PKM2 promotes Th17 cell differentiation and autoimmune inflammation by fine-tuning STAT3 activation

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Th17 cell differentiation and pathogenicity depend on metabolic reprogramming inducing shifts toward glycolysis. Here, we show that the pyruvate kinase M2 (PKM2), a glycolytic enzyme required for cancer cell proliferation and tumor progression, is a key factor mediating Th17 cell differentiation and autoimmune inflammation. We found that PKM2 is highly expressed throughout the differentiation of Th17 cells in vitro and during experimental autoimmune encephalomyelitis (EAE) development. Strikingly, PKM2 is not required for the metabolic reprogramming and proliferative capacity of Th17 cells. However, T cell–specific PKM2 deletion impairs Th17 cell differentiation and ameliorates symptoms of EAE by decreasing Th17 cell–mediated inflammation and demyelination. Mechanistically, PKM2 translocates into the nucleus and interacts with STAT3, enhancing its activation and thereby increasing Th17 cell differentiation. Thus, PKM2 acts as a critical nonmetabolic regulator that fine-tunes Th17 cell differentiation and function in autoimmune-mediated inflammation.

Introduction

Th17 cells are critical components of the adaptive immunity that contribute to the host defense against extracellular pathogens, but they are also implicated in the pathogenesis of autoimmune-mediated inflammatory diseases (Korn et al., 2009). Consignaling of IL-6 and TGF-β induces the differentiation of Th17 cells (Veldhoen et al., 2006; Bettelli et al., 2006; Mangan et al., 2006). IL-6 drives the phosphorylation of STAT3 that translocates into the nucleus and induces the expression of the transcription factors Rora and Rorγt (Ivanov et al., 2006; Yang et al., 2007, 2008). TGF-β inhibits IL-6–induced SOCS3 expression, thus prolonging STAT3 activation (Qin et al., 2009; Chen et al., 2006). In combination with other transcription factors, STAT3 and retinoic acid orphan receptor gamma T synergize to regulate transcription of the T-helper type 1 (Th1) cell–signature genes IL-17A, IL-17F, IL-22, and IL-23R (Korn et al., 2009). Another cytokine, IL-23, mediates the final differentiation, stabilization, and induction of GM-CSF production by Th17 cells, making these cells pathogenic (El-Behi et al., 2011; Codarri et al., 2011; McGeachy et al., 2009). However, much remains unclear about the regulatory signaling pathways that control the differentiation and pathogenicity of Th17 cells.

Recent studies have shown that immune cells undergo a dynamic metabolic reprogramming to support the bioenergetic and biosynthetic requirements for proper activation, proliferation, and differentiation. Mammalian target of rapamycin complex 1 (mTORC1) and hypoxia-inducible factor 1α (HIF1α) are critical regulators of cellular metabolism and also have a central role in controlling immune cell activation and functions (O’Neill et al., 2016; Buck et al., 2015; Almeida et al., 2016). Indeed, the HIF1α- and mTORC1-dependent metabolic reprogramming toward aerobic glycolysis, a phenomenon that resembles the well-described Warburg effect in tumor cells, is also especially important for Th17 cell development (Shi et al., 2011; Delgoffe et al., 2011; Dang et al., 2011; Kurebayashi et al., 2012). Consistent with this, the blockade of glycolysis with 2-deoxyglucose inhibits Th17 cell generation in vitro and ameliorates the development of experimental autoimmune encephalomyelitis (EAE; Shi et al., 2011).
Pyruvate kinase (PK) is a glycolytic enzyme that converts phosphoenolpyruvate to pyruvate (Israelens and Vander Heiden, 2015; Gui et al., 2013). Four isoforms of PK are present in mammals and differentially distributed according to the cell type. Particularly, expressions of the PK isoforms M1 (PKM1) and M2 (PKM2) are derived through alternative splicing of the Pkm gene (Noguchi et al., 1986). PKM1 is constitutively expressed at a constant level in most tissues, while PKM2 is mainly expressed in proliferating and tumor cells. Structurally, PKM1 forms constitutive and stable tetramers with high metabolic activity, whereas the PKM2 tetrameric conformation requires allosteric modulation, being mostly expressed as metabolically inactive monomeric and dimeric forms (Israelens and Vander Heiden, 2015; Gui et al., 2013). Although the dimeric PKM2 has low metabolic activity, it gains the ability to translocate into the nucleus and act as a nuclear transcriptional coactivator, regulating gene expression by interaction with some transcriptional factors, including HIF1α (Yang et al., 2011; Luo et al., 2011; Yang et al., 2012a). Pharmacological inhibition of PKM2 nuclear translocation or its silencing decreases aerobic glycolysis and the proliferation of tumor cells (Christofk et al., 2008; Anastasiou et al., 2012). Moreover, recent reports show that PKM2 regulates the production of inflammatory cytokines in LPS-activated macrophages (Shirai et al., 2016; Yang et al., 2014; Palsson-McDermott et al., 2015).

In this study, we demonstrated that PKM2 mediates the differentiation of Th17 cells, but not Th1, Th2, or regulatory T (T reg) cells, through activation of STAT3. We found that the dimeric PKM2 translocates into the nucleus and interacts with STAT3, enhancing its phosphorylated status throughout the differentiation of Th17 cells. T cell–specific PKM2 deletion impairs the development of Th17 cells and ameliorates symptoms of EAE by decreasing Th17 cell–mediated inflammation and demyelination. PKM2 therefore represents a potential therapeutic target for autoimmune-mediated inflammation.

Results

Th17 cells express PKM2 throughout differentiation

To determine the role of PKM2 in the activation, proliferation, and differentiation of T cells, we initially analyzed the expression of PKM splice isoforms in Th cell subtypes. To this end, we cultured naive CD4+CD25− T cells from C57BL/6 mice under Th1, Th2, Th17, and induced T reg (iT reg) cell–polarizing conditions in vitro to obtain T cells with selective expression of Ifng, Il4, Il17a, and Foxp3, respectively (Fig. S1 A). As controls, naive CD4+CD25− T cells were activated with anti-CD3ε/CD28 antibodies without the addition of differentiating cytokines (Th0 cells). We found that Pkm1 mRNA is constitutively expressed in freshly isolated naive T cells and did not increase substantially in Th cell subtypes, whereas Pkm2 mRNA expression was upregulated in all Th cell subtypes when compared with naive T cells at 48 h of culture. However, significantly more Pkm2 mRNA expression was observed in Th17 cells (Fig. 1 A). Of note, effector/memory CD62LloCD44hi CD4 T cells in homeostatic conditions show a slight increase in Pkm2 mRNA expression, but not Pkm1, in comparison to naive cells, but lower than that observed in fully differentiated Th17 cells in vitro (Fig. S1 B).

In a kinetic analysis of Th17 cell differentiation, Pkm2 mRNA expression was detectable at 24 h, and it reached a peak at 48 h of culture, whereas Pkm1 expression remained constant throughout differentiation (Fig. 1 B). Immunoblot analysis confirmed that PKM2 protein levels were very low or undetectable in naive T cells. However, it markedly increased throughout Th17 cell differentiation, while PKM1 protein expression is constitutively expressed in resting naive T cells showing a slight increase in differentiated Th17 cells (Fig. 1 C). According to our findings on Pkm2 gene expression, PKM2 protein expression was higher in Th17 cells than Th1 cells (Fig. S1 C). Additionally, using the flow cytometry approach, we observed that IL-17A+ CD4 T cells exhibited higher intracellular staining for PKM2 than IL-17A− CD4 T cells from the same culture wells after Th17 differentiation. Moreover, the addition of IL-23 to the cell cultures concomitantly increased both Th17 cell differentiation and PKM2 expression in IL-17A−producing T cells (Fig. 1 D).

mTORC1 signaling up-regulates the expression of PKM2 in tumor cells (Sun et al., 2011), whereas T cell–specific deletion of mTORC1 activity impairs Th17 differentiation in vitro and in vivo (Kurebayashi et al., 2012; Delgoffe et al., 2011). We therefore investigated whether mTORC1 signaling is involved in the expression of PKM2 during the differentiation of Th17 cells. As expected, inhibition of mTOR with rapamycin dramatically reduced IL-17A and increased Foxp3 expression in CD4 T cells cultured under Th17 cell–polarizing conditions (Fig. S1, D and E). Rapamycin did not affect Pkm1 expression, but it significantly reduced PKM2 mRNA and protein expression (Fig. 1, E and F).

These observations led us to determine if the expression of PKM2 changes in a Th17 cell–mediated inflammatory disease model (Sie et al., 2014). Its expression profile was evaluated throughout the course of EAE development. This was done by immunizing mice with myelin oligodendrocyte glycoprotein (MOG)35–55 peptide (Fig. 2, A and B). We found that Pkm2 mRNA expression had increased in draining LNs (DLNs) before disease onset (day 10) and in the spinal cord at the peak of the EAE symptoms (day 15) following the expression profile observed with the transcription of Th17 cell–related genes, such as Il17a, Csf2, and I23r (Fig. 2 C). Immunoblot analysis confirmed the increased PKM2 protein levels in the spinal cord of EAE mice (Fig. 2 D). Moreover, histopathological analysis with H&E and immunofluorescent staining of spinal cord lesions in EAE mice showed that PKM2 expression was confined almost exclusively into the inflammatory cell infiltration region, while absent in spinal cord sections of naive mice (Fig. 2 E). Consistent with this, CD4 T cells isolated from the spinal cords of EAE mice expressed significantly higher Pkm2 mRNA transcription levels along with the Th17 cell–related genes Il17a, Csf2, I23r, Rora, and Rorc compared with CD45+ cells from the spinal cord of naive mice (Fig. 2 F). Taken together, these results indicate that PKM2 expression is induced in Th17 cells in vitro and in vivo, suggesting that it might affect their differentiation.

Th17 cells require PKM2 for the complete differentiation

Activated T cells undergo a dynamic metabolic reprogramming to support the bioenergetic and biosynthetic demands for proper proliferation and differentiation (Buck et al., 2015; Almeida Damasceno et al. Nonmetabolic role of PKM2 drives Th17 development
Cancer cells and macrophages require PKM2 expression for their metabolic reprogramming toward aerobic glycolysis (Palsson-McDermott et al., 2015; Christofk et al., 2008), whereas defective glycolysis dramatically impairs Th17 cell differentiation and proliferation (Shi et al., 2011). We hypothesized that similar to macrophages and tumor cells, PKM2 up-regulation would also be required for metabolic reprogramming of T cells. To test this hypothesis, we crossed mice carrying the LoxP-flanked Pkm2-specific exon 10 (Pkm2<sup>fl/fl</sup>; Israelsen et al., 2013) with CD4Cre mice (Lee et al., 2001) to generate T cell-specific PKM2 deficient mice (CD4Cre<sup>Pkm2</sup>fl/fl). Littermates Pkm2<sup>fl/fl</sup> and CD4Cre mice were used as WT controls.

No significant difference in LN and spleen sizes were observed between WT or CD4Cre<sup>Pkm2</sup>fl/fl mice (Fig. S2 A), and CD4Cre<sup>Pkm2</sup>fl/fl pups did not display any apparent abnormalities, presenting a grossly healthy development (data not shown).

**Figure 1.** Th17 cell differentiation accompanies high PKM2 expression levels. (A) Pkm1 and Pkm2 gene expression were evaluated by RT-qPCR in freshly isolated CD4 T cells (naive) and polyclonally activated CD4 T cells (Th0) and Th1, Th2, Th17, and iT reg cells at 48 h after culture (n = 3). (B) Naive CD4 T cells were differentiated into Th17 cells, and gene expression of Pkm1 and Pkm2 was determined at different time points by RT-qPCR (n = 3). (C) Protein expression levels of PKM1 and PKM2 during Th17 cell differentiation were detected by immunoblot; β-actin was used as a loading control. (D) Th17 cells were differentiated in the presence or absence of IL-23, and PKM2 expression was determined by flow cytometry. MFI, mean fluorescence intensity. (E) Rapamycin (0.1 µM), an mTOR inhibitor, was added to the Th17 cell cultures. After 96 h, cells were collected, and the expression of Pkm1 and Pkm2 was determined by RT-qPCR. For gene expression analysis, the cycle threshold values were normalized to Gapdh; fold change was calculated relative to untreated cells (n = 3). (F) Rapamycin-treated Th17 cells were also collected for immunoblot analysis of PKM2 protein levels (n = 3); β-Actin was used as a loading control. Data are representative of two independent experiments. Error bars show mean ± SEM. P values were determined by one-way ANOVA followed by Tukey’s post hoc test (A and F), two-way ANOVA followed by Tukey’s post hoc test (B and D), or two-tailed Student’s t test (E). *, P < 0.05; ns, not significant.
PKM2 expression increases during EAE development. (A and B) EAE was induced in WT C57BL/6 mice by subcutaneous immunization with MOG35–55; the clinical score was evaluated throughout the days after immunization. (C) DLNs (top) and spinal cord samples (bottom) were collected at the indicated time points depicted in B (red arrows) for analysis of Pkm2, Il17a, Csf2, and Il23r gene expression by RT-qPCR (n = 7 per time point). Cycle threshold values were normalized to Gapdh. (D) PKM2 total protein levels in the spinal cord of EAE-bearing mice were determined by immunoblot; β-actin was used as a loading control. (E) Inflammatory cell infiltration was observed in the spinal cord by using H&E staining (left; black arrows). Scale bar indicates 500 and 50 µm. PKM2 protein expression in the spinal cord was analyzed by immunofluorescence (red); DAPI was used as a nuclear marker (blue), and myelin was stained with fluoromyelin stain probe (green; n = 3). Scale bar represents 50 µm. (F) Mononuclear cells were isolated from CNS of naive and EAE mice (n = 9 per group) followed by magnetic separation of CD4 T cells. Expression of Pkm2, Il17a, Csf2, Il23r, Rora, and Rorc was analyzed by RT-qPCR. Each sample was a pool of three
Moreover, the proportion of CD4 or CD8 T cells in the thymus and the frequency of naive or memory CD4 T cell populations in LNs and spleen was not significantly different between WT and CD4Cre<Pkm2fl/fl> mice (Fig. S2, B–D).

We then cultured naive PKM2-deficient CD4 T cells under a Th17 cell-polarizing condition and examined the expression levels of key molecules required for glycolysis (Fig. 3 A). The loss of Pkm2 in CD4 T cells was confirmed by immunoblot analysis of PKM2 protein expression in Th17 cells after 72 h in culture (Fig. 3 B). Consistent with previous studies, WT Th17 cells showed increased expression of Slc2a1 (the gene encoding glucose transporter GLUT1), Ldha (lactate dehydrogenase [LDH]), and Hif1a (HIF1α) compared with naive CD4 T cells (Fig. 3 C). Strikingly, PKM2 deficiency did not affect the expression of those proglycolytic genes (Fig. 3 C). Correspondingly, LDH and HIF1α proteins levels were also comparable between WT and PKM2-deficient Th17 cells (Fig. 3 D). Nevertheless, deficiency of PKM2 led to compensatory up-regulation of PKM1 expression (Fig. 3, B and C). Thus, we next examined whether PKM2 affects the glucose metabolism of Th17 cells. This evaluation was performed by monitoring the uptake of fluorescent glucose analogue 2-NBDG in concert with glucose consumption and lactate secretion in vitro. WT Th17 cells were highly glycolytic, showing increased glucose uptake when compared with naive T cells. Consistent with the normal expression of the main proglycolytic molecules in the absence of PKM2 expression, glucose uptake by PKM2-deficient Th17 cells was not different from that of WT Th17 cells, even in the presence of IL-23 (Fig. 3 E and Fig. S2 E).

Figure 3. PKM2 deficiency does not alter Th17 cell metabolic reprogramming. (A) Naive CD4 T cells were obtained from CD4Cre<Pkm2fl/fl> or control littermates (WT) and cultured under Th17 cell–skewing conditions. (B) PKM1 protein expression in PKM2-deficient Th17 cells was determined by immunoblot; PKM2 deficiency was also confirmed by immunoblot analysis. (C) Th17 cells were harvested to evaluate the expression of glycolysis-related genes (Slc2a1, Ldha, Hif1a, and Pkm1) by RT–qPCR; data were normalized to Gapdh and fold change calculated relative to freshly isolated naive CD4 T cells (n=3). (D) WT and PKM2-deficient Th17 cells were harvested at 96 h to determine protein levels of LDHA and HIF1α by immunoblot. β-Actin was used and loading control. (E) Th17 cells were incubated with a fluorescent glucose analogue (2-NBDG; 30 µM) for glucose uptake evaluation by flow cytometry; dotted lines indicate fluorescence-minus-one (FMO) control values (n=3–4). MFI, mean fluorescence intensity. (F) Glucose consumption and lactate production were measured in Th17 cell-culture supernatants (n=4–5). Data are representative of two (D, E and F) or three (B and C) independent experiments. Error bars show mean ± SEM. P values were determined by one-way ANOVA followed by Tukey’s post hoc test (C), two-way ANOVA followed by Tukey’s post hoc test (E), or two-tailed Student’s t test (F). *, P < 0.05; ns, not significant.
Moreover, glucose consumption and lactate production were not significantly different between WT and PKM2-deficient Th17 cells (Fig. 3 F and Fig. S2 F). Collectively, these results indicate that PKM2 deficiency did not impair the metabolic reprogramming of Th17 cells toward aerobic glycolysis.

To assess whether loss of PKM2 function affects T cells proliferative capacity, we next performed flow cytometric analysis of fluorescent dye dilution in CD4 T cells stimulated with anti-CD3ε and anti-CD28 in the presence of Th17 cell–polarizing cytokines. PKM2-deficient CD4 T cells showed normal proliferative capacity after 72 h in culture (Fig. 4 A). Additionally, we evaluated the proliferation of PKM2-deficient T cells cultured under a Th0 condition, and again no significant differences were detected, even in the presence of IL-2 (Fig. S2 G). However, proliferating PKM2-deficient Th17 cells showed significantly reduced expression of IL-17A (Fig. 4 B), suggesting that PKM2 is required for differentiation, but not Th17 cell proliferation. Consistent with this association, expression of the Th17 cell–related genes Il17a, Csft2, Il22, Il23r, Rora, and Rorc were also markedly reduced in PKM2-deficient Th17 cells (Fig. 4 C). Further analysis confirmed that PKM2 deficiency impaired rises in IL-17A expression in Th17 cells, even after they had been differentiated in the presence of IL-23 and frequency of IL-17A+ CD4 T cell population determined by flow cytometry (n = 3). (E) Supernatants of Th17 cultures were collected, and IL-17A levels detected were by ELISA (n = 3). Data are representative of two (A–C) or more than five (D and E) independent experiments. Error bars indicate mean ± SEM. P values were determined by two-way ANOVA followed by Tukey’s post hoc test (D and E) or two-tailed Student’s t test (A and B). *, P < 0.05.

Loss of PKM2 in T cells ameliorates autoimmune-mediated neuroinflammation

To determine the in vivo relevance of these findings, we next examined whether the loss of PKM2 in T cells influenced the pathogenesis of EAE. To this end, CD4CrePkm2fl/fl mice were

Figure 4. PKM2 deficiency impairs Th17 cell differentiation. (A) Naive CD4 T cells from WT or conditional knockout (CD4CrePkm2fl/fl) mice were stained with CellTrace Violet proliferation dye (CTV; 5 µM). Cells were then cultured under Th17 cell–skewing conditions and after 96 h cell proliferation was evaluated by flow cytometry. MFI, mean fluorescence intensity (n = 5). (B) Cells were stained with 5 µM eFluor 670 proliferation dye and cultured under Th17 cell–polarizing conditions for 96 h. Cells were intracellularly stained for IL-17A after 4 h of PMA/ionomycin stimulation (n = 3). (C) The expression of Th17 cell–signature genes was evaluated by RT-qPCR and displayed in a heatmap. Gene expression correlates with color intensity, data normalized by Z-score (row); cycle threshold values were normalized to Gapdh (n = 3). (D) Naive CD4 T cells from WT or CD4CrePkm2fl/fl were also differentiated in the presence of IL-23 and frequency of IL-17A+ CD4 T cell population determined by flow cytometry (n = 3). (E) Supernatants of Th17 cultures were collected, and IL-17A levels detected were by ELISA (n = 3). Data are representative of two (A–C) or more than five (D and E) independent experiments. Error bars indicate mean ± SEM. P values were determined by two-way ANOVA followed by Tukey’s post hoc test (D and E) or two-tailed Student’s t test (A and B). *, P < 0.05.
immunized with MOG35-55 peptide. Notably, the loss of PKM2 in T cells not only significantly reduced the clinical severity but also decreased the incidence of EAE (Fig. 5, A–C). Consistently, histopathological analysis with H&E and fluoromyelin staining of spinal cords showed that CD4^{+}CD8^{+}Pkm2^{fl/fl} mice had lower inflammatory cell infiltration and decreased demyelination than WT mice (Fig. 5 D). To evaluate the impact of PKM2 deficiency on Th17 differentiation during EAE, we next analyzed the expression of Th17 cell–associated genes in DLNs. Deficiency of PKM2 in T cells resulted in a significant reduction of the Th17 cell–related genes Il17a, Rora, and Rorc in DLNs before disease onset (day 6; Fig. 5 E), suggesting that T cell–specific deletion of PKM2 inhibits Th17 cell differentiation in the EAE model. Indeed, loss of PKM2 in T cells significantly reduced the frequency of IL-17A^{+}CD4 T cells in DLNs at the peak of the disease (day 15), whereas the population of Foxp3^{+} CD4 T cells remained unaltered (Fig. S4 A). Moreover, when we restimulated cells isolated from DLNs of EAE mice with MOG35-55 ex vivo, the frequency of IL-17A^{+}Roryt^{+} CD4 T cells and the production of IL-17A, GM-CSF, and IFN-γ by PKM2-deficient T cells were significantly lower compared to WT cells (Fig. 5 F and Fig. S4 B). PKM2 deficiency also reduced the transcription of mature pathogenic Th17 cell effector genes Csf2 and Il17a in the total spinal cord tissue at the peak of EAE symptoms (Fig. S4 C). Consistent with this, mRNA transcription levels in Pkm2 and Th17 cell–related genes, including Il17a, Il21, Csf2, Il17r, Il23r, Rora, and Rorc, were lower in CD4 T cells isolated from the spinal cords of CD4^{+}CD8^{+}Pkm2^{fl/fl} mice than in those obtained from WT mice (Fig. 5 G). Accordingly, mice lacking PKM2 in T cells had significantly decreased frequencies of IL-17A^{+} CD4 T cells coproducing the pathogenic Th17 cytokines GM-CSF or IFNγ in their spinal cords (Fig. 5 H). Of note, we noticed that deficiency of PKM2 did not affect the frequency of IFNγ^{+}GM-CSF^{+} Th17 cells in vitro, while the generation of IL-17A^{+}GM-CSF^{+} Th17 cells was impaired (Fig. S3 E).

To further confirm the role of PKM2 for the development of encephalitogenic Th17 cells, we adoptively transferred enriched MOG-specific WT or PKM2-deficient Th17 cells in vitro into Ragg^{−/−} mice. We found that the loss of PKM2 in Th17 cells significantly reduced their ability to induce passive EAE when compared with WT cells (Fig. S4 D). Collectively, these data indicate that PKM2 is required for Th17 cell differentiation in vitro and in vivo, contributing to the pathogenesis of EAE.

PKM2 promotes STAT3 phosphorylation in Th17 cells

In its tetrameric form, PKM2 has high metabolic activity converting phosphoenolpyruvate to pyruvate in the glycolytic pathway. However, its less enzymatically active dimeric form has the potential to translocate into the nucleus and act as a transcriptional coactivator (Yang et al., 2011; Gao et al., 2012). In this context, phosphorylation of PKM2 at tyrosine 105 (Y105) prevents tetramer conformation, favoring the dimeric state (Hitosugi et al., 2009). To investigate how PKM2 regulates Th17 cell differentiation, we initially examined its phosphorylation status and conformational state. We found that increases in total PKM2 expression preceded parallel increases in its phosphorylation at Y105 during Th17 cell differentiation, peaking at 72 h of culture (Fig. 6 A). Phosphorylated PKM2 at Y105 was also increased in the spinal cord tissue of EAE mice and was positively associated with the clinical score of the disease (Fig. S4 E). Moreover, immunoblot analysis of protein cross-linking assay revealed that all oligomeric forms of PKM2 were up-regulated in Th17 cells when compared with Th0 cells after 72 h of culture. However, the dimeric PKM2 was the most prevalent conformation detected, mainly when Th17 cells were differentiated in the presence of IL-23 (Fig. 6 B), suggesting that PKM2 can be translocated into the nucleus. Indeed, confocal immunofluorescence microscopy analysis revealed a punctate staining pattern of PKM2 in Th17 cells, with both cytoplasmic and nuclear localization, whereas Th0 cells showed an evenly distributed pattern of PKM2 mainly in the cytoplasm (Fig. 6 C). Immunoblot analysis of cytoplasmic and nuclear fractions further confirmed the nuclear translocation of PKM2 in Th17 cells (Fig. 6 D).

To investigate the functional importance of the PKM2 nuclear translocation in mediating Th17 cell differentiation, we next used the small molecule TEPP-46, which is a well-characterized PKM2-specific allosteric activator that promotes tetramer formation and inhibits nuclear translocation (Anastasiou et al., 2012). Notably, treating CD4 T cells with TEPP-46 significantly reduced Th17 cell differentiation to the same level as that observed in PKM2-deficient T cells (Fig. 6 E and Fig. S5 A), suggesting that dimeric PKM2 nuclear translocation is required for the regulation of Th17 cell differentiation. Indeed, immunoblot analysis of nuclear fractions of Th17 cells showed that TEPP-46 completely abrogated the translocation of PKM2 into the nucleus (Fig. 6 F). Of note, we found that PKM2 also translocates into the nucleus of Th1 cells, which is also inhibited by TEPP-46 (Fig. S5 B). Nevertheless, TEPP-46 did not affect the differentiation of Th1 cells (Fig. S5 C).

IL-6 and IL-23 induce Th17 cell differentiation through activation of the STAT3 signaling (Korn et al., 2009; Dong, 2008). Moreover, the phosphorylation of STAT3 (phospho-STAT3) at Y705 residue is known to be required for Th17 cell differentiation (Guizzi et al., 2018; Renner et al., 2008). Interestingly, the nuclear dimeric PKM2 form can act as a protein kinase and phosphorylate STAT3 at Y705 in the nucleus, enhancing its transcriptional activity and promoting tumor growth (Gao et al., 2012). We then examined whether PKM2 and STAT3 proteins can interact during the differentiation of Th17 cells. Confocal immunofluorescence images indicated that STAT3 and PKM2 colocalize in the nucleus of Th17 cells (Fig. 7 A). Indeed, immunoprecipitation coupled to immunoblot analysis showed that PKM2 coimmunoprecipitated with STAT3 in WT Th17 cells (Fig. 7 B). The specific PKM2–STAT3 interaction was supported by immunoprecipitating STAT3 in PKM2-deficient Th17 cells and using a control IgG antibody for the immunoprecipitation assay. To further confirm the direct interaction between PKM2 and STAT3, we also conducted a proximity ligation assay (PLA) in Th17 cells. We found a robust fluorescent signal generated by PLA probes targeting STAT3 and PKM2 in WT, but not PKM2-deficient, Th17 cells, indicating a nuclear PKM2–STAT3 interaction (Fig. 7 C and Fig. S5 D).

We then evaluated whether the absence of PKM2 could affect the phosphorylation status of STAT3. Immunoblot analysis demonstrated that acute phosphorylation of STAT3 at Y705 by
Figure 5. T cell–specific PKM2 deletion ameliorates autoimmune-mediated inflammation. (A–C) WT or CD4CrePkm2fl/fl mice were immunized with MOG35–55 and monitored daily for clinical signs (n = 18–24 per group). (A) Cumulative EAE clinical scores. (B) Representation by linear regression curves; dashed lines indicate the 95% confidence intervals. (C) Disease incidence by severity is represented on a bar chart as no EAE (score <1), mild EAE (score 1–2), and severe EAE (score ≥2.5). (D) Inflammatory cell infiltration in the spinal cord (top; black arrowheads) was observed by using H&E staining; the number of inflammatory cells in transverse spinal cord sections was determined in a blinded fashion (right; n = 7 per group). Scale bars represent 500 and 50 µm. Fluoromyelin staining (green) was performed to detect demyelination sites (bottom; white arrowheads); nuclei labeled with DAPI (blue). Scale bar indicates 50 µm. (E) Analysis of Il17a, Rora, and Rorc gene expression in DLN cells collected 6 d after immunization. Data were normalized to Gapdh; fold-change is relative to naive controls (n = 5 per group). (F) DLN cells were harvested 6 d after immunization and restimulated in vitro with MOG35–55; the frequencies of IL-17A+Rorc+ CD4 T cells were then determined by flow cytometry (n = 3). (G) CNS-infiltrating CD4 T cells were isolated. Each sample was a pool of cells from two mice and analyzed for expression of Th17 cell–associated genes (n = 6 per group). Cycle threshold values were normalized to Gapdh; fold change is relative to CNS CD45+ cells from naive mice. Data were normalized by Z score (row) and depicted in a heatmap. (H) Spinal cord–infiltrating mononuclear cells were collected from WT
IL-6 stimulation was reduced in TCR-activated PKM2-deficient CD4 T cells when compared with WT CD4 T cells (Fig. 7D). Additionally, fully differentiated PKM2-deficient Th17 cells (96 h culture) showed significantly lower levels of phosphorylated STAT3 at Y705, while the total STAT3 protein expression was not altered (Fig. 7E and Fig. S5E). In accordance, TEPP-46 also substantially reduced the levels of nuclear Y705-phosphorylated STAT3 in Th17 cells (Fig. 7F). Of note, although the abundance of phosphorylated STAT3 in Th1 cells is markedly lower than that observed in Th17 cells, PKM2-deficient Th1 cells showed reduced levels of phosphorylated STAT3 when compared with WT cells (Fig. S5E). In vivo, the deficiency of PKM2 in T cells resulted in a significant reduction of Y705-phosphorylated STAT3 in the spinal cord tissue of EAE mice compared to

![Figure 6](https://doi.org/10.1084/jem.20190613)

**Figure 6.** PKM2 translocates into the nucleus of Th17 cells. (A) The degree of PKM2 phosphorylation at Y105 was determined by immunoblot at different time points of Th17 cell culture; β-actin was used as a loading control. (B) Th0 or Th17 cells underwent protein cross-linking using disuccinimidyl suberate followed by immunoblot analysis to identify PKM2 oligomer states. (C) Naive CD4 T cells were cultured under Th17 cell-inducing conditions for 96 h and prepared for confocal immunofluorescence analysis. Cells were stained with fluorophore-conjugated anti-PKM2 (red) and nuclei labeled with DAPI (blue). Confocal images were acquired; scale bar indicates 5 µm. (D) Cytoplasmic and nuclear protein extracts from Th17 cell culture were obtained and analyzed by immunoblot to determine PKM2 levels in these compartments. GAPDH and NPM were used as cytoplasm and nuclear loading controls, respectively. (E) WT or PKM2-deficient CD4 T cells were cultured under Th17 cell-skewing conditions in the presence of or absence of TEPP-46 (100 µM), a PKM2 activator, followed by flow cytometry analysis of IL-17A+ CD4 T cells frequencies (n = 3–5). (F) Nuclear fractions from Th17 cells were obtained and analyzed by immunoblot to determine PKM2 protein expression. GAPDH and NPM were used as cytoplasm and nuclear loading controls, respectively. Data are representative of two independent experiments. Error bars are mean ± SEM. P values were determined by two-way ANOVA followed by Tukey's post hoc test (E). *, P < 0.05.
WT mice (Fig. S5 F). Finally, we examined the importance of STAT3 activation by PKM2 in the differentiation of Th17 cells. To this end, we investigated the effect of a suboptimal concentration of Stattic, a small-molecule inhibitor of STAT3 activation (Schust et al., 2006), on the differentiation of Th17 cells. As expected, a suboptimal concentration of Stattic partially reduced the differentiation of WT Th17 cells, whereas it had no additive inhibition on the Th17 differentiation observed in PKM2-deficient T cells (Fig. 7 G). Of note, the inhibition of STAT3 activation did not affect Th1 cell differentiation in WT or PKM2-deficient T cells (Fig. S5 G). Taken together, our results provide strong evidence for the nonmetabolic role of PKM2 in the Th17 cell differentiation program.
Discussion
Recent studies connecting the fields of cellular metabolism and immunology have dramatically improved our understanding of how immune cells benefit from a metabolic reprogramming to support their activation, proliferation, and differentiation (O’Neill et al., 2016; Buck et al., 2015; Almeida et al., 2016). Emerging evidence proposes that metabolic enzymes, rather than solely being components of biochemical pathways, are also proteins that mediate many other biological functions, including gene transcription and cell cycle progression (Yu and Li, 2017; Seki and Gaultier, 2017). The enzyme PK is a critical rate-limiting enzyme in the glycolytic pathway that catalyzes the formation of pyruvate from phosphoenolpyruvate. Notably, the PK isoform M2 is not only present in the cytoplasm as a metabolic enzyme but also can translocate into the nucleus, indicating that it has additional noncanonical or nonmetabolic functions unrelated to glycolysis (Israelsen and Vander Heiden, 2015; Gui et al., 2013).

In the present study, we have shown that PKM2 acts as a transcriptional coactivator during Th17 cell differentiation by fine-tuning STAT3 nuclear activation. We have shown that PKM2 is hardly detectable in naive T cells, whereas the TCR activation of T cells substantially increases its expression, at least in part, through mTORC1 signaling. mTORC1 is a well-known metabolic sensor that promotes aerobic glycolysis by inducing the expression of several glycolysis-related genes (Saxton and Sabatini, 2017). Consistent with our results, it has been previously reported that the mTORC1-HIF1α signaling axis up-regulates the expression of PKM2 in tumor cells (Sun et al., 2011; Iqbal et al., 2013). Importantly, T cell–specific deletion of mTORC1 activity or HIF1α impairs Th17 differentiation (Delgoffe et al., 2011; Kurebayashi et al., 2012; Shi et al., 2011; Dang et al., 2011).

In this context, we found that PKM2 mRNA and protein expression are higher in differentiated Th17 cells than other Th cell subtypes. Consistent with this, we found that CD4 T cells isolated from the spinal cords of mice undergoing EAE, a well-characterized animal autoimmune disease model for the effector function of Th17 cells (Sie et al., 2014), express high levels of PKM2 in parallel with the up-regulation of the Th17 cell–related genes Il17a, Csf2, Il23r, Rora, and Rorc. This association implies a potential role of this glycolytic enzyme in supporting Th17 cell differentiation. It is noteworthy that while we did not detect alteration in Pkm1 mRNA levels throughout Th17 cell differentiation, we observed a particular increase in PKM1 protein levels in fully differentiated Th17 cells, which might result from reduced proteasomal degradation. Thus, it will be important to determine if the later up-regulation PKM1 expression is solely part of the differentiation process or a response to the metabolic demand of mature Th17 cells.

PKM2 acts as a transcriptional coactivator for β-catenin and HIF1α in tumor cells, promoting the expression of genes involved in glycolysis and proliferation (Yang et al., 2011; Luo et al., 2011). Moreover, LPS-induced glycolytic reprogramming and IL-1β production by macrophages require nuclear interaction of PKM2 with HIF1α (Palsson-McDermott et al., 2015). We therefore hypothesized that PKM2 would also be required for the metabolic reprogramming and proliferation of Th17 cells. Strikingly, PKM2 deficiency neither impaired the metabolic reprogramming toward aerobic glycolysis nor affected the proliferative capacity of Th17 cells. Nonetheless, the loss of PKM2 in T cells selectively inhibited Th17 differentiation without altering Th1, Th2, or iT reg cell differentiation in vitro.

As described above, the neuroinflammation observed in mice undergoing EAE is mainly mediated by autoantigen-specific Th17 cells (Sie et al., 2014). Consistent with our in vitro results, the specific loss of PKM2 in T cells not only significantly reduced the clinical severity but also decreased the incidence of EAE. These declines were associated with a lower frequency of IL-17A+ CD4 T cells and less Th17 cell–related cytokine production by T cells upon ex vivo stimulation with MOG35–55. Nevertheless, while we did not find a role for PKM2 in Th1 cell polarization in vitro, mice lacking PKM2 in T cells showed a reduced frequency of T cells expressing IFNγ during EAE. A plausible explanation for these last findings is the potential conversion of Th17 cells into IL-17A+ IFNγ+ CD4 T cells, which is one of the signatures of pathogenic Th17 cells and the dominant T cell population found in the spinal cord of EAE mice (Kurschus et al., 2010; Hirota et al., 2011). In support of this, we also demonstrated that GM-CSF, another pathogenic Th17 cell signature cytokine (Codarri et al., 2011; El-Beih et al., 2011), was similarly reduced in mice lacking PKM2 in T cells during EAE. However, cytokine-driven T cell polarization in vitro is different from pathophysiological differentiation in vivo, where other mediators might be directly or indirectly involved. Thus, we cannot rule out the possibility that PKM2 might regulate Th1 cell differentiation in vivo. Indeed, it was reported that homocysteine stimulation of T cells increases glycolytic metabolism and IFN-γ expression in a PKM2–dependent manner (Lü et al., 2018).

In agreement with our findings, during the revision process of this article, a study was published supporting the role of PKM2 in the generation of Th7 cells (Kono et al., 2019). However, using shikonin, a pharmacological inhibitor of PKM2, it was proposed that inhibition of PKM2 impairs Th17 cell differentiation by reducing glycolysis, which contrasts with our metabolic findings obtained with the genetic approach (Cre/LoxP). The differences in the metabolic profile might be explained by off-target effects of shikonin in other enzymes that regulate glycolysis, including the inhibition of glycogen synthase kinase 3β (GSK-3β) and cell division cycle 25 (Cdc25) phosphatases (Chen et al., 2019; Zhang et al., 2019; Liang et al., 2016). Furthermore, we found that the deficiency of PKM2 in Th17 cells led to a compensatory up-regulation of PKM1 expression, which might explain the normal glycolytic profile that we have observed and also supports the hypothesis of a nonmetabolic mechanism of PKM2 in mediating Th17 cell differentiation. The differential role of PKM1 in Th17 cell differentiation is currently unclear and merits further investigation.

PKM1 and PKM2 isoforms are products of alternative splicing of the same Pkm gene (Noguchi et al., 1986). PKM2, in its tetrameric form, has high metabolic activity in the glycolysis pathway, similar to PKM1. However, the less enzymatically active dimeric form of PKM2 can translocate into the nucleus and act as a transcriptional coactivator (Yang et al., 2011; Luo et al., 2011; Yang et al., 2012a). Phosphorylation of PKM2 on Y105 is indicative of the dimeric form of PKM2, as it prevents the
tetrameric conformation (Hitosugi et al., 2009). Herein, we found that the expression of total PKM2 was followed by increased phosphorylation at Y105 during Th17 cell differentiation. Consistently, the dimeric form of PKM2 was the most prevalent conformation detected in Th17 cells, localized in both cytoplasmic and nuclear compartments. Other posttranslational modifications of PKM2, including acetylation and succinylation, have been reported to affect PKM2 conformation favoring the dimeric form (Lv et al., 2013; Wang et al., 2017). Therefore, we cannot exclude the role of other posttranslational modifications affecting the translocation of PKM2 into the nucleus of Th17 cells. However, consistent with the well-characterized effect of small compound TEPP-46 in promoting PKM2 tetramer formation and inhibiting its nuclear translocation (Anastasiou et al., 2012), we observed that TEPP-46 reduces Th17 cell differentiation, suggesting that nuclear translocation of PKM2 is required for the generation of Th17 cells. Indeed, this hypothesis was recently supported by Angiari et al. (2020). Since the nuclear translocation of PKM2 requires the binding of its nuclear localization signal sequence to the importin α5, an adaptor protein that imports proteins into the nucleus (Yang et al., 2012b), further analysis showing the interaction of the PKM2 nuclear localization signal with importin α5 in Th17 cells may help to confirm our conclusions.

Nuclear PKM2 has been shown to interact with and enhance STAT3 phosphorylation at Y705, contributing to increases in cancer cell proliferation (Gao et al., 2012) and inflammatory cytokine production by macrophages (Shirai et al., 2016). Moreover, it was reported that the mutation of PKM2 at residue R399 locks it in dimeric conformation, enhancing its ability to phosphorylate STAT3 (Yang et al., 2012b). Interestingly, integrative phosphoproteomics analysis of IL-23 reported that PKM2 interacts with STAT3 in the nucleus of Th17 cells, and food ad libitum. Mice used in experiments were sex and age matched. All experiments were performed in accordance with protocols approved by the Ethics Committee on Animal Use of Ribeirao Preto Medical School, University of Sao Paulo.

In vitro T cell differentiation

Naive CD4+CD25− T cells were purified from LNs and spleen of WT C57BL/6, CD4CrePkm2fl/fl or control littermate (CD4Cre and Pkm2fl/fl) mice with the untouched CD4 T cell isolation kit (Miltenyi Biotec) and a biotinylated CD25 monoclonal antibody (eBioscience) by using an AutoMACS magnetic cell sorter (Miltenyi Biotec) according to the manufacturer’s protocol. Purified cells were activated with soluble anti-CD3ε:CD28 (both 1 µg/ml; BD Biosciences) on U-bottomed plates (105/well). Skewing conditions were as follows: Th1, 2.5 ng/ml rhIL-12 β1 (eBioscience) plus 20 ng/ml rmIL-6 (R&D Systems) with or without or 20 ng/ml rmIL-12 (R&D Systems); Th1, rmIL-12, and rmIL-2 (both 20 ng/ml; R&D Systems); Th2, anti-IFN-γ (10 µg/ml), rmIL-4, and rmIL-2 (both 20 ng/ml; R&D Systems). For iT reg cell polarization, naive T cells were cultured with plate-bound CD3ε:CD28 (both 1 µg/ml; BD Biosciences) in the presence of 1 ng/ml rhTGF-β1 (eBioscience). When indicated, 0.1 µM rapamycin (Cayman Chemical), 100 µM TEPP-46 (Millipore), or 2 µM Stattic (Tocris) was used.

Induction and assessment of EAE

EAE was induced by subcutaneously immunizing mice in the flanks with MOG35–55 (Proteimax). The 300 µg of administered MOG35–55 was composed of 100 µl PBS and 100 µl CFA (Sigma-Aldrich) supplemented with 5 mg/ml heat-inactivated Mycobacterium tuberculosis H37Ra (Difco). Additionally, mice received 200 ng pertussis toxin (Sigma-Aldrich) i.p. followed on the day of immunization as well as 2 d later. For adoptive transfer experiments, DLNs cells were harvested from WT or CD4crePkm2fl/fl donor 8 d after immunization and cultured in vitro with MOG35–55 under Th17 cell–polarizing conditions for 72 h. CD4 T cells were isolated by magnetic separation (Miltenyi Biotec), and a total of 106 CD4 T cells were injected i.v. into naive Rag1−/− recipients. 1 d later, the recipient mice were immunized with MOG35–55 plus pertussis toxin, as previously described. Clinical signs of EAE were scored on a standard 0–5 scale, according to previous recommendations (Stromnes and Goverman, 2006), as follows: 0 = unaffected; 0.5 = partial limp tail; 1 = paralyzed tail; 1.5 = loss of coordinated movements; 2 = hindlimb paresis; 2.5 = one hindlimb paralyzed; 3 = both hindlimbs paralyzed; 3.5 = hindlimbs paralyzed and weakness in forelimbs; 4 = one forelimb paralyzed; 4.5 = both forelimbs paralyzed; and 5 = moribund/death.

RNA isolation and quantitative real-time PCR (RT-qPCR)

Total RNA from cultures or sorted CD4 T cells were isolated using the RNeasy Isolation Kit according to the manufacturer’s instructions (Qiagen). Total RNA from spinal cords was harvested...
following the TRIzol Reagent (Invitrogen) protocol. The RNA was quantified and then converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using a StepOnePlus Real-Time PCR machine (Applied Biosystems). Gene expression was determined relative to Gapdh and fold change calculated by using the 2−ΔΔCT threshold cycle method. In some cases, gene expression was represented as heatmaps generated by using the open-source software Morpheus (https://software.broadinstitute.org/morpheus). A list of primers is presented in Table S1.

Flow cytometry
For intracellular cytokine staining, cells were stimulated in culture medium with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) for 4 h in the presence of monensin (GolgiStop 1.5 µg/ml; BD Biosciences) at 37°C in a humidified 5% CO2 chamber. The cells were then washed and stained for 10 min at room temperature with fixable viability dye (Invitrogen) for dead cells exclusion and fluorochrome-labeled monoclonal antibodies against surface cell markers. Afterward, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and Perm/Wash buffer (BD Biosciences), followed by intracellular staining with monoclonal antibodies for 20 min. Intracellular staining of transcription factors was done without stimulation, with the eBioscience Foxp3 Fixation/Permeabilization Kit. Data were acquired on FACSVerse or FACSCanto II machines (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Cell proliferation assay
Naïve CD4+CD25− T cells were labeled with Cell Proliferation Dye eFluor 670 or CellTrace Violet (both 5 µM; Invitrogen) following the manufacturer’s protocol. Cells were then resuspended in culture medium and activated with anti-CD3ε:CD28 (both 1 µg/ml; BD Biosciences) in the presence or absence of rmIL-2 (20 ng/ml; R&D Systems) or cultured under Th17 cell–skewing conditions for 3 d. The stepwise dilution of the fluorescence in daughter cells as indicative of cell proliferation was assessed by flow cytometry.

Cytokine measurement
Supernatants from cell cultures were collected after centrifugation and IFN-γ, IL-17A, GM-CSF, and IL-13 levels were measured by ELISA according to the manufacturer’s instructions (R&D Systems).

Glucose uptake, consumption, and lactate production
For flow cytometry–based glucose uptake assay, naïve CD4 T cells or differentiated Th17 cells were stimulated (1 µg/ml anti-CD3ε:CD28) and incubated with 30 µM 2-NBDG (Invitrogen), a fluorescent glucose analogue, diluted in the glucose-free medium for 30 min at 37°C before measuring fluorescence by flow cytometry. Lactate and glucose concentrations in the cell culture supernatants were measured with colorimetric kit assays according to the manufacturer’s instructions (Bioclin).

Immunoprecipitation and immunoblot analysis
Whole-cell lysates were prepared using radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich) supplemented with

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Figure 8. Schematic representation describing how PKM2 induces Th17 cell differentiation. The cooperation between TCR activation and costimulatory signals per se leads to a significant increase of Pkm2 expression (1), which is highly augmented by the presence of IL-6 and IL-23, important cytokines for controlling the Th17 cell phenotype program. This cascade boosts the activity of the metabolic sensor mTOR that, in turn, contributes to Pkm2 transcription (2). IL-6R and IL-23R signaling cascade promote STAT3 phosphorylation/activation (3), concomitantly with an accumulation of PKM2 dimers in Th17 cells (4). The dimeric oligomer state facilitates PKM2 translocation into the nucleus (5) and its interaction with STAT3, increasing its transcriptional activity (6). This process culminates in enhanced transcription of Th17 cell–associated genes, contributing to the development of autoimmune neuroinflammation.
protease and phosphatase inhibitor cocktail (Cell Signaling). Protein concentrations were determined with a bicinchoninic acid protein assay reagent kit (Sigma-Aldrich). For separation by electrophoresis, 10 μg total protein was loaded onto SDS-polyacrylamide gels according to standard protocols (SDS-PAGE) and then transferred to nitrocellulose membrane (GE Healthcare). Membranes were blocked with 5% (wt/vol) nonfat milk (Cell Signaling) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature and then incubated overnight at 4°C with 1:1,000 dilutions of primary antibodies against PKM1, PKM2, phospho-PKM2 (Y105), STAT3, phospho-STAT3 (Y705), LDHA, or HIF1α (all from Cell Signaling). Subsequently, membranes were repeatedly washed with TBST and incubated for 2 h with the appropriate HRP-conjugated secondary antibody (1:5,000 dilution; Sigma-Aldrich). Immunoreactivity was detected using the ECL prime reagent (GE Healthcare), and then the chemiluminescence signal was recorded on the ChemiDoc XRS Imager (Bio-Rad Laboratories). Data were analyzed with Image Lab software (Bio-Rad Laboratories). Total β-actin levels were used as a loading control. Immunoprecipitation was performed using the Pierce coIP kit (Thermo Scientific) following the manufacturer’s protocol. Briefly, control IgG antibody and mouse anti-STAT3 (Cell Signaling) were immobilized using AminoLink Plus coupling resin. Equal amounts of Th17 cell lysates were pre-cleared and subsequently incubated with the antibody-coupled resin overnight at 4°C. Afterward, the resin was washed, and proteins were eluted using elution buffer. The immunoprecipitated samples were analyzed for PKM2 and STAT3 protein expression by immunoblot, as described above.

Subcellular fractionation

Nuclear and cytosolic fractionation was performed by using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit according to the manufacturer’s recommendations (Thermo Scientific). Protein levels were quantified (bicinchoninic acid) and samples separated by SDS-PAGE before immunoblot analysis. The housekeeping proteins GAPDH and nucleophosmin (NPM) were used as cytosolic and nuclear loading controls, respectively.

Cross-linking reaction

Th0 cells (no cytokines) or differentiated Th17 cells were cross-linked with 500 μM disuccinimidyl suberate (Sigma-Aldrich) for 30 min, and then cell lysates were prepared with radiolimmunoprecipitation assay buffer. The subsequent steps were performed as described in the immunoblot analysis section.

Antigen-specific T cell response

Cells from DLNs and spleen of EAE-bearing mice were isolated and cultured in 96-well round-bottom plates (3 × 10^5 cells/well) with MOG 35-55 (50 μg/ml) in culture medium for 4 d at 37°C. The concentration of IL-17A, GM-CSF, and IFN-γ in the culture supernatants was measured using ELISA kits (R&D Systems).

Preparation of central nervous system (CNS) mononuclear cells

EAE mice were deeply anesthetized and transcardially perfused with ice-cold PBS. The spinal cord was collected and minced with a sharp razor blade, following digestion for 30 min at 37°C with collagenase D (2.5 mg/ml; Roche Diagnostics). Mononuclear cells were isolated by the passage of the tissue through a cell strainer (70 μm), followed by centrifugation through a 37/ 70% Percoll gradient (GE Healthcare). For intracellular cytokine staining, isolated cells were stimulated as previously described, followed by flow cytometric analysis. Conversely, cell suspensions were labeled with anti-CD4 (L3T4) microbeads (Miltenyi Biotec) and separated using an AutoMACS magnetic cell sorter (Miltenyi Biotec). The purity of cell preparations was >90%, and total RNA was extracted for RT-qPCR analysis.

Histology

Mice were anesthetized and perfused with cold PBS, followed by 4% paraformaldehyde (PFA). Spinal cords were collected, post-fixed in 4% PFA, and then cryoprotected in 30% sucrose solution for 72 h. Tissues were then embedded in OCT compound (Tissue-Tek; Sakura Finetek) and snap-frozen on dry ice. Spinal cords were cryostat-cut (Leica) into 20-μm-thick transverse sections, mounted on glass slides, and stained with H&E. A pathologist assessed transverse spinal cord tissue sections for inflammatory cell infiltration in a blinded fashion.

Immunofluorescence

For immunofluorescence analysis, spinal cord cryosections were permeabilized with 0.2% Triton X-100 in PBS for 20 min, blocked with 2% BSA in PBS for 30 min, and then incubated overnight at 4°C with primary antibodies. Subsequently, sections were incubated for 2 h at room temperature with species-specific Alexa Fluor–conjugated secondary antibodies (Abcam). CNS tissue sections were incubated for 1 h with the fluorescent myelin stain FluoroMyelin Green (1:200; Invitrogen). Slides were rinsed in PBS and coverslipped in ProLong Gold antifade reagent with DAPI (Invitrogen). For T cell immunofluorescence, cells were incubated on poly-L-lysine–coated coverslips, fixed (4% PFA), and permeabilized. After incubation with primary and secondary antibodies, coverslips were washed and mounted onto microscope slides using a DAPI-containing mounting medium. The following primary antibodies were used: anti-PKM2 (1:200; Abcam) and anti-STAT3 (1:200; Cell Signaling). The slides were visualized with a high-resolution SP5 confocal microscope (Leica) and image analysis performed on Fiji software.

PLA

PLA was performed using a Duolink In Situ Kit Mouse/Rabbit according to the manufacturer’s instructions (Sigma-Aldrich). Briefly, naive CD4 T cells were cultured under Th17 cell-skewing conditions for 96 h. Cells were attached to poly-L-lysine–coated cover slips, fixed with 2% PFA (10 min at room temperature), washed with PBS, and blocked with blocking buffer (30 min at room temperature). Cells were permeabilized (0.1% Triton X-100) and intracellularly stained with primary mouse anti-STAT3 and rabbit anti-PKM2 (overnight at 4°C; both from Cell Signaling), followed by incubation with oligonucleotide-labeled secondary antibodies. Ligase was added for the hybridization of PLA probes (30 min at 37°C) to form a circularized DNA strand if in close proximity. Samples were incubated
with amplification solution containing fluorescently labeled oligonucleotides plus polymerase (100 min at 37°C) for the rolling-circle amplification reaction. Fluorescent signals indicating proximity was visualized by confocal microscopy.

Statistical analysis
GraphPad Prism 7.0 software was used for statistical analysis. Multiple-group comparisons were performed with either one-way ANOVA or two-way ANOVA followed by Tukey’s post hoc test. Unpaired two-tailed Student’s t test was used for comparison of two conditions. Data are expressed as means ± SEM. P value < 0.05 was considered significant.

Online supplemental material
Fig. S1 validates the expression of signature genes of CD4 T cell subsets and exhibits the differential expression of Pkm1 and Pkm2 among naive, effector/memory, and Th17 cells, and it also shows the PKM2 protein levels in Th1 and Th17 cells. Fig. S2 includes data showing that T cell-specific Pkm2 deletion in mice does not cause gross defect and present data reinforcing that loss of PKM2 does not affect glucose uptake, lactate production, and proliferation of CD4 T cells. Fig. S3 demonstrates that loss of Pkm2 in CD4 T cells does not impair Th1, Th2, or iT reg cell differentiation. Fig. S4 displays additional data confirming that PKM2 boosts Th17 cell–mediated EAE pathogenesis. Fig. S5 shows that both STAT3 activation and PKM2 are dispensable for Th1 differentiation and provide data showing reduced levels of STAT3 activation in the spinal cord of EAE Pkm2-deficient mice. Table S1 contains the sequences of mouse primer pairs used for RT-qPCR analysis. Table S2 lists the reagents used in the study.

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Figure S1. Expression of signature genes of CD4 T cell subsets and differential expression of Pkm1 and Pkm2 among naive, effector/memory, and Th17 cells. (A) Naive CD4 T cells were isolated and cultured under Th1, Th2, Th17 or iT reg cells polarizing-conditions; cells were collected, and expression of *Ifng*, *Il4*, *Il17a*, and *foxp3* was determined by RT-qPCR. (B) Naive (CD4+CD62LhiCD44lo) or effector/memory CD4 T cells (CD4+CD62LloCD44hi) were sorted from LNs and spleen of C57BL/6 WT mice (n = 3). Naive cells were also cultured under Th17 cell–polarizing conditions (96 h). Cells were collected, and total mRNA extracted for RT-qPCR analysis. (C) WT or CD4CrePkm2fl/fl CD4 T cells were differentiated into Th1 or Th17 cells for 96 h and collected for immunoblot analysis of Pkm2 protein expression. β-actin was used as a loading control. (D and E) Rapamycin (0.1 µM), an mTOR inhibitor, was added to the Th17 cell cultures. Cells were collected and intracellularly stained for IL-17A and Foxp3, followed by flow cytometric analysis; *Il17a* and *Foxp3* gene expression levels were determined by RT-qPCR (n = 3). For gene expression analysis, the cycle threshold values were normalized to *Gapdh*; fold change was calculated relative to naive cells (in A and B) or untreated cells (medium; in D and E). Data are representative of two independent experiments. Error bars show mean ± SEM. P values were determined by two-way ANOVA followed by Tukey’s post hoc test (B) or two-tailed Student’s t test (D and E). *, P < 0.05; ns, not significant.
Figure S2. T cell–specific PKM2 deletion in mice does not cause gross defects or affect glucose uptake, lactate production, and proliferation of CD4 T cells. (**A**) Photograph of spleen and LNs isolated from WT and CD4<sup>Cre</sup>Pkm2<sup>fl/fl</sup> mice (n = 3). (**B**) Flow cytometric analyses of thymic CD4<sup>+</sup> and CD8<sup>+</sup> frequencies (n = 3). (**C and D**) Proportion of activated (CD62L<sup>lo</sup>CD44<sup>hi</sup>) and naive (CD62L<sup>hi</sup>CD44<sup>lo</sup>) CD4 T cells in LNs and spleen (n = 3). SSC, side scatter. (**E**) WT or PKM2-deficient Th17 cells differentiated in the presence or absence of IL-23 were incubated with 2-NBDG (30 µM) for 30 min. The glucose uptake ability of Th17 cells was evaluated by flow cytometry; MFI, mean fluorescence intensity. (**F**) Levels of lactate produced by Th17 cells were determined in culture supernatants (n = 3). (**G**) Naive CD4 T cells were labeled with 5 µM proliferation dye and then activated with anti-CD3ε/CD28 and cultured in the presence or absence of IL-2 for 72 h. Flow cytometric analyses were performed to determine their proliferative capacity (n = 3). Data are representative of two (E and F) or three (B–D) independent experiments. Error bars show mean ± SEM. P values were determined by two-tailed Student’s t test.
Loss of PKM2 in CD4 T cells does not impair Th1, Th2 or iT reg differentiation. (A) WT or PKM2-deficient CD4 T were cultured under Th17 cell-skewing conditions and stained for both IL-17A and Foxp3, followed by flow cytometric analysis (n = 5). (B–D) Naive CD4 T cells were also cultured under Th1, Th2, or iT reg-skewing conditions and analyzed for expression of IFN-γ, IL-4, and Foxp3, respectively, by flow cytometry (n = 3). In addition, IFN-γ and IL-13 levels in supernatants of Th1 and Th2 cultures, respectively, were measured by ELISA (n = 3). (E) Naive CD4 T cells were cultured under Th1 or Th17 cell-polarizing conditions for 96 h. Intracellular staining for IFN-γ and GM-CSF in Th1 cells (top) and both IL-17A and GM-CSF in Th17 cells (bottom) was performed, followed by flow cytometric analysis (n = 5). Data are representative of at least three independent experiments. Error bars show mean ± SEM. P values were determined by two-tailed Student’s t test. *, P < 0.05.
Figure S4. PKM2 boosts Th17 cell-mediated EAE pathogenesis. (A) EAE was induced in WT or CD4CrePkm2fl/fl mice and DLN cells collected on day 15 (n = 5 per group). Cells were stimulated and intracellularly stained for IL-17A or Foxp3, followed by flow cytometric analysis. (B) DLN cells were harvested and restimulated with MOG35–55 in vitro for 72 h. The supernatants were collected, and the levels of IL-17A, GM-CSF, and IFN-γ were measured by ELISA (n = 5). (C) Lumbar spinal cord sections were collected from naive or EAE mice with PKM2 deficiency in CD4 T cells. Homogenates were obtained and mRNA extracted, followed by cDNA conversion. RT-qPCR was performed to analyze the expression of Il17a, Csf2, and Ifng. Gapdh was used for normalization (n = 5). (D) DLN cells were collected from WT or CD4CrePkm2fl/fl EAE mice (day 8) and cultured in the presence of MOG35–55 under Th17 cell-skewing conditions for 72 h. CD4 T cells were sorted and intravenously transferred (10^6) into Rag1−/− mice. 1 d later, EAE was induced in the recipient mice (n = 6 per group). Mice were monitored for clinical signs of EAE and CNS inflammatory cell infiltrate analyzed by H&E staining. Scale bar represents 50 µm. (E) PKM2 and phospho-PKM2 (Y105) protein levels in the spinal cord of EAE-bearing mice were determined by immunoblot. β-actin was used as a loading control. Data are representative of two (A–D) or three (E) independent experiments. Error bars show mean ± SEM. P values were determined by two-way ANOVA followed by Tukey’s post hoc test (B–D) and two-tailed Student’s t test (A). *, P < 0.05.
Tables S1 and S2 are provided online as separate Word documents. Table S1 lists mouse primer pairs used for RT-qPCR analysis. Table S2 lists reagents used in this study.

Figure S5. **STAT3 activation and PKM2 are dispensable for the generation of Th1 cells.** (A) Naive CD4 T cells were cultured under Th17 cell-skewing conditions in the presence or absence of TEPP-46 (100 µM), followed by flow cytometric analysis (n = 3). (B and C) Th1 cells were differentiated with or without TEPP-46, and then cytoplasmic and nuclear fractions collected to determine PKM2 protein expression by immunoblot. NPM was used as a nuclear loading control. Flow cytometric analysis of Th1 cell differentiation was conducted (n = 3). (D) PLA assay was performed in WT or PKM2-deficient Th17 cells, followed by confocal microscopy analysis. The close proximity of STAT3 and PKM2 is represented in green. The blue signal indicates DAPI-stained nuclei. Scale bar indicates 10 µm. (E) WT or PKM2-deficient Th1 or Th17 cell lysates were subjected to immunoblot analysis of total and phospho-STAT3 (Y705) expression. GAPDH was used as a loading control. (F) STAT3 and phospho-STAT3 (Y705) levels were determined in spinal cords of WT or CD4\(^{+}\)Pkm2\(^{-/-}\) EAE-bearing mice by immunoblot analysis. β-Actin was used as a loading control. (G) Naive WT and PKM2-lacking Th1 cells were differentiated in the presence or absence of Statick (2 µM); flow cytometric analysis of IFN-γ-producing cells was performed (n = 3). Data are representative of two (B–G) or three (A) independent experiments. Error bars show mean ± SEM. P values were determined by two-way ANOVA followed by Tukey’s post hoc test (G) and two-tailed Student’s t test (A and C). *, P < 0.05; ns, not significant.