Here we report a previously unrecognized requirement for the serine-threonine phosphatase PP2A in the function of T<sub>reg</sub> cells. T<sub>reg</sub> cells exhibited high PP2A activity, and T<sub>reg</sub> cell–specific ablation of the PP2A complex resulted in a severe, multi-organ, lymphoproliferative autoimmune disorder. Mass spectrometry revealed that PP2A associated with components of the mTOR metabolic-checkpoint kinase pathway and suppressed the activity of the mTORC1 complex. In the absence of PP2A, T<sub>reg</sub> cells altered their metabolic and cytokine profile and were unable to suppress effector immune responses. Therefore, PP2A is required for the function of T<sub>reg</sub> cells and the prevention of autoimmunity.

Immunological tolerance is achieved through the elimination of self antigen–specific T cell clones generated in the thymus and through the active suppression, by regulatory T cells (T<sub>reg</sub> cells), of autoreactive T cells that have escaped the thymus to the periphery<sup>1</sup>. T<sub>reg</sub> cells express the signature transcription factor Foxp3 and have a distinct metabolic, proliferation and cytokine profile<sup>2,3</sup>. These characteristics are inherent to their ability to suppress, which allows them to maintain immunological homeostasis. Loss of T<sub>reg</sub> cell function leads invariably to autoimmunity in mice<sup>4</sup> and humans<sup>5</sup>.

PP2A is a highly conserved serine-threonine phosphatase that is the assembly product of three distinct subunits—the scaffold A subunit (PP2A<sub>A</sub>), the regulatory B subunit (PP2A<sub>B</sub>) and the catalytic C subunit (PP2A<sub>C</sub>)—into a trimolecular complex<sup>6,7</sup>. The heterodimer of the scaffold A subunit and the catalytic C subunit (PP2A<sub>A</sub>-PP2A<sub>C</sub>) forms the PP2A core enzyme that associates with one of the regulatory B subunits. The PP2A holocomplex regulates key cellular processes, such as cell-cycle progression, apoptosis, cellular metabolism and migration<sup>7</sup>. PP2A is involved in the development of cancer<sup>8</sup>, neurodegenerative diseases<sup>9</sup> and systemic lupus erythematosus<sup>10</sup>. In systemic lupus erythematosus, PP2A has been linked to regulation of the production of interleukin 2 (IL-2) and IL-17 by CD4<sup>+</sup> T cells and to the control of T cell apoptosis induced by IL-2 deprivation<sup>10,11</sup>. Furthermore, PP2A has a central role in tolerance to endotoxins that is dependent on the adaptor MyD88 (ref. 12), in T cell–mediated anti-tumor responses<sup>13</sup> and in the termination of transcription factor IRF3–dependent signaling via type I interferons after viral infection<sup>14</sup>.

T<sub>reg</sub> cells depend on several activating signals, including the T cell antigen receptor (TCR), coreceptor CD28 and IL-2 signaling pathways, for their survival and function. Specifically, T<sub>reg</sub> cells are agonist selected by high-affinity TCR ligands in the thymus<sup>15</sup>, and continuous TCR engagement is required for their maintenance in the periphery<sup>16</sup>. Loss of signaling via CD28 (ref. 17) or via IL-2 and its receptor<sup>18,19</sup> results in profound T<sub>reg</sub> cell impairment and autoimmunity. Paradoxically, while T<sub>reg</sub> cell function requires the constant presence of such activating signals, T<sub>reg</sub> cells display diminished activity of several key downstream signaling pathways, including the metabolic-checkpoint kinase mTOR pathway<sup>21,22</sup> and the phosphatidylinositol-3-OH kinase (PI(3)K)–kinase mTOR pathway<sup>21,22</sup>, compared with that of other antigen-experienced T cells. Therefore, T<sub>reg</sub> cells utilize additional negative regulators that are not active in conventional T cells (T<sub>conv</sub> cells) to ‘rewire’ these downstream signaling relays. Published reports have established that negative regulation of the PI(3)K–AKT pathway by the Nrp1–SEMA4a axis<sup>23</sup> and of the mTORC2 pathway by the inositol phosphatase PTEN<sup>24</sup> in T<sub>reg</sub> cells is indispensable for the maintenance of their suppressive function. However, very little is known about how T<sub>reg</sub> cells control the mTORC1 complex in a cell-intrinsic manner and whether this regulation is integral for their function.

In this report, we found that the serine-threonine phosphatase PP2A controlled the activity of the mTORC1 complex in T<sub>reg</sub> cells. This allowed them to maintain a metabolic and cytokine profile essential for their suppressive function. T<sub>reg</sub> cell–specific loss of PP2A caused a severe lymphoproliferative and autoimmune disorder with spontaneous activation of the immune system and autoantibody production.

**RESULTS**

Ablation of PP2A in T<sub>reg</sub> cells leads to autoimmunity

As noted above, the PP2A holoenzyme structurally consists of three different proteins: the catalytic C subunit (PP2A<sub>C</sub>), the scaffold A...
subunit (PP2A$_\alpha$) and the regulatory B subunit (PP2A$_\beta$)\textsuperscript{16-17}. We compared the catalytic activity of the PP2A complex in the T$_{reg}$ cells with that in T$_{conv}$ cells and found that T$_{reg}$ cells displayed greater PP2A activity (Supplementary Fig. 1a). The nascent catalytic PP2A$_{C}$ subunit, which is generated via one of two isoforms (C$_\alpha$ and C$_\beta$), is produced in an inactive state and undergoes an activation process that is coupled to its incorporation with the scaffold PP2A$_{A}$ subunit into the heterodimeric PP2A$_{A}$-PP2A$_{C}$ complex\textsuperscript{24-26}. The absence of PP2A$_{A}$ prevents maturation of the catalytic subunit into its active state and thus the catalytic activity of PP2A is impaired\textsuperscript{24}. The scaffold PP2A$_{A}$ subunit is also generated via one of two isoforms, A$\alpha$ and A$\beta$ (encoded by $Ppp2r1a$ and $Ppp2r1b$ respectively), with the former being the dominant in primary and secondary lymphoid organs\textsuperscript{27}, as well as in isolated CD4$^+$ T cells (Supplementary Fig. 1b). Accordingly, to study the role of PP2A in T$_{reg}$ cell function, we deleted the dominant A$\alpha$ isoform of the scaffold PP2A$_{A}$ subunit in a T$_{reg}$ cell-specific manner by crossing Foxp3$^{YFP-cre}$ mice (which express yellow fluorescent protein (YFP) and Cre recombinase via the T$_{reg}$ cell-specific gene Foxp3) with Ppp2r1a$^{flox/flox}$ mice (in which exons 5–6 of $Ppp2r1a$ are lost)\textsuperscript{28} to generate Foxp3$^{YFP-cre}$Ppp2r1a$^{flox/flox}$ mice (called ‘PP2A$^{flox}$ mice’ here) and Foxp3$^{YFP-cre}$Ppp2r1a$^{flox/+}$ or Foxp3$^{YFP-cre}$Ppp2r1a$^{+/+}$ mice (collectively called ‘PP2A$^{+/+}$ mice’ here, because we did not appreciate any haploinsufficiency of $Ppp2r1a$ in the T$_{reg}$ cell population).

By the age of 10–14 weeks, the PP2A$^{flox}$ mice spontaneously developed severe, progressive, multi-organ autoimmunity characterized by wasting, dermatitis, scaly tails and ears, eyelid crusting and, in some cases, overt skin rash and ulcerations (Fig. 1a and Supplementary Fig. 1c–f). The clinical picture displayed similarities to the phenotype of scurvy mice, which harbor a spontaneous mutation mapped to Foxp3 (ref. 28) and develop early-onset, multi-organ autoimmunity. Upon macroscopic examination of the organs, it was evident that the PP2A$^{flox}$ mice suffered a lymphoproliferative syndrome with enlargement of the secondary lymphoid organs (Fig. 1b and Supplementary Fig. 1g,h). Histological examination of the PP2A$^{flox}$ mice revealed extensive inflammatory infiltrates in the lungs, stomach, pancreas, salivary glands and skin (Fig. 1c and Supplementary Fig. 1d). In the lungs, extensive lymphocytic infiltrates were appreciable, especially in the perivascular and peribronchial areas. This cellular infiltration was accompanied by parenchymal consolidation and thickening of the alveolar walls. In the skin, dermal thickening and epidermal hyperplasia with spongiosis, hyperkeratosis and parakeratosis were noted. Lymphoid and polymorphonuclear aggregates in the dermis were abundant and occasionally extended into the epidermis. Focal micro-abscess formation in the stratum corneum and ulceration were also observed. Periductal and perivascular lymphocytic infiltrates were evident in the pancreas and salivary glands and around the pancreatic islets of the PP2A$^{flox}$ mice. In the stomach, inflammatory aggregates, composed of lymphocytes, polymorphonuclear leukocytes and eosinophils, were observed in the submucosa protruding into the adjacent mucosal layer.

The spontaneous autoimmune phenotype of the PP2A$^{flox}$ mice prompted us to assess the status of their immune system compared with that of their PP2A$^{wt}$ littermates. We observed greater activation of both CD4$^+$ T cells and CD8$^+$ T cells in PP2A$^{flox}$ mice relative to that of their PP2A$^{wt}$ littermates, as indicated by the presence of a greater frequency of CD4$^+$CD62L$^-$CD4$^+$, CD4$^+$CD62L$^-$CD8$^+$ and CD4$^+$CD62L$^-$CD8$^+$ T cells in the spleen and peripheral and mesenteric lymph nodes of PP2A$^{flox}$ mice (Fig. 2a). When stimulated ex vivo, CD4$^+$ T cells from PP2A$^{flox}$ mice produced significantly larger amounts of IL-17 and IL-2, and CD8$^+$ T cells from PP2A$^{flox}$ mice produced greater amounts of interferon-$\gamma$ and tumor-necrosis factor (TNF), relative to that of their PP2A$^{wt}$ counterparts (Fig. 2b). Incorporation of the thymidine analog EdU by CD4$^+$ T cells was greater in PP2A$^{flox}$ mice than in their PP2A$^{wt}$ littermates (Supplementary Fig. 2), which demonstrated their enhanced proliferative state. PP2A$^{flox}$ mice had higher concentrations of all immunoglobulin classes.

Figure 1 PP2A$^{flox}$ mice develop multi-organ autoimmunity. (a,b) Body weight (a) and spleen weight (b) of 10- to 14-week-old PP2A$^{wt}$ mice ($n = 17$ (a) and $n = 5$ (b)) and PP2A$^{flox}$ mice ($n = 9$ (a) and $n = 5$ (b)). Body weight (a) is presented relative to that of PP2A$^{wt}$ mice, set as 100%. (c) Hematoxylin-and-eosin staining of the lungs, skin, pancreas, salivary glands and stomach of PP2A$^{wt}$ and PP2A$^{flox}$ mice. Scale bars, 200 $\mu$m. *$P < 0.001$ (unpaired, two-tailed t-test). Data are representative of three experiments (a,b; mean ± s.e.m.) or are from one experiment representative of three independent experiments with similar results (c; $n = 3$ mice per group in each).
(IgM, IgG, IgE and IgA) than those of their PP2A<sup>wt</sup> littermates (Fig. 2c). Congruent with these findings was the greater frequency of follicular helper T cells (CD3<sup>+</sup>CD4<sup>+</sup>PD1<sup>+</sup>CXCR5<sup>+</sup>) and germinal center B cells (CD19<sup>+</sup>FAS<sup>+</sup>GL7<sup>+</sup>) in the spleens of the PP2A<sup>flex</sup> mice than in those of their PP2A<sup>wt</sup> littermates (Fig. 2d). To determine whether PP2A<sup>flex</sup> mice produced autoantibodies, we used an autoantigen array that assess 128 known autoantigens. We detected the presence of IgG autoantibodies to 76 autoantigens that included both tissue-restricted autoantigens and lupus-associated nuclear autoantigens (Fig. 2e). These results indicated that the dominant tolerance exerted by T<sub>reg</sub> cells was lost in the PP2A<sup>flex</sup> mice. Additionally, the absence of any signs of autoimmunity or spontaneous activation of the immune system in female Foxp3<sup>YFP<sup>+</sup>cre<sup>+</sup>/PP2r2a<sup>flex</sup>/flex</sup> mice (data not shown), in which approximately half of the T<sub>reg</sub> cells were lost, suggests that the absence of this regulatory mechanism is not sufficient to induce autoimmunity.

**Figure 2** Spontaneous activation of T cells and B cells in PP2A<sup>flex</sup> mice. (a, b) Flow cytometry analyzing CD4<sup>+</sup> and CD62L (a) and IL-17 and interferon-γ (IFN-γ), IL-4 and IL-2, or CD8 and TNF (b) in CD4<sup>+</sup> or CD8<sup>+</sup> T cells from the spleen, peripheral lymph nodes (pLN) and mesenteric lymph nodes (mLN) (a) or spleen (b) of 10- to 14-week-old PP2A<sup>wt</sup> and PP2A<sup>flex</sup> mice (n = 3 per group). Numbers in quadrants indicate percent cells in each throughout; numbers adjacent to outlined areas (b, right) indicate percent TNF<sup>+</sup> cells among CD8<sup>+</sup> T cells. (c) Enzyme-linked immunosorbent assay of IgM, IgG, IgA and IgE in serum from PP2A<sup>wt</sup> mice (n = 8) and PP2A<sup>flex</sup> mice (n = 5). Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.e.m.). (d) Flow cytometry of splenocytes from PP2A<sup>wt</sup> and PP2A<sup>flex</sup> mice. Numbers adjacent to outlined areas indicate percent PD-1<sup>+</sup> CXCR5<sup>+</sup> cells (follicular helper T cells (T<sub>FH</sub> cells) (left) or FAS<sup>+</sup>GL7<sup>+</sup> cells (germinal center (GC) B cells) (right). (e) Abundance of autoantibodies (right margin) in serum from PP2A<sup>wt</sup> and PP2A<sup>flex</sup> mice (one column per mouse; four mice per group). *P < 0.01 (unpaired, two-tailed t-test). Data are from one experiment representative of three (a, b) or two (c, d) independent experiments, or are pooled from two independent experiments (e).
Figure 3 TCR activation induces SET-mediated phosphorylation of PP2A at Tyr307 in T<sub>reg</sub> cells but not in T<sub>conv</sub> cells. (a,b) Immunoblot analysis of PP2A<sub>C</sub> phosphorylated at Tyr307 (p-PP2A<sub>C</sub> (Y307)), total PP2A<sub>C</sub> and β-actin (loading control) in naive CD4<sup>+</sup> T cells (a) or T<sub>conv</sub> or T<sub>reg</sub> cells (b) stimulated for various times (above lanes; a) or left unstimulated (−) or stimulated for 24 h (+) (b) with anti-CD3 plus anti-CD28 (α-CD3 + α-CD28). (c,f) Intracellular staining of PP2A<sub>C</sub> (c) or SET (f) in splenocytes isolated from Foxp3<sup>Cre</sup>-GFP mice and left unstimulated (US) or stimulated for 24 h with anti-CD3 plus anti-CD28 (Stim). Isotype, isotype-matched control antibody. (d) Intracellular staining of SET and PP2A<sub>C</sub> phosphorylated at Tyr307 in naive CD4<sup>+</sup> T cells stimulated for 0–24 h (left margin) with anti-CD3 plus anti-CD28 (left) and mean fluorescent intensity (MFI) of such staining (right). Dashed vertical lines (left) indicate median fluorescence at 0 h. (e) Intracellular staining of PP2A<sub>C</sub> phosphorylated at Tyr307 in naive CD4<sup>+</sup> T cells activated for 24 h with anti-CD3 plus anti-CD28, then spin-injected with lentiviral vector expressing the red fluorescent dye mCherry alone (empty vector (EV)) or SET and mCherry (Set), followed by analysis of mCherry<sup>*</sup> (infected) T cells. *P < 0.01 (unpaired, two-tailed t-test). Data are from one experiment representative of two (a,b,d,e) or three (c,f) independent experiments with similar results (n = 3 mice in each (a,b,d,e) or n = 5 mice (e); mean ± s.e.m. in d,e).

Cell population was PP2A sufficient, further suggested that loss of T<sub>reg</sub> cell–mediated suppression was responsible for the phenotype of the PP2A<sub>fl</sub> mice.

Ceramide accumulation activates PP2A in T<sub>reg</sub> Cells

Our results suggested that PP2A was necessary for T<sub>reg</sub> cells to maintain their suppressive function. Thus, we investigated the mechanisms that regulate the activity of the PP2A complex in T<sub>reg</sub> cells. Whereas the abundance of the catalytic PP2A<sub>C</sub> subunit is stable in a given cellular state<sup>29</sup>, its activity is controlled through post-translational modifications at its carboxy-terminal tail<sup>30</sup>. Specifically, phosphorylation of the Tyr307 residue at the carboxy-terminal end of PP2A<sub>C</sub> results in the inactivation of PP2A<sub>30</sub>,<sup>31</sup>. When we activated wild type CD4<sup>+</sup> T<sub>conv</sub> cells with antibody to the invariant signaling protein CD3 (anti-CD3) plus anti-CD28, we detected phosphorylation of PP2A<sub>C</sub> at Tyr307 within 24 h of activation (Fig. 3a). However, we did not detect the same phosphorylation in T<sub>reg</sub> cells, which retained small amounts of Tyr307 phosphorylation after stimulation with anti-CD3 plus anti-CD28 (Fig. 3b and Supplementary Fig. 3a), consistent with their enhanced PP2A activity (Supplementary Fig. 1a). Notably, the

Figure 4 T<sub>reg</sub> cells have a greater ceramide content than that of T<sub>conv</sub> cells, through Foxp3-mediated inhibition of Sgms1. (a,b) Staining of ceramide (a) or PP2A<sub>C</sub> phosphorylated at Tyr307 (b) in naive CD4<sup>+</sup> T cells stimulated for 24 h with anti-CD3 plus anti-CD28, then treated for 1 h with SMase (0.5 units/ml) or vehicle (50% glycerol in PBS) (Veh) (left), and mean fluorescent intensity of such staining (right). (c) Quantification of ceramide species (horizontal axis) in T<sub>conv</sub> and T<sub>reg</sub> cells stimulated for 24 h with anti-CD3 plus anti-CD28, analyzed by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. (d) Ceramide content of CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>−</sup> cells, analyzed by flow cytometry. (e) ChIP and real-time quantitative PCR analysis of the binding of IgG (control) or Foxp3 at Sgms1 in T<sub>reg</sub> cells; results are normalized to input. (f) Ceramide content of mCherry<sup>*</sup> (infected) cells among naive CD4<sup>+</sup> T cells stimulated for 24 h with anti-CD3 plus anti-CD28, then ‘spin-injected’ with lentiviral vector expressing mCherry alone (EV) or the Sgms1 coding sequence and mCherry (Sgms1), analyzed by flow cytometry (left), and quantification of those results (right). *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired, two-tailed t-test). Data are from one experiment representative of two (a–e,f) or three (d) independent experiments with similar results (n = 3 mice in a,b,d; n = 3 mice per group in e; mean ± s.e.m. in a,b,d,e).
total amount of the PP2A_c subunit in Treg cells was similar to that in Tconv cells both before stimulation with anti-CD3 plus anti-CD28 and after such stimulation (Fig. 3c).

The phosphorylation of PP2A_c at Tyr307 is under the control of the ceramide-SET pathway. Set (originally named ‘I’-PP2A’ for ‘inhibitor 2 of PP2A’) has been identified as an inducer of the phosphorylation of PP2A_c at Tyr307 and thus as an inhibitor of PP2A activity.)–34. We found that in CD4+ T cells, Set was a highly TCR-responsive gene with kinetics of induction that paralleled those of the phosphorylation of PP2A_c at Tyr307 following stimulation of CD4+ T cells with anti-CD3 plus anti-CD28 (Fig. 3d). Lentiviral infection of CD4+ T cells with a Set-expressing vector and knockdown of Set expression via short hairpin RNA promoted the phosphorylation of PP2A_c at Tyr307 and decreased such phosphorylation, respectively, after stimulation of the cells with anti-CD3 plus anti-CD28 (Fig. 3e and Supplementary Fig. 3b); this indicated that Set was responsible for the Tyr307-phosphorylation of PP2A_c after activation of CD4+ T cells. However, Treg cells and Tconv cells exhibited similar induction of Set upon stimulation with anti-CD3 plus anti-CD28 (Fig. 3f). Thus, to investigate the cause of differential Tyr307-phosphorylation of PP2A_c in Treg cells and Tconv cells, we turned our focus to ceramide, the upstream regulator of the ceramide-SET pathway. Specifically, the SET-mediated induction of the phosphorylation of PP2A_c at Tyr307 can be abolished by an increase in the intracellular abundance of ceramide, because ceramide interacts with SET and constrains its inhibitory action on the PP2A complex and thus represents an important endogenous PP2A activator)–37. Indeed, treatment of T cells with sphingomyelinase (SMase), which increased the endogenous ceramide content (Fig. 4a), diminished the association of SET with PP2A_c (Supplementary Fig. 3c). Accordingly, the treatment of activated CD4+ T cells with SMase decreased the Tyr307-phosphorylation of PP2A_c (Fig. 4b and Supplementary Fig. 3d). To quantify the abundance of the ceramide species present in Treg CD4+ cells and Tconv CD4+ cells, we used high-performance liquid chromatography coupled with electrospray-ionization tandem mass spectrometry and found that Treg cells had larger amounts of several ceramide species than did Tconv cells (Fig. 4c). Flow cytometry analyzing total ceramide content confirmed those results at the single-cell level (Fig. 4d).

We subsequently explored the mechanism that led to the Treg cell-specific accumulation of intracellular ceramide. The sphingomyelin S1M1 (encoded by Sgms1) is an enzyme of the sphingolipid-metabolism pathway that has been shown to be part of the Treg cell signature)–38 and is underexpressed in Treg cells. S1M1 catalyzes the conversion of ceramide and phosphatidylcholine to diacylglycerol and sphingomyelin. Small amounts of S1M1 result in the intracellular accumulation of ceramides)–39,40. Chromatin immunoprecipitation (ChIP) experiments demonstrated that Sgms1 was a direct target of Foxp3 (Fig. 4e), and retroviral overexpression of FOXP3 in Jurkat human T lymphocytes decreased the expression of Sgms1 at the level of mRNA and protein (Supplementary Fig. 4a,b), in agreement with published data41. Accordingly, lentiviral infection of mouse CD4+ T cells with an Sgms1-expressing vector reduced the intracellular abundance of ceramide (Fig. 4f). Therefore, the Treg cells had a cell-intrinsic mechanism for maintaining their increased PP2A activity. Foxp3-mediated suppression of Sgms1 resulted in the accumulation of ceramide in Treg cells that led to activation of the PP2A complex.

**PP2A inhibits mTORC1 in Treg cells**

To delineate the specific contribution of ceramide-mediated activation of PP2A to Treg cell function, we activated the PP2A complex in Jurkat T cells through the use of SMase. Immunoprecipitation of PP2A_c was performed, and bands that represented increased association of PP2A_c with Raptor, one of the proteins identified by Ingenuity Pathway Analysis (IPA) revealed that components of the mTORC2 complex, and retroviral overexpression of FOXP3 in Jurkat human T lymphocytes decreased the expression of Sgms1 at the level of mRNA and protein (Supplementary Fig. 4a,b), in agreement with published data41. Accordingly, lentiviral infection of mouse CD4+ T cells with an Sgms1-expressing vector reduced the intracellular abundance of ceramide (Fig. 4f). Therefore, the Treg cells had a cell-intrinsic mechanism for maintaining their increased PP2A activity. Foxp3-mediated suppression of Sgms1 resulted in the accumulation of ceramide in Treg cells that led to activation of the PP2A complex.

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**Figure 5** PP2A inhibits the mTORC1 pathway in Treg cells. (a) Flow cytometry analyzing AKT phosphorylated at Thr308 (p-AKT(T308)) or Ser473 (p-AKT(S473)) and phosphorylated S6 (p-S6) in Jurkat T cells treated for 1 h with SMase (0.5 units/ml) or vehicle (50% glycerol in PBS). (b) Staining of AKT phosphorylated at Thr308 or Ser473 and phosphorylated S6 in naive CD4+ T cells (n = 3 mice per treatment group) stimulated for 24 h with anti-CD3 plus anti-CD28 (2 µg/ml), then incubated for 3 h with okadaic acid (+) or DMSO (−) and treated with SMase (+) or vehicle (−) during the final hour (below plots). (c) Staining of phosphorylated S6 in Foxp3+ Treg cells isolated from PP2A_c and PP2A_c mice (n = 3 per group) (left), and quantification of those results (right). NS, not significant (P > 0.05); *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired, two-tailed t-test). Data are from one experiment representative of two (a,b) or three (c) independent experiments with similar results (mean ± s.e.m. in b,c).
the activity of mTORC1 complex and a negligible effect on mTORC2 and PI(3)K (Fig. 5a). We obtained similar results when we used isolated primary mouse CD4+ T cells. Specifically, treatment of the CD4+ T cells with SMIase significantly diminished the phosphorylation of S6, and pharmacological inhibition of PP2A with okadaic acid increased the phosphorylation of S6 (Fig. 5b). Notably, the SMIase-induced decrease in the phosphorylation of S6 was completely abrogated when PP2A was inhibited with okadaic acid (Fig. 5b). We observed a slight decrease in the phosphorylation of AKT at Ser473 and no change in its phosphorylation at Thr380 upon treatment of mouse CD4+ T cells with SMIase (Fig. 5b). Flow cytometry analyzing phosphorylated S6 in T<sub>reg</sub> cells immediately after isolation from PP2A<sup>wt</sup> and PP2A<sup>fox</sup> mice (directly ex vivo) showed that PP2A<sup>fox</sup> T<sub>reg</sub> cells had significantly more phosphorylation of S6 than did PP2A<sup>wt</sup> T<sub>reg</sub> cells (Fig. 5c).

mTOR is centrally involved in the regulation of several key cellular processes, including nutrient sensing, cell proliferation and metabolism. In T cells, mTOR additionally exerts control over cytokine production and T cell differentiation. The increased activity of the mTORC1 complex in PP2A<sup>fox</sup> T<sub>reg</sub> cells (Fig. 5c), coupled with the loss of T<sub>reg</sub> cell–mediated tolerance in PP2A<sup>fox</sup> mice (Figs. 1 and 2 and Supplementary Figs. 1 and 2), prompted us to assess the phenotypic characteristics of PP2A<sup>fox</sup> and PP2A<sup>wt</sup> T<sub>reg</sub> cells. Specifically, we quantified the glycolytic and mitochondrial respiratory capacity of these cells with an extracellular flux analyzer. PP2A<sup>fox</sup> T<sub>reg</sub> cells had a significantly higher baseline and maximum glycolytic rate than that of PP2A<sup>wt</sup> T<sub>reg</sub> cells (Fig. 6a). Similarly, PP2A<sup>fox</sup> T<sub>reg</sub> cells displayed an increased oxidative-phosphorylation rate both at baseline and at maximum capacity, relative to that of PP2A<sup>wt</sup>.
Figure 7 Rapamycin reverses the abnormal profile of PP2A\textsuperscript{flox} T\textsubscript{reg} cells. (a) Flow cytometry of PP2A\textsuperscript{wt} and PP2A\textsuperscript{flox} T\textsubscript{reg} cells (gated on Foxp3-YFP\textsuperscript{+}CD4\textsuperscript{+}T cells) stimulated with anti-CD3 plus anti-CD28, treated for 4 d with IL-2 (100 IU/ml) for population expansion, with treatment with rapamycin (Rap) (100 nM) or DMSO during the final 24 h (left margin), then re-stimulated for 6 h with PMA plus ionomycin. Numbers adjacent to outlined areas indicate percent IL-17\textsuperscript{+} cells (left) or IL-2\textsuperscript{+} cells (right) among the Foxp3-YFP\textsuperscript{+}CD4\textsuperscript{+} T cells. (b) Extracellular acidification rate and oxygen-consumption rate of PP2A\textsuperscript{wt} and PP2A\textsuperscript{flox} T\textsubscript{reg} cells \((n = 3 \text{ mice per group})\) sorted, stimulated and treated as in a (without re-stimulation with PMA plus ionomycin), then sorted again as Foxp3-YFP\textsuperscript{+}CD4\textsuperscript{+} T cells at the end of the culture. (c) Hematoxylin-and-eosin staining of the lungs and salivary glands of PP2A\textsuperscript{wt} and PP2A\textsuperscript{flox} mice treated with vehicle or rapamycin (left), and combined clinical scores of inflammation in the liver, skin, stomach, salivary glands, lungs and pancreas of such mice (right). Scale bar (left), 100 µm. (d,e) Flow cytometry analyzing phosphorylated S6 (d) and CD98 (e) ex vivo in T\textsubscript{reg} (Foxp3-YFP\textsuperscript{+}) cells from mice as in c. (f) In vitro suppression of CD45.1\textsuperscript{+}CD4\textsuperscript{+} effector T cells by T\textsubscript{reg} cells from mice- or rapamycin-treated PP2A\textsuperscript{wt} and PP2A\textsuperscript{flox} mice (incubated together at a ratio of 1:1, T\textsubscript{reg} cells/T\textsubscript{eff} cells), presented as dilution of the cytosolic dye CFSE. Numbers at ends of lines indicate percent divided cells. \(*P < 0.05, **P < 0.01 \text{ and } ***P < 0.001\) (unpaired, two-tailed t-test (b) or one-way analysis of variance followed by Tukey’s multiple comparison test (c)). Data are from one experiment representative of two independent experiments with similar results (mean and s.e.m. in b,c).

T\textsubscript{reg} cells (Fig. 6a). The augmented glycolytic and oxidative-phosphorylation rates of the PP2A\textsuperscript{flox} T\textsubscript{reg} cells were comparable to or higher than the corresponding rates in PP2A\textsuperscript{wt} T\textsubscript{conv} cells before and after stimulation with anti-CD3 plus anti-CD28 (Supplementary Fig. 6a). In vivo determination of the proliferation rate of T\textsubscript{reg} cells was done by intraperitoneal injection of EdU into PP2A\textsuperscript{wt} and PP2A\textsuperscript{flox} mice. PP2A\textsuperscript{flox} T\textsubscript{reg} cells displayed increased incorporation of EdU (Fig. 6c), which indicated that they proliferated more than the PP2A\textsuperscript{wt} T\textsubscript{reg} cells did. These results were congruent with our observation of a greater frequency of T\textsubscript{reg} cells in the spleen and peripheral and mesenteric lymph nodes of the PP2A\textsuperscript{flox} mice than in those of PP2A\textsuperscript{wt} mice (Fig. 6d). In addition, ex vivo stimulation with the phorbol ester PMA and ionomycin resulted in greater production of IL-2 and IL-17 in PP2A\textsuperscript{flox} T\textsubscript{reg} cells than in PP2A\textsuperscript{wt} T\textsubscript{reg} cells (Fig. 6e). PP2A\textsuperscript{flox} T\textsubscript{reg} cells and PP2A\textsuperscript{wt} T\textsubscript{reg} cells exhibited similar expression of the surface markers CD25, CTLA-4, PD-1, LAP, CD73, GITR and FR-4 (Fig. 6f and Supplementary Fig. 6b), but PP2A\textsuperscript{flox} T\textsubscript{reg} cells had higher expression of the large neutral amino acid–transporter CD98 (LAT1), which depends on mTORC1 activity\textsuperscript{43}, than that of PP2A\textsuperscript{wt} T\textsubscript{reg} cells (Fig. 6g). In an in vitro suppression assay, PP2A\textsuperscript{flox} T\textsubscript{reg} cells did not suppress the proliferation of effector T cells to the same extent that PP2A\textsuperscript{wt} T\textsubscript{reg} cells did (Fig. 6h).

To determine the functional importance of the deregulation of the mTORC1 pathway in PP2A-deficient T\textsubscript{reg} cells, we assessed the ability of the mTORC1 inhibitor rapamycin to reverse the abnormal phenotype of the PP2A\textsuperscript{flox} T\textsubscript{reg} cells. Treatment of PP2A\textsuperscript{flox} T\textsubscript{reg} cells...
with rapamycin in vitro diminished the production of IL-2 and IL-17 (Fig. 7a). In addition, rapamycin normalized the increased glycolytic and oxidative-phosphorylation rates of these cells to the rates of the PP2A<sup>wt</sup> T<sub>reg</sub> cells (Fig. 7b). In vivo administration of rapamycin (3 mg per kg body weight, intraperitoneally, every other day<sup>44</sup>) to PP2A<sup>flox</sup> mice reduced the infiltration of leukocytes into the affected peripheral tissues (Fig. 7c). Such in vivo administration of rapamycin also normalized the proportion and number of effector CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells in PP2A<sup>flox</sup> mice such that these were comparable to those of untreated PP2A<sup>wt</sup> mice (Supplementary Fig. 7a,b). T<sub>reg</sub> cells from the rapamycin-treated PP2A<sup>flox</sup> mice displayed less phosphorylation of S6 than that of T<sub>reg</sub> cells from untreated PP2A<sup>flox</sup> mice (Fig. 7d), indicative of diminished mTOR signaling. This inhibition was also indicated by a reduction in the surface expression of CD98 (Fig. 7e). Notably, T<sub>reg</sub> cells from rapamycin-treated PP2A<sup>flox</sup> mice effectively suppressed the proliferation of PP2A<sup>wt</sup> naive CD4<sup>+</sup> T cells activated in vitro with anti-CD3 plus anti-CD28 (Fig. 7f). In addition, rapamycin treatment decreased the proportion of follicular helper T cells and germinal center B cells in PP2A<sup>flox</sup> mice compared with that in untreated PP2A<sup>flox</sup> mice (Supplementary Fig. 7c,d), such that these were comparable to those of untreated PP2A<sup>wt</sup> mice. We concluded that PP2A-mediated inhibition of mTORC1 was necessary for the suppressive function of T<sub>reg</sub> cells and that the treatment of PP2A<sup>flox</sup> T<sub>reg</sub> cells with rapamycin in vitro and in vivo re-established their suppressive function and metabolic and cytokine profile.

**DISCUSSION**

With the exception of the role of PTEN, the role of intracellular phosphatases in the control of T<sub>reg</sub> cell function is poorly understood. In this article, we reported that T<sub>reg</sub> cells required PP2A, an evolutionarily conserved serine-threonine phosphatase, to suppress the effector function of cells of the immune system. Mass spectrometry, pathway-enrichment analysis and intracellular signaling analysis revealed that PP2A operated through inhibition of the mTORC1 signaling pathway. Published studies have shown that increased activity of the mTOR pathway negatively affects the generation and function of T<sub>reg</sub> cells<sup>20,22,45,46</sup>. Interestingly, abrogation of the mTORC1 complex in T<sub>reg</sub> cells in Foxp3<sup>YFP-cre</sup>Raptor<sup>flox/flox</sup> mice (which undergo deletion of loxP-flanked alleles encoding Raptor via Cre expressed via Foxp3)<sup>13</sup> also disrupts T<sub>reg</sub> cell function, which indicates that fine regulation of the activity of this pathway, rather than its complete absence, is the determining factor for T<sub>reg</sub> cell ‘operational fitness.’ Ceramide-mediated activation of PP2A provided T<sub>reg</sub> cells with the requisite phosphatase activity for control of mTORC1 and established their tolerogenic metabolic and cytokine profile.

Foxp3, the main transcriptional regulator of T<sub>reg</sub> cells, utilizes the pre-existent transcriptional landscape of the activated CD4<sup>+</sup> T cell to establish T<sub>reg</sub> cell function<sup>47</sup> and acts largely as a repressor<sup>41</sup>. However, understanding of the ‘translation’ of Foxp3-dependent epigenetic regulation into intracellular signaling events is limited. Ceramides have been recognized as important intracellular bioactive metabolites and second messengers<sup>48</sup>. A major effect of ceramide is activation of the PP2A complex<sup>35–37</sup>. SMS1, an enzyme of sphingolipid metabolism, regulates the amount of ceramide in a cell and has consistently been recognized in microarray studies as being encoded by one of the main genes repressed in T<sub>reg</sub> cells<sup>38</sup>. Here we found that Sgms1 was a direct target of Foxp3 and thus we have provided a link between Foxp3 function and PP2A-mediated signaling effects.

PP2A has been linked to the regulation of key intracellular signaling pathways, including but not limited to the AKT, MAPK and JAK-STAT pathways<sup>7,8</sup>. The relative importance of each of these associations depends on the cellular type and the biological response investigated. Thus, it was crucial in our system to define the main downstream signaling events following the activation of PP2A. Our experiments have highlighted the importance of the interaction between PP2A and the mTOR pathway in T<sub>reg</sub> cells. Given the well-established association of PP2A with AKT<sup>49</sup> and of AKT with T<sub>reg</sub> cells<sup>21–23,50</sup>, we independently investigated the effect of PP2A activation on AKT phosphorylation.

We found that ceramide-mediated activation of PP2A had minimal effect on the mTORC2-dependent phosphorylation of AKT at Ser473 and had no effect on the PI(3)K-mediated phosphorylation of AKT at Thr308. We concluded that the activation of PP2A in T<sub>reg</sub> cells in a ceramide-dependent manner targeted mainly the mTORC1 signaling.

In this report, we have provided evidence that T<sub>reg</sub> cell function depends on the presence of PP2A. Moreover, in its absence, mice developed profound lymphoproliferation and autoimmunity. We propose that therapeutic enhancement of PP2A activity in T<sub>reg</sub> cells should mitigate autoimmunity and transplant rejection and that inhibition of the PP2A complex in T<sub>reg</sub> cells should compromise their function and promote the fight of the immune system against cancer.

**METHODS**

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

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**AUTHOR CONTRIBUTIONS**

S.A.A. designed, performed and analyzed experiments and wrote the manuscript; N.R.-R., A.S.-F., N.D., E.O. and I.C.C. performed and analyzed experiments; M.G.T. analyzed the histopathology samples; and G.C.T. designed the overall study, analyzed the data and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Antibodies and reagents. Anti-PP2A-α (1D6; Millipore), antibody to PP2A-α phosphorylated at Tyr307 (E155; Epitomics), anti-SET (EPR12972(B); Abcam), anti-Raptor (24C12; Cell Signaling), anti-Foxp3 (150D (BioLegend) and D608R (Cell Signaling)), anti-ceramide (MID 15B4; Sigma-Aldrich), Pacific Blue–conjugated antibody to phosphorylated S6 (D57.2.E; Cell Signaling), Alexa Fluor 647–conjugated antibody to AKT phosphorylated at Ser473 (9E12; Cell Signaling), Alexa Fluor 488–conjugated antibody to AKT phosphorylated at Thr308 (C31E5E; Cell Signaling) and anti-SGSM1 (PA1-12761; Thermo Scientific) were used for immunoblot analysis, immunoprecipitation, ChIP and flow cytometry experiments. Staining for phospho-flow experiments was performed using the Beckman Coulter PerFix Expose kit (B26976). Antibodies for T cell and B cell sorting and analysis of cytokine production by flow cytometry were as follows (all from BioLegend): anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-CD62L (5D6), anti-CD25 (3C7), anti-CD25 (5C6) and anti-CD69 (M1/70.4). Antibodies and reagents are listed below. Cell Signaling Technologies. For all other experiments, T cell isolation was performed with the magnetic separation Naive CD4+ T cell isolation kit (130-104-453) from Miltenyi Biotech. In all instances, cells were sorted to more than 95% purity.

Glycolytic and mitochondrial respiration rate measurement. For metabolic experiments, a Seahorse XF24 instrument was used. Foxp3-eGFP+ or Foxp3-YFP+CD4+ T cells sorted by flow cytometry (Fig. 6a,b) and CD4+CD25+ T cells (isolated as described below; Supplementary Fig. 6a) were seeded at a density of 5 × 10^4 per well. The extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) for each well were calculated, while the cells were subjected to the XF Cell Mito or the XF Glycolytic stress test protocols. The XF Cell Mito and the XF Glycolytic stress test kits were purchased from Seahorse Biosciences.

T cell isolation. Treg cell isolation for immunoblot analysis, mass spectrometry and ChIP experiments was performed using the magnetic separation Naive CD4+ T cell isolation kit (11463D) from Life Technologies. For all other experiments, Treg cells were sorted on a FACSAria II (Becton, Dickinson) as CD3+CD4+CD25+Foxp3-eGFP+ T cells. Naive CD4+ T cell isolation was performed with the magnetic separation Naive CD4+ T Cell Isolation kit (130-104-453) from Miltenyi Biotech. In all instances, cells were sorted to more than 95% purity.

Mass spectrometry. Ceramide species were quantified by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry at the Lipidomics Analytical Core Facility of the Medical University of South Carolina, as previously described. 3 × 10^5 Treg cells and 3 × 10^5 Treg cells were analyzed, after lipid extraction, with internal sphingolipid controls, by a Thermo Fisher Scientific triple quadrupole mass spectrometer operating in a multiple reaction monitoring (MRM) positive ionization mode for the quantification of ceramide species.

For the identification of PP2A-α–interacting proteins, Jurkat T cells were treated with sphingomyelinase (0.5 units/ml) or vehicle (50% glycerol in PBS) for 1 h and then were lysed for protein immunoprecipitation with a PP2A-α–specific antibody (1D6; Millipore) or a mouse IgG control antibody (MG2b; Biolegend). Immunoblot analysis was performed under denaturing conditions with the immunoprecipitates separated on different gels: a 4–12% Bis-Tris gel (SDS-PAGE, pH 7.0; NuPage Novex Life Technologies) with effective range of separation of 2–200 kilodaltons (Supplementary Fig. 5a) and a 3–8% Tris-Acid gel (pH 8.1; NuPage Novex Life Technologies) with effective range of separation of 36–400 kilodaltons (data not shown). Bands that were represented differentially by the SMase- and vehicle-treated groups were subjected to mass spectrometry for identification of PP2A-α–interacting proteins after ceramide-mediated PP2A activation. The bands with corresponding height at the IgG control lane were also excised and any proteins identified were excluded. The results were controlled for false-discovery rate and the proteins identified were analyzed with QIAGEN’s Ingenuity Pathway Analysis (IPA) software for the identification of pathway enrichment in the data set.

Autoantigen microarray. Serum from 10- to 14-week-old PP2A-I and PP2A-I0 mice was analyzed for the presence of autoantibodies using the Autoantigen Microarray Super Panel (128-antigen panel) at the Genomics and Microarray Core facility of the University of Texas Southwestern Medical Center. Autoantibodies with a statistically significant difference between the two groups were used to create a row-normalized heat-map analysis with R software.

Code availability. The pathway analysis for the mass spectrometry data in Supplementary Figure 5b was done with the use of QIAGEN’s Ingenuity Pathway Analysis (QIAGEN: http://www.qiagen.com/ingenuity; IPA Winter Release 2014).

R software was used to analyze and generate the results of the autoantigen array (Fig. 2e).

PP2A-α enzymatic activity. PP2A-α enzymatic activity was assessed using the PP2A Immunoprecipitation Phosphatase Assay Kit (17–313; Millipore) per the manufacturer’s instructions. Protein extracts were immunoprecipitated with a PP2A-α–specific antibody (1D6; Millipore). An appropriate phosphorylated peptide (amino acid sequence K-R-pT-I-R-R, where ‘pT’ indicates phosphorylated threonine) was added to the immunoprecipitated immunocomplexes as a substrate for PP2A-α, and samples were incubated at 30 °C in a shaking incubator for 10 min. Supernatants (25 μl) were transferred to a 96-well plate and released phosphate was measured by the addition of 100 μl malachite green phosphate detection solution in a 15-min colorimetric reaction. Phosphate concentrations were calculated from a standard curve created using serial dilutions of a standard phosphate solution.

In vitro suppression assay. CD45.1+CD4+CD62L+CD25+CD44+ T cells (1 × 10^6; Teff cells) were stained with CFSE (carboxyfluorescein diacetate succinimidyl ester; Life Technologies) and were cultured for 4 d with PP2A-I or PP2A-I0 Treg cells in serially decreasing ratios (1:1, 1:2, 1:4, 1:8, only Treg cells) in the presence of 1 × 10^5 irradiated spleenocytes and plate-bound anti-CD3 (2 μg/ml; 145–2C11; BioXcell). Treg cell proliferation was assessed by flow cytometry as the dilution of CFSE at the end of the 4-d culture.

Plasmid, retroviral and lentiviral infection. For SET-silencing experiments, the psi-mU6 mCherry-expressing vector was used, which harbors SET-specific or scrambled control short hairpin RNA (Geneceopia). For lentiviral overexpression of SMS1 and SET, the full coding sequences of mouse Sms1 and Set were inserted into the psi-hu8 lentivector (Addgene 25417) and packaged into lentivirus particles (InGenious Pathway Analysis (IPA) software for the identification of pathway enrichment in the data set.
α5′-5′αT cells were stimulated in complete RPMI-1640 medium 6 αStudent’s two-tailed g (5′+FOXP3 controls regulatory T cell function through cooperation with 5′-5′c

gating on mCherry

Downstream flow cytometry analysis of the infected cells was performed by

fuged at 900

µg/ml) using spin-infection, during which cells were centri

Viral particles were generated as described above, using pCG-GagPol and

pVSVg helper plasmids, and Jurkat T cells were infected two consecu

Viral supernatants were concentrated 100-fold with

Lenti-X Concentrator (63123; Clontech Laboratories). Lentiviral infection of naive CD4+ T cells was performed 24 h after activation with anti-CD3 plus

anti-CD28 (2 µg/ml) using plate-bound anti-CD3 (2 µg/ml; 145-2C11; BioXcell) and anti-CD28

µg/ml; 145-2C11; BioXcell) and anti-CD28

for 6 h in the

presence of GolgiPlug (BD Biosciences) and were stained using the Cytofix/ Cytoperm kit from BD Biosciences (555028).

Cell lines. The Jurkat human acute T cell leukemia cell line (E6-1) was purchased from ATCC (ATCC TIB-152) and has been tested for mycoplasma by ATCC.

Histology. Formalin-fixed tissues were processed, stained with hematoxylin and eosin and evaluated blindly.

The clinical scores in Figure 7c represent the combined inflammation score of the following organs: liver, skin, stomach, salivary glands, lungs and pancreas. The inflammation was scored blindly. No infiltration was scored as 0, some cellular infiltration was scored as 0.5, and clear abundant infiltrates were scored as 1. All the scores were added for each organ to produce the combined clinical score.

Statistical analysis. Student’s two-tailed t-test, two-way analysis of variance (ANOVA) followed by Bonferroni’s test and one-way ANOVA, followed by Tukey’s multiple comparison test, were used to calculate statistical significance among groups. A P value of less than 0.05 was considered significant.