**IMMUNOLOGY**

**Phosphofructokinase P fine-tunes T regulatory cell metabolism, function, and stability in systemic autoimmunity**

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by defective regulatory T (Treg) cells. Here, we demonstrate that a T cell–specific deletion of calcium/calmodulin-dependent protein kinase 4 (CaMK4) improves disease in B6.1pr lupus-prone mice and expands Treg cells. Mechanistically, CaMK4 phosphorylates the glycolysis rate-limiting enzyme 6-phosphofructokinase, platelet type (PFKP) and promotes aerobic glycolysis, while its end product fructose-1,6-biphosphate suppresses oxidative metabolism. In Treg cells, a CRISPR-Cas9–enabled Pfkp deletion recapitulated the metabolism of Camk4−/− Treg cells and improved their function and stability in vitro and in vivo. In SLE CD4+ T cells, PFKP enzymatic activity correlated with SLE disease activity and pharmacologic inhibition of CaMK4-normalized PFKP activity, leading to enhanced Treg cell function. In conclusion, we provide molecular insights in the defective metabolism and function of Treg cells in SLE and identify PFKP as a target to fine-tune Treg cell metabolism and thereby restore their function.

**INTRODUCTION**

Systemic lupus erythematosus (SLE) is an autoimmune disease that involves all organ systems and affects young women (1). Despite available treatments, SLE is responsible for substantial morbidity and mortality worldwide (2). Current treatment relies on the use of immunosuppressive drugs that control the immune dysregulation at the price of increased risk of infections and cardiovascular disease, which represent the main causes of morbidity and mortality in SLE (3).

In recent years, the study of immune cell metabolism has transformed our understanding of immunobiology and autoimmune diseases. Different cellular subsets, classically identified using surface or intracellular markers, are characterized by distinct metabolic profiles, which also adapt on the basis of the immune cell activation status and localization (4). SLE CD4+ T cells are characterized by an increased glycolytic metabolism and dysregulated oxidative phosphorylation (5). Normalization of T cell metabolism using the glycolysis inhibitor 2-deoxyglucose and the adenosine monophosphate (AMP)–activated kinase (AMPK) agonist metformin reverses the T cell–aberrated phenotype in vitro and improves lupus-like disease in mice (5). Furthermore, a recent proof-of-concept clinical trial of metformin in patients with SLE yielded encouraging results (6).

Among the proposed therapeutic strategies for SLE, reinforcing the regulatory T (Treg) cell compartment is particularly attractive (7) because the Treg cell compartment has consistently been described as quantitatively reduced (8, 9) and/or functionally impaired (10, 11). The mechanisms that drive Treg cell dysfunction in SLE are multifactorial and involve systemic inflammation, altered T cell signaling (7), and platelet–Treg cell interaction (12). During the past two decades, our team has studied the role of the calcium/calmodulin-dependent protein kinase 4 (CaMK4), a serine/threonine kinase (13). CaMK4 activity is regulated by intracellular calcium levels, which are higher in human SLE T cells compared to healthy donors (HDs) because of aberrant TCR signaling (14, 15). Consequently, SLE T cells are characterized by an increased CaMK4 activity (16), and Camk4 global knockdown improves induced and spontaneous autoimmunity in mice (17). CaMK4 inhibition enhanced mouse Treg cell differentiation and function in vitro, impaired T helper 17 (Th17) cell differentiation, and increased interleukin-2 (IL-2) production by conventional T cells (18). Recent data suggest that CaMK4 controls several aspects of immune cell metabolism. Studies from our team identified that CaMK4 advances aerobic glycolysis and promotes Th17 cell differentiation by controlling the activity of pyruvate kinase M2 (19). However, the exact mechanisms whereby CaMK4 negatively affects Treg cell function are still unknown.

Here, we present evidence that the rate-limiting glycolysis enzyme 6-phosphofructokinase (PFK) platelet-type (PFKP) activity is controlled through serine phosphorylation by CaMK4. We demonstrate that, in parallel to the control of the glycolytic pathway, PFKP, through its end product fructose-1,6-biphosphate (F-1,6P), controls oxidative phosphorylation in an opposite manner. Furthermore, the modulation of Treg cell metabolism using a CRISPR-Cas9 Pfkp deletion led to enhanced Treg cell function and stability in vitro and in vivo systems. At the translational level, we demonstrate increased PFKP activity in SLE CD4+ T cells that, when corrected, improves Treg cell activity. Our studies bring forward PFKP as a target molecule to control Treg cell function.

**RESULTS**

T cell–specific CaMK4 deficiency expands the Treg cell compartment and ameliorates disease in lupus-prone mice

CaMK4-deficient MRL/lpr18+ show improved lupus-like features (18), but because CaMK4 is expressed in other cells, including podocytes (20), the contribution of CaMK4 deficiency in T cells in
the expression of pathology is unclear. To address this question, we crossed B6.lpr mice with B6.Camk4^fl/fl.dlck-Cre^ mice expressing the Cre recombinase only in T cells (B6.lpr.Camk4^fl/fl.dlck-Cre^), leading to depletion of CaMK4 in T cells (fig. S1A). Camk4^fl/fl.dlck-Cre^ mice had decreased cervical lymph node volume, as well as spleen size, weight, and cellularity, compared to Camk4^fl/fl^ littermates (Fig. 1A to C). Because both conventional T (T_{conv}) cells and T_{reg} cells express CaMK4 (fig. S1B), we studied these subpopulations in B6.lpr mice. The percentage of IL-17A–producing CD4^+ T cells was decreased in the spleen (P < 0.05) but not in the peripheral blood of the Camk4^fl/fl.dlck-Cre^ mice (Fig. 1D and fig. S1C, gating), while the percentage of interferon-γ (IFN-γ)–producing CD4^+ T cells remained unchanged (fig. S1, C and D). Conversely, Camk4^fl/fl.dlck-Cre^ mice were characterized by an expanded T_{reg} cell compartment in the spleen and the peripheral blood compared to CaMK4-sufficient littermates (P < 0.01 for both comparisons; Fig. 1E and fig. S1E, gating). CaMK4 deficiency in T cells led to decreased double-negative T cell in the blood, the spleen, and the kidney of B6.lpr mice (fig. S1, F and G). Furthermore, serum double-stranded DNA (dsDNA) antibody levels were significantly decreased in mice with CaMK4-deficient T cells as compared to CaMK4-sufficient littermates (P < 0.01; Fig. 1F). At the organ level, B6.lpr mice with CaMK4-deficient T cells were characterized by decreased proteinuria (Fig. 1G) and improved kidney glomerular (Fig. 1H) and perivascular pathology scores (Fig. 1I). Collectively, these results indicate that a T cell–specific CaMK4 deficiency in B6.lpr mice results in the improvement of hallmark features of lupus-like disease with a simultaneous expansion of the T_{reg} cell compartment.

**CaMK4 affects the glycolytic and oxidative metabolism of inducible T_{reg} cells**

In accordance with previous findings that CaMK4 is expressed in T_{reg} cells and affects their differentiation (18, 21), we found that...
CaMK4 deficiency enhanced murine in vitro inducible T_reg (iT_reg) cell differentiation (Fig. 2A) and Forkhead Box P3 (FoxP3) expression (fig. S2A). Conversely, the transfection of a CaMK4 overexpression (Camk4-OE) vector suppressed iT_reg cell differentiation and FoxP3 expression (P < 0.01; Fig. 2B and fig. S2B). To evaluate the impact of CaMK4 on the iT_reg cell metabolic profile, we conducted real-time metabolic studies of iT_reg cells at 8, 24, and 72 hours of differentiation using a Seahorse XF analyzer. We found that Camk4<sup>−/−</sup> T cells were characterized by decreased extracellular acidification rate (glycolytic activity) at all time points of iT_reg cell differentiation (Fig. 2C). In contrast, oxidative metabolism assessed by the oxygen consumption rate was increased in Camk4<sup>−/−</sup> iT_reg cells at the 24- and 72-hour time points (Fig. 2, D and E). Similar results were found when comparing wild-type and Camk4<sup>−/−</sup> conventional (nondifferentiating) CD4<sup>+</sup> T cells (Fig. S2, C and D). Consistent with these findings, Camk4<sup>−/−</sup> iT_reg cells were characterized by increased levels of mitochondrial reactive oxygen species (mtROS) (P < 0.01; Fig. 2F) and mitochondrial DNA content (Fig. 2G). Collectively, these results suggest that during iT_reg cell differentiation, CaMK4 affects the glycolytic and mitochondrial cellular metabolism and negatively affects iT_reg differentiation.

**CaMK4 posttranslationally controls the activity of the rate-limiting enzyme PFKP**

To investigate how CaMK4 affects the iT_reg cell metabolic profile during differentiation, we conducted a comprehensive metabolomic analysis. At early steps of iT_reg cell differentiation (8 hours), the classical glycolysis pathway was enriched (P < 0.05), while the pentose phosphate pathway was not (fig. S3A). Close evaluation of the glycolysis metabolites of the Camk4<sup>−/−</sup> iT_reg cells differentiated for 8 hours showed that fructose-6-phosphate (F-6P) was increased, while F-1,6P was decreased (Fig. 3, A and B). The dysbalanced levels of the two metabolites suggested that the activity of PFK, the rate-limiting glycolysis enzyme catalyzing the transformation of F-6P to F-1,6P, is impaired in CaMK4-deficient T_reg cells (Fig. 3B). PFK has three isoforms in humans and mice: PFKP, PFK liver-type (PFKL), and PFK muscle-type (PFKM). Because we found that in mouse iT_reg cells, PFKP was expressed at levels 10 to 100 times higher compared to PFKM and PFKL (Fig. 3C), we focused on the PFKP isoform. The expression of PFKP was similar between wild-type and Camk4<sup>−/−</sup> iT_reg cells at the mRNA and the protein levels (Fig. 3, D and E). However, Camk4<sup>−/−</sup> iT_reg cells had significantly decreased PFK enzyme activity compared to wild-type counterparts (P < 0.01; Fig. 3F). Conversely, transfection of iT_reg cells with a Camk4-OE vector led to increased PFK activity (P < 0.01; Fig. 3G). Collectively, these results suggest that CaMK4 enhances T_reg cell glycolysis by controlling PFKP enzymatic activity at a posttranslational level.

**CaMK4 phosphorylates serine-539 of PFKP and affects iT_reg cell differentiation**

Previously published mass spectrometry data suggested that CaMK4 may physically interact with PFKP (19). To confirm this, we transfected Camk4<sup>−/−</sup> T cells with a FLAG-tagged CaMK4-OE vector. We subsequently immunoprecipitated the CaMK4 protein using a FLAG antibody and were able to detect PFKP by immunoblotting (Fig. 4A), confirming physical interaction. Because CaMK4 is a serine/threonine kinase, we hypothesized that CaMK4 may phosphorylate serine residues on PFKP. To address this, we immunoprecipitated PFKP from protein lysates of wild-type or Camk4<sup>−/−</sup> iT_reg cells. After blotting the precipitate, while the signal for PFKP (86 kDA) was similar in both conditions (Fig. 4B, bottom), the signal for phosphoserine at 86 kDA was decreased in lysates from Camk4<sup>−/−</sup> cells (Fig. 4B, top), suggesting that CaMK4 affects the phosphorylation of PFKP serine residues. To further substantiate this hypothesis, we conducted a phosphoproteomics study of the PFKP immunoprecipitates from wild-type and Camk4<sup>−/−</sup> iT_reg cells. We identified that two serine residues (S162 and S539) were phosphorylated in wild-type, but not in Camk4<sup>−/−</sup>, cells (Fig. 4C; full phosphoproteomics results available in the Supplementary Materials). Serine-539 (S540 in humans) is a highly conserved amino acid among mammals including humans and may harbor an N-acetylglucomosamine (GlcNAc) residue that negatively affects PFKP activity (22). Furthermore, amino acids 537 to 541 of PFKP represent the binding site of fructose-2,6-biphosphate, an allosteric activator of PFKP (23), reinforcing the importance of this site as a modulator of the PFKP enzymatic activity. To evaluate the potential impact of these phosphorylated serine residues on T_reg cell biology, we conducted a site-directed mutagenesis of a PFKP-OE vector. We substituted serine-162 or serine-539 with an alanine residue that cannot be phosphorylated (S162A-OE and S539A-OE, respectively), thus blocking the potential impact of CaMK4 on PFKP. While the transfection of PFKP and PFKP-S162A-OE vector resulted in a reduction in iT_reg cell differentiation and FoxP3 expression, the PFKP-S539A-OE vector did not (Fig. 4D and fig. S4A). Furthermore, compared to PFKP-OE–transfected iT_reg cells, PFKP-S539A-OE–transfected iT_reg cells demonstrated lower glycolysis and higher Oxidative phosphorylation (OXPHOS) (fig. S4, B and D). Recaptulating the differences observed between wild-type and Camk4<sup>−/−</sup> T cells (Fig. 2, C and D). These results suggest that serine-539 phosphorylation is responsible for CaMK4/PFKP effect on the T_reg cell biology. Collectively, CaMK4 physically interacts with PFKP and phosphorylates serine residue 539, thereby affecting PFKP activity and iT_reg cell metabolism and differentiation.

**The CaMK4/PFKP axis affects iT_reg cell metabolic rheostat and differentiation through PFKP end product F-1,6P**

We have shown that Camk4<sup>−/−</sup> iT_reg cells are characterized by enhanced oxidative metabolism and mitochondrial DNA content after 24 hours of differentiation (Fig. 2, D and E). Furthermore, metabolomic studies of iT_reg cells showed that metabolites from the tricarboxylic acid cycle were enriched at 24 hours of differentiation (fig. S5, A and B). To understand the mechanism underlying the impact of CaMK4 on oxidative phosphorylation, we studied the phosphorylation (i.e., activation) status of kinases known to control the metabolic rheostat. We found that Camk4<sup>−/−</sup> iT_reg cells were characterized by increased phosphorylation of AMPK, a master regulator of the oxidative metabolism (Fig. 5A), and reduced molecular target of rapamycin (mTOR) pathway activation, as assessed by decreased phospho-p70S6K levels (fig. S5C). Conversely, the transfection of iT_reg cells with a CaMK4-OE vector resulted in reduced AMPK phosphorylation (Fig. 5B). PFKP catalyzes the glycolysis rate-limiting step of F-6P conversion to F-1,6P. F-1,6P was demonstrated to inhibit AMPK phosphorylation in fibroblast cell lines, thereby regulating the metabolic cellular rheostat (24). Because F-1,6P levels were decreased in Camk4<sup>−/−</sup> iT_reg cells (Fig. 2, A and B), we hypothesized that the impact of CaMK4 on the mitochondrial metabolism is mediated by reduced F-1,6P levels driving increased AMPK phosphorylation. To evaluate this hypothesis, we differentiated Camk4<sup>−/−</sup> iT_reg cells together with the glycolysis intermediates F-6P or F-1,6P. We
Fig. 2. CaMK4 affects T cell glycolytic and oxidative metabolism during T<sub>reg</sub> cell differentiation. (A) CD62L<sup>+</sup>CD4<sup>+</sup> T cells were isolated from the spleen of C57Bl/6 or Camk4<sup>−/−</sup> mice and differentiated to iT<sub>reg</sub> cells. The percentage of CD25<sup>+</sup>FoxP3<sup>+</sup> iT<sub>reg</sub> cells was evaluated using flow cytometry at day 3. Representative cytometry results (left) and the cumulative results (right; n = 4) are shown. (B) CD62L<sup>+</sup>CD4<sup>+</sup> T cells of C57Bl/6 mice were differentiated to iT<sub>reg</sub> cells and transfected at day 2 of culture with an empty vector or a CaMK4-OE vector. iT<sub>reg</sub> cell differentiation was evaluated at day 3 using flow cytometry on viable transfected (GFP<sup>+</sup>) cells (n = 4). (C and D) CD62L<sup>+</sup>CD4<sup>+</sup> T cells were isolated from the spleen of C57Bl/6 or Camk4<sup>−/−</sup> mice were differentiated to iT<sub>reg</sub> cells. Representative results of Seahorse XFe glycolysis stress test (C) or mitochondrial stress test (D) at 8, 24, or 72 hours. ECAR, extracellular acidification rate; 2-DG, 2-deoxyglucose; OCR, oxygen consumption rate. (E) Cumulative results of the basal respiration rate (left), the adenosine triphosphate (ATP)-linked respiration rate (middle), and the maximal respiration rate (right) at 8, 24, and 72 hours of wild-type (WT) and Camk4<sup>−/−</sup> (KO) iT<sub>reg</sub> cells (n = 3 independent experiments per time point with three technical replicates each). (F) WT and Camk4<sup>−/−</sup> (KO) iT<sub>reg</sub> cells were stained with MitoSOX (2 μM) and evaluated using flow cytometry. Representative (left) and cumulative data (right) of MitoSOX mean fluorescence intensity (MFI) are shown (n = 3 biological replicates per time point). (G) Total DNA from iT<sub>reg</sub> from WT and Camk4<sup>−/−</sup> mice were extracted, and a quantitative polymerase chain reaction (qPCR) was conducted using NADH dehydrogenase subunit 1 gene (ND1) probe to evaluate mitochondrial DNA (mtDNA). MtDNA copies per cell were calculated by the formula 2<sup>−ΔΔCt</sup>. *P < 0.05 and **P < 0.01 using unpaired bilateral Student's t test (A, F, and G) or paired bilateral Student’s t test (B and E).
found that the PFKP end product F-1,6P, but not its precursor F-6P, reduced AMPK phosphorylation (Fig. 5C), mtROS levels (Fig. 5D), and cellular mitochondrial DNA content (Fig. 5E). Furthermore, F-1,6P reduced \( \text{Camk4}^{-/-} \) iT reg cell differentiation to the same level of wild-type (\( P < 0.001 \); Fig. 5F) and blunted FoxP3 expression (fig. S5D). Glycolysis metabolites or selected amino acids did not affect CaMK4 activation nor CamK4 and PFKP expression (fig. S5, E and F). Collectively, these results suggest that \( \text{Camk4}^{-/-} \) fine-tunes iT reg cell oxidative metabolism by affecting the AMPK/mTOR metabolic rheostat through the PFKP end product, F-1,6P, thereby affecting iT reg cell differentiation.

**CRISPR-Cas9–mediated PFKP knockdown alters the metabolism and enhances iT reg cell differentiation and function in vitro**

To confirm the importance of PFKP in iT reg cell biology, we silenced PFKP by transfecting CRISPR-Cas9–expressing T cells with single guide RNA (sgRNA) targeting the \( Pfkp \) gene. Transfection of \( Pfkp \) target sgRNA led to marked reduction of PFKP expression (Fig. 6A), as well as cellular PFK activity and extracellular acidification rate (i.e., glycolysis; Fig. 6, B and C) compared to control sgRNA. In addition, PFKP knockdown led to increased AMPK phosphorylation (Fig. 6D), mtROS (Fig. 6E), and cellular oxygen consumption rates (Fig. 6F). The impact of these metabolic changes translated to enhanced iT reg cell differentiation upon PFKP knockdown (\( P < 0.01 \); Fig. 6G), as well as increased FoxP3 expression (fig. S6A). To evaluate the impact of PFKP modulation on T reg cell functions, we conducted in vitro immunosuppression assay with control or \( Pfkp \) target sgRNA-transfected iT reg cells. The silencing of PFKP in iT reg cells led to enhanced in vitro immunosuppressive capacity (Fig. 6H). Collectively, these results demonstrate that PFKP fine-tunes the glycolytic and oxidative metabolisms of T reg cells and thereby affects their differentiation and suppressive function in vitro.

**CRISPR-Cas9–mediated PFKP knockdown improves T reg cell function and stability in vivo**

To gain further insight on the impact of PFKP modulation on iT reg cell function in vivo, we transferred \( 4 \times 10^6 \) syngeneic CD45RB high CD4+ T conv cells ± \( 5 \times 10^5 \) control or \( Pfkp \) target sgRNA-transfected iT reg cells to C57BL/6. \( \text{Rag1}^{-/-} \) mice to induce inflammatory colitis (Fig. 7A). Without iT reg cell transfer, the mice developed severe colitis leading to massive weight loss within 7 weeks mandating euthanasia (purple line; Fig. 7B). The transfer of iT reg cells blunted the weight loss and the colitis severity, with \( Pfkp \) target–transfected
iT reg cells showing the best efficiency (blue line; Fig. 7, B and C). Transferred iT reg cells constitutively expressed green fluorescent protein (GFP), allowing the lineage tracking of these cells. The proportion of CD25+ FoxP3+ cells among splenic and mesenteric lymph node GFP+ CD4+ T cells was higher in the mice transferred with PFKP-deficient iT reg cells (Fig. 7D), suggesting higher stability of the PFKP-deficient iT reg cells. Similarly, transferred PFKP-deficient iT reg cells expressed lower levels of IL-17A compared to control iT reg cells (Fig. 7E). To further investigate whether PFKP deficiency improved T reg cell stability, we cultured control or PFKP-deficient iT reg cells for 24 hours under T H 17-differentiating conditions. We found that PFKP-deficient iT reg cells retained higher FoxP3 expression (fig. S7A and B) while expressing lower levels of IL17A RNA (fig. S7C). Furthermore, these differences translated in higher immunosuppressive functions in vitro (fig. S7D). To evaluate whether these differences were associated with a different methylation profile in the FoxP3 conserved noncoding sequences (CNS), we evaluated CpG methylation in control and PFKP-deficient iT reg cells cultured under TH17-differentiating conditions (fig. S7E). The CpG methylation profile was similar in the Treg-specific demethylation region and in the proximal promoter of FoxP3, while it was decreased in evolutionary conserved region 3 located in the upstream enhancer in the PFKP-deficient iT reg cells (fig. S7F). However, the biological relevance of these differences is unknown. Collectively, these results demonstrate that the modulation of PFKP in iT reg cells improves their immunosuppressive capacity in vivo and their stability in an inflammatory environment.

**CaMK4 regulates PFKP activity in CD4+ T cells from patients with SLE and impairs human T reg function**

To evaluate the translational value of our findings, we first confirmed that PFKP was the main PFK expressed in CD4+ T cells from HDs and patients with SLE (Fig. 8A). Furthermore, sorted primary human Tconv and Treg cells expressed similar mRNA levels of PFKP and minimal levels of PFKM and PFKL (fig. S7A). In accordance with our data in mice and with previous reports of increased CaMK4 activity in SLE T cells (16), especially in patients with high disease activity (25), we found that human CD4+ T cells were characterized by increased PFK activity compared to age- and sex-matched HDs (P < 0.01; Fig. 8B). Moreover, the PFK activity of CD4+ T cells from patients with SLE correlated significantly with the SLE disease activity index (SLEDAI; r = 0.579, P < 0.005; Fig. 8C). Supporting the role of CaMK4 as a driver of PFKP activity in SLE CD4+ T cells, the culture of SLE CD4+ T cells with the CaMK4 inhibitor KN93 led...
to a significant decrease in PFK activity \((P < 0.01; \text{Fig. } 8D)\), to similar levels as compared with HD CD4+ T cells (fig. S7B). To confirm that CaMK4 may increase PFKP enzymatic activity in healthy CD4+ T cells, we stimulated HD CD4+ T cells with ionomycin to induce CaMK4 activation (fig. S8D), which resulted in an increase in cellular PFK activity (fig. S8E). Similar to our findings with SLE CD4+ T cells, treatment of healthy CD4+ T cells with KN93 prevented the increase of PFK activity mediated by CaMK4 activation.

Last, to evaluate whether the decrease of PFKP activity through CaMK4 inhibition translated in enhanced human Treg cell function, we conducted in vitro immunosuppression assay using freshly sorted HD Treg cells (fig. S7D). While KN93 did not affect Tconv cell proliferation compared to the solvent dimethyl sulfoxide (Fig. 8E, top), KN93 led to improved Treg cell–mediated suppression \((P < 0.01; \text{Fig. } 8F)\). Collectively, these results suggest that in human CD4+ T cells, CaMK4 controls PFK activity and is responsible for impaired Treg cell immunosuppressive functions.

**DISCUSSION**

In the current study, we demonstrate that the rate-limiting enzyme PFKP fine-tunes Treg cell metabolism and that its activity is controlled by CaMK4. Using immunoprecipitation and phosphoproteomics studies, we showed that CaMK4 binds and phosphorylates PFKP, most likely on the serine-539 residue, thereby mediating its effect on AMPK. S539 residue has been linked to the enzymatic regulation of PFKP either through GlcNacetylation, which inhibits PFKP activity \((22)\), or through the binding of fructose-2,6-biphosphate, an allosteric activator of PFKP \((23)\). We have linked decreased PFKP activity of CaMK4-deficient Treg cells to increased activation of the AMPK axis, a master regulator of the mitochondrial metabolism, through reduced levels of the PFKP end product F-1,6P, which is a potent inhibitor of AMPK \((26)\).

As a result, CaMK4-deficient iTreg cells displayed enhanced mitochondrial respiration, increased mitochondrial content, and impaired mTOR signaling. Outside its effect on AMPK, CaMK4 controls PFK activity and is responsible for impaired Treg cell immunosuppressive functions.
Fig. 6. CRISPR-Cas9–mediated PFKP knockdown alters the metabolism of iTreg cells and affects their in vitro immunosuppressive function. CD62L^+ CD4^+ T cells from CRISPR-Cas9–expressing mice were differentiated into iTreg cells and transfected at day 1 with control or Pfkp target sgRNAs. (A) Western blot showing PFKP expression in iTreg cell lysates. (B) Phosphofructokinase activity of Treg cell lysates (n = 3). (C) Representative of a Seahorse glycolysis stress test of control (red) or Pfkp target (blue) iTreg cells. (D) Representative Western blot (left) and cumulative densitometry result (right; n = 4) showing phospho-AMPK and total AMPK in control and PFKP target iTreg cells. (E) Mean fluorescence intensity of MitoSOX marker in control and Pfkp target iTreg cells (n = 3). (F) Representative of a Seahorse mitochondrial stress test of control (red) and Pfkp target (blue) iTreg cells. (G) Percentage of CD25^+ FoxP3^+ among control and Pfkp target iTreg cells was measured using flow cytometry at day 3 of differentiation. Representative flow plot (left) and cumulative results (right) are shown (n = 4). (H) Control or Pfkp target iTreg cells were cocultured for 3 days with CellTrace Violet–stained Tconv cells (Treg/Tconv cell ratio of 1:1 to 1:4) and antigen-presenting cells together with CD3/CD28 stimulation. Representative proliferation of Tconv cells under different coculture ratio (left) and cumulative results of iTreg cell immunosuppression activity (right; n = 3 independent experiments of three replicates each). Each dot represents the value of one biological replicate, and bars indicate means and SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 using unpaired bilateral Student's t test.
may also negatively affect oxidative metabolism by promoting mitophagy through its effect on the PTEN-induced putative kinase 1 (PINK1)/PARKIN axis (27). In accordance with an increased mitochondrial content and enhanced oxidative metabolism, CaMK4-deficient iTreg cells were characterized by increased levels of mtROS. While ROS are often considered as pathogenic intermediates (28), their effect on Treg cell may be beneficial through the stabilization of Nuclear factor of activated T-cells (NFAT), which in turn stabilizes FoxP3 expression through a direct interaction with its CNS2 region (29, 30). However, very high levels of mtROS from damaged mitochondria may lead to DNA damage and Treg cell death in autoimmunity (31), emphasizing the importance of a tight regulation of mtROS in Treg cells.

Close links exist between Treg cell metabolism and their regulatory program. The transcription factor FoxP3, which is the main driver of the Treg cell regulatory program, promotes mitochondrial metabolism and fatty acid oxidation (32, 33). The reverse link also exists because the metabolic modulation of Treg cells affects their differentiation, stability, and immunosuppressive functions (34). Although glycolysis is necessary during Treg cell proliferation (35) and migration (36), Treg cell immunosuppressive properties rely on their mitochondrial metabolism (33, 37). In accordance with these findings, we found that the modulation of glycolysis in Treg cells using a CRISPR-Cas9–mediated Pfkp knockdown enhanced mitochondrial oxidative metabolism of and ultimately led to improved Treg cell function and stability in vitro and in an in vivo model of adoptive transfer colitis. Because PFKP knockdown in iTreg cells recapitulated the metabolism of Camk4−/− iTreg, we hypothesize that PFKP enzymatic modulation represents a major mechanism underlying the effect of CaMK4 on Treg cells metabolism. Other studies from our group have shown the importance of CaMK4 in the differentiation of other T cell subsets, such as the proinflammatory Th17 cells (17). In the B6.1pr lupus-like mouse, we found that a T cell–specific Camk4 deletion led to increased circulating and splenic Treg cells, while Th17 cells were decreased (Fig. 1, D and E). This finding is concordant with a previous report showing that a CaMK4 inhibitor (KN93) or somatic Camk4 deletion expanded the Treg cell compartment in the MRL/lpr mouse (18, 21). However, the use of CD4-tagged nanolipogel loaded with KN93 in MRL/lpr mice led to clinical
improvement without affecting the splenic Treg and T\textsubscript{H}17 compartment (38). This discrepancy may be explained by the lower levels of inhibition of CaMK4 with nanolipogels compared to genetic deletion (dose effect) or by the fact that nanolipogels mainly affect circulating CD4\textsuperscript{+} T cells (mature), while genetic deletion was effective early during T cell differentiation (time effect).

Enzymes involved in immunometabolism and their end products represent attractive therapeutic targets in autoimmune diseases. Current SLE pharmacopeia relies on nonspecific antiproliferative and immunosuppressive drugs, or more recently, on anticytokine biologics. These therapeutic strategies have limited clinical value either because of the redundancy of the immune signaling when blocking one single cytokine axis or because of the side effect of immunosuppressive drugs such as infections, which rank among the first causes of mortality in SLE (3). Modulation of the metabolism of immune cells allows the targeting of a broad range of cells without total disruption of their function, which may come with better safety profile. Recently, the well-tolerated AMPK agonist metformin was shown to have beneficial clinical effects in a proof-of-concept clinical study in SLE (6). Drugs that affect immunometabolism through other mechanisms (e.g., PPAR agonists) have also shown promising results in inflammatory disease animal models (39). Our current study identifies PFKP as a potential therapeutic target in autoimmunity as it impairs Treg cell function and stability. Furthermore, our results in humans suggest that targeting PFKP activity through CaMK4 inhibition improves Treg cell immunosuppressive function and may therefore represent a promising adjuvant therapy in SLE. Targeting the glucose metabolism through PFKP offers several advantages. First, PFKP modulation does not affect cellular entry of glucose, which remains crucial for cellular proliferation and the production of nucleotides via the pentose phosphate pathway (40). Second, because PFKP is a rate-limiting enzyme, the modulation of its activity affects downstream metabolites, including F-1,6P that we and the others have demonstrated to affect the metabolic rheostat through the AMPK/mTOR axis (24). Last, the targeting of the PFKP isoform limits off-target impact outside the immune system, especially in highly glycolytic organs such as the liver and the muscle, which mainly express PFKL and PFKM, respectively (41). However, the central nervous system and the heart highly express PFKP inhibition of which could have unwanted effects. In this regard, inhibition of CaMK4 would lead to more physiological inhibition of PFKP and parallel suppression of T\textsubscript{H}17 cells (17). However, since CaMK4 is also expressed in the central nervous system, inhibitors that do not cross the blood-brain barrier should be developed.

In conclusion, we describe a new mechanism whereby CaMK4 fine-tunes Treg cell metabolism through the phosphorylation of the glycolysis rate-limiting enzyme PFKP, thereby affecting Treg cell function and stability in inflammatory diseases. These results
identify the CaMK4/PFKP axis as a potential therapeutic target in autoimmunity, able to correct Treg cell dysfunction, which characterizes patients with SLE.

MATERIALS AND METHODS

Study design

The aim of this study was to evaluate how CaMK4 affects cellular metabolism of Treg cells and therefore affects their differentiation, stability, and function in human SLE. Cellular metabolism was assessed using Seahorse XFE, metabolomics studies, and cytometry (ROS quantification). We conducted phosphoproteomics study [liquid chromatography–mass spectrometry (LC-MS)] to identify that PFKP was phosphorylated by CaMK4. We then used in vitro and in vivo experiments to evaluate the importance of PFKP and CaMK4 in Treg cell biology and in autoimmunity. Our animal protocol was approved by the Beth Israel Deaconess Medical Center institutional animal care and use committee (approvals 088-2015 and 063-2021). Patients with SLE and HDs gave informed and written consent and provided peripheral blood for research purposes. Our human research protocol was approved by the Beth Israel Deaconess Medical Center Institutional Review Board (2006-00298).

Human samples

Adult patients diagnosed with SLE according to the ACR/EULAR 2019 classification criteria and followed at the Beth Israel Deaconess Medical Center (BIDMC) rheumatology outpatient clinic were offered to participate to the study. HDs were recruited by the rheumatology outpatient clinic. For human immunosuppressive assays, leukocytes from healthy blood donors were retrieved using apheresis outpatient clinic. For human immunosuppressive assays, leukocytes to participate to the study. HDs were recruited by the rheumatology outpatient clinic were offered 2019 classification criteria and followed at the Beth Israel Deaconess Medical Center Institutional Review Board (2006-000298).

T cell culture and in vitro Treg cell differentiation

Murine CD62L−CD4+ T cells were magnetically sorted from freshly isolated splenocytes using a Miltenyi CD62L− CD4+ T cell sorting kit according to the manufacturer’s instructions. Naïve CD4+ T cells (3 × 10^6) were cultured on with RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and β-mercaptoethanol in a 48-well plate precoated with goat anti-hamster cross-linking antibody. To obtain iTreg cells in vitro, the medium was supplemented with anti-CD3 (0.25 μg/ml, 145-2C11; BioLegend, San Diego, CA), anti-CD28 (0.5 μg/ml, 37.51; BioXcell, Lebanon, NH), IL-2 (20 ng/ml; R&D Systems), transforming growth factor–β1 (TGF-β1; 1.0 ng/ml; R&D Systems), anti–IFN-γ (10 μg/ml; BioXcell), and anti–IL-4 (10 μg/ml; BioXcell). To evaluate iTreg stability, day 3–differentiated iTreg cells were cultured 24 hours with Tg17-differentiating conditions: anti-CD3 (0.25 μg/ml), anti-CD28 (0.5 μg/ml), IL-6 (3 ng/ml; R&D Systems), TGF-β1 (0.3 μg/ml), anti–IFN-γ (10 μg/ml), and anti–IL-4 (10 μg/ml).

Transfection

For overexpression experiments, the transfection of 15 μg of the vector (listed in table S3) was conducted using the Amaza Mouse T Cell Nucleofector Kit with the X-001 program (Lonza, Basel, Switzerland) following the manufacturer’s instructions. Electroporation was conducted on day 2 of differentiation, and the transfected cells were evaluated at day 3 using flow cytometry and gating on viable transfected (GFP+ or DsRed+). Cells for CRISPR-Cas9 gene knockdown experiments, 50 pmol of each targeting sgRNA was transfected in CAS9-expressing cells at day 1 of differentiation using the X-001 program.

Site-directed mutagenesis

Site-directed mutagenesis of PFKP-OE vector was conducted using a Q5 site-directed mutagenesis kit (New England Biolabs, Ipswich, MA) following the manufacturer’s instructions. The primers used for mutagenesis were designed using the New England Biolabs mutagenesis primer design online tool (table S3). After mutagenesis, the products were Sanger-sequenced (Azenta, Chelmsford USA) to verify correct mutagenesis.

FoxP3 promoter methylation study

Genomic DNA was extracted from 10^6 iTreg cells using a Qiamp DNA mini kit (Qiagen) according to the manufacturer’s instructions. Genomic DNA (500 ng to 1 μg) was converted using an Epitect Fast bisulfite conversion kit (Qiagen) according to the manufacturer’s instructions. Bisulfite-treated DNA (500 ng) was submitted to EpigenDx (Hopkinton MA, USA) for amplification and next-generation sequencing.

Flow cytometry and cell sorting

Surface staining was conducted in phosphate-buffered saline (PBS) for 15 min at room temperature. Intracellular staining was conducted by fixing cells with Cytofix/Cytoperm buffer (15 min, +4°C) and then staining with the antibody in Perm/wash buffer overnight at +4°C (BD Biosciences, Franklin Lakes, NJ, USA). For cytokine evaluation, the cells were stimulated in complete RPMI 1640 medium with phorbol myristate acetate (500 ng/ml; Sigma-Aldrich, St. Louis, MI, USA), ionomycin (1.4 μg/ml; Sigma-Aldrich), and GolgiStop (BD Biosciences). Zombie Aqua and Zombie ultraviolet fixable viability markers (BioLegend) were used to gate on living cells. Flow cytometry acquisition was conducted with Cytoflex (Beckman Coulter, Brea, CA) and cell sorting using FACSaria (BD). For cell sorting, the purity of
the sorted population was over 95%. Flow cytometry analysis was conducted using FlowJo software (BD Biosciences).

Enzyme-linked immunosorbent assay
dsDNA antibody levels were measured using a homemade enzyme-linked immunosorbent assay as previously described (43). Briefly, sera were diluted 1:50 with 1% PBS bovine serum albumin and incubated at room temperature with shaking for 2 hours on 96-well plates precoated with 1-lysine (0.05 mg/ml) for 2 hours at room temperature, then coated with Calf thymus DNA (0.1 mg/ml, Sigma-Aldrich), +4°C overnight. Standard was made using serial dilution of the serum of a 16-week-old MRL/lpr mouse. After washing, the wells were incubated with an alkaline phosphatase–conjugated goat anti-mouse immunoglobulin G antibody (1:5000; Jackson ImmunoResearch) for 1 hour, and colorimetric reaction was provoked by adding diethanolamine substrate buffer (Thermo Fisher Scientific) and PNPP (p-Nitrophenyl Phosphate)-phosphatase substrate (Sigma-Aldrich) and read at 405 nm. The results are expressed as units per volume.

Metabolic assays and metabolomics study
Glycolysis stress test and mitochondrial stress test were conducted using a Seahorse XF mini device (Agilent). A total of 2 × 10^5 cells per well were plated on an Xp culture miniplate precoated with Celltak (Corning). The assay was conducted following the manufacturer’s instructions. Cellular phosphofructokinase 1 enzymatic activity was measured using the PKF assay (Abcam) from cell lysates of murine or human CD4^+ T cell. The cell lysates were prepared using 100 μl of assay buffer, snap-frozen in liquid nitrogen, and stored at −80°C until further processing. Protein concentration of the cell lysate was measured using Coomassie protein assay (Sigma-Aldrich) conducted with TaqMan probes (see list in table S3), Taqman reaction buffer, and CDNA, according to the manufacturer’s instructions, with a LightCycler 480 device (Roche). Relative gene expression was calculated using the ΔΔCt method with TATA-box Binding Protein (TBP) as the housekeeper gene.

Immunosuppressive assay
For mouse immunosuppression assay, 5 × 10^4 magnetically sorted CD4^+ T cells (Miltenyi) stained with CellTrace Violet (5 μM; Thermo Fisher Scientific) were cocultured with iT_reg cells (ratio of 1:1 to 1:4) together with 10^5 antigen-presenting cells (splenocytes treated with mitomycin C) under CD3-activating condition (5 μg/ml)/CD28-activating condition (3 μg/ml). After 3 days of culture, the proliferation of CD4^+ T cells was assessed using CellTrace Violet dilution and FlowJo V10 (Ashland, OR, USA) proliferation module.

Statistical analysis
Statistical analysis was conducted using GraphPad Prism Version 9. In figures, points show individual data points, bars represent the mean, and error bars indicate SEM. Quantitative data were compared between two groups using Student’s two-tailed t test or one-way analysis of variance (ANOVA) with Holm-Sidak’s correction for multiple analysis when comparing more than two groups, unless indicated otherwise. Correlation was computed using the non-parametric Spearman correlation test. In figures, statistical results are indicated as follows: not significant (ns), P ≥ 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.adc9657.

View/request a protocol for this paper from Bio-protocol.

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The authors declare that they have no competing interests. **Data and materials availability:**

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

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