DUSP6 mediates T cell receptor-engaged glycolysis and restrains T_{FH} cell differentiation

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Activated T cells undergo metabolic reprogramming and effector-cell differentiation but the factors involved are unclear. Utilizing mice lacking DUSP6 (DUSP6^{-/-}), we show that this phosphatase regulates T cell receptor (TCR) signaling to influence follicular helper T (T_{FH}) cell differentiation and T cell metabolism. In vitro, DUSP6^{-/-} CD4⁺ T_{FH} cells produced elevated IL-21. In vivo, T_{FH} cells were increased in DUSP6^{-/-} mice and in transgenic OTII-DUSP6^{-/-} mice at steady state. After immunization, DUSP6^{-/-} and OTII-DUSP6^{-/-} mice generated more T_{FH} cells and produced more antigen-specific IgG2 than controls. Activated DUSP6^{-/-} T cells showed enhanced JNK and p38 phosphorylation but impaired glycolysis. JNK or p38 inhibitors significantly reduced IL-21 production but did not restore glycolysis. TCR-stimulated DUSP6^{-/-} T cells could not induce phosphofructokinase activity and relied on glucose-independent fueling of mitochondrial respiration. Upon CD28 costimulation, activated DUSP6^{-/-} T cells did not undergo the metabolic commitment to glycolysis pathway to maintain viability. Unexpectedly, inhibition of fatty acid oxidation drastically lowered IL-21 production in DUSP6^{-/-} T_{FH} cells. Our findings suggest that DUSP6 connects TCR signaling to activation-induced metabolic commitment toward glycolysis and restrains T_{FH} cell differentiation via inhibiting IL-21 production.

DUSP6 | follicular helper T cells | IL-21 | glycolysis | T cell metabolism

DUSP6 (also known as MKP-3 or Pyst1) is a MAPK phosphatase that can reverse the activation of ERK1/2 by dephosphorylating tyrosine and threonine residues (1–3). Studies of gene-targeted DUSP6-deficient (DUSP6^{-/-}) mice have revealed essential roles for this phosphatase in embryogenesis (4), heart development (5), metabolism (6–9), colitis (10), and T cell immunity (11, 12). In this study, we demonstrate a role for DUSP6 in follicular helper T (T_{FH}) cell differentiation and in the metabolic reprogramming implemented by activated T cells.

Upon stimulation through the T cell receptor (TCR), naive T cells switch off the oxidative phosphorylation used to maintain their quiescent state (13) and immediately up-regulate genes required for nutrient uptake. They also engage metabolic pathways such as glycolysis, glutaminolysis, and the pentose phosphate pathway to meet their increased bioenergetic demands (14, 15). During the terminal differentiation, memory T cells turn to utilize oxidative phosphorylation with a remodeled mitochondrial membrane structure and a greater spare respiratory capacity (16, 17). Understanding the factors that determine the metabolic reprogramming appears to be important for immune regulation. The differentiation of T_{FH} cells is crucial for germinal center (GC) formation, induction of the humoral response, and immune memory (18–20). T_{FH} differentiation of activated CD4⁺ T cells is instructed by IL-6 (21), IL-12 (22), IL-21 (23), STAT3 signaling (23, 24), and the master transcription factor Bcl6 (21, 25, 26). T_{FH} cells are characterized by the surface markers inducible T cell costimulatory receptor (ICOS), pro-

grammed cell death protein-1 (PD-1), and CXC chemokine receptor 5 (CXCR5). CXCR5 mediates the migration of T_{FH} cells into B cell follicles of secondary lymphoid organs, where ICOS and PD-1 sustain T_{FH} cell interaction with GC B cells by binding to ICOS ligand and PD-1 ligand, respectively (27–29).

It has been proposed that T_{FH} differentiation requires a strong and sustained TCR–antigen interaction (30–33). Engagement of TCR and CD28 activates the MAPK pathway, which is crucial for the induction of glucose uptake, glycolysis, and hexokinase (HK) activity (34–36). Activation of glycolysis in CD4⁺ T cells is required to sustain IL-21 production in T_{FH} cells in vitro (37). However, it has also been shown that expression of Bcl6 represses the glycolytic gene program (38). Consistent with this, inhibition of glycolysis pathway or glucose deprivation does not block Bcl6-expressing T_{FH} cell differentiation (39). The findings reveal a controversial role of glycolysis during T_{FH} differentiation and suggest that glycolysis might be required for early T cell activation, but optional for differentiated mature T_{FH} cells. In our study, we utilized DUSP6^{-/-} mice to identify a contribution by this phosphatase to T_{FH} differentiation and metabolic switching. T cells lacking DUSP6 exhibited strong TCR-mediated JNK and p38 signaling that promoted IL-21 production. Surprisingly, the increased capacity of DUSP6^{-/-} T cells to undergo T_{FH} differentiation was associated with a severe defect in glycolysis. Inhibition of

Significance

Naive T cells at quiescent state utilize mitochondrial respiration to generate ATP. In response to antigen, activated T cells through T cell receptor (TCR) and CD28 differentiate to T_H1, T_H2, or T_H17 effector cells with an induction of aerobic glycolysis. Here, we demonstrate a role for DUSP6 in the glycolysis commitment during T cell activation. DUSP6 deficiency prompts activated T cells to rely on glucose-independent fuels. We show that DUSP6 fine-tunes TCR-MAPK signaling, which determines follicular helper T (T_{FH}) effector-cell differentiation regardless of a glycolytic defect. Our findings imply that glycolysis is required for survival of activated T cells but optional for IL-21 production in T_{FH} cell differentiation.

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TCR-triggered p38 or JNK activity reduced IL-21 production by DUSP6-deficient T cells but did not rescue their glycolytic defect. We further identified that the failure in glycolysis in activated DUSP6^{-/-} T cells due to a loss of phosphofructokinase (PFK) activity was associated with reduced viability. Thus, DUSP6 is a key regulator of TCR-induced metabolic commitment to glycolysis, which is essential for T cell survival but optional for IL-21 production. Our work reveals an important regulatory aspect of the metabolic reprogramming implemented by activated T cells and suggests that down-regulation of DUSP6 accelerates T_{FH} differentiation.

Results

DUSP6^{-/-} T Cells Produce Increased IL-21. To determine the effect of DUSP6 deficiency on T cell-mediated immune responses, we characterized T cell development in DUSP6^{-/-} mice and found that CD4⁺ and CD8⁺ T cells in the thymus, spleen, or lymph nodes (LNs) of DUSP6^{-/-} mice were comparable to DUSP6^{+/+} control mice (WT) in the cellularities or the flow cytometric staining profiles (SI Appendix, Fig. S1A–E). In addition, B cell development in DUSP6^{-/-} mice was normal (SI Appendix, Fig. S1F). We bred DUSP6^{-/-} mice with OTII-transgenic mice, whose CD4⁺ T cells recognize chicken ovalbumin peptide 323–339 (OVA^{323–339}) in the context of MHCII molecule, to generate OTII-DUSP6^{-/-} mutants and their control OTII-DUSP6^{+/+} littermates. These mice were immunized with ovalbumin emulsified in complete Freund's adjuvant (OVA-CFA), an adjuvant that promotes T_H1-biased immune responses. On day 8 postimmunization, OTII-DUSP6^{-/-} T cells produced more IFN- γ than OTII-DUSP6^{+/+} T cells at 12 h postrestimulation and sustained this increase until 24 h postrestimulation (SI Appendix, Fig. S2A). In contrast, the levels of IL-17 and IL-2 produced by activated OTII-DUSP6^{-/-} T cells were comparable to those of control T cells. Upon induction of T cell differentiation in vitro, DUSP6^{-/-} T cells produced more IFN- γ under both T_H1-inducing and T_H2-inducing differentiation conditions, while IL-4 production by DUSP6^{-/-} T cells was not significantly altered (SI Appendix, Fig. S2B). The ability of activated DUSP6^{-/-} T cells to differentiate into either T_H17 cells or Treg cells in vitro was similar to that of DUSP6^{+/+} cells (SI Appendix, Fig. S2C and D). These data suggest that DUSP6 deficiency sensitizes activated T cells to preferentially undergo T_H1 differentiation and increase IFN- γ production, consistent with previous studies (11, 10).

We next examined in vitro IL-21 and IL-6 production by activated OTII-T cells and found that the mutant cells synthesized significantly more IL-21 by 24 h postrestimulation than did controls (Fig. 1A). We therefore used an established assay (23) to compare the abilities of DUSP6^{+/+} and DUSP6^{-/-} CD4⁺ T cells to differentiate into T_{FH} cells. Under T_H0 conditions, IL-21 was undetectable in cultures of DUSP6^{+/+} CD4⁺ T cells, whereas a trace amount of IL-21 was detected in cultures of DUSP6^{-/-} CD4⁺ T cells (Fig. 1B, Left). Under T_{FH} conditions, DUSP6^{+/+} CD4⁺ T cells secreted a moderate amount of IL-21 but DUSP6^{-/-} CD4⁺ T cells produced much more of this cytokine. IL-6 production by DUSP6^{-/-} CD4⁺ T cells under both T_H0 and T_{FH} conditions was comparable to that of controls (Fig. 1B, Right). These findings indicate that DUSP6 deficiency promotes T_{FH} cell differentiation via IL-21 production in a T cell-intrinsic manner.

In our T_{FH} differentiation assay, we cultured naive CD4⁺ T cells with low dose of IL-2 to improve the viability of DUSP6^{-/-} T cells. Nevertheless, IL-2 has been shown to reduce the early commitment of T_{FH} cell lineage and impair T_{FH} response due to the suppressive effect on Bcl6 expression (40–42). To explore the effect of IL-2 on IL-21 production in DUSP6^{-/-} T cells, we performed a T_{FH} differentiation assay in vitro and compared IL-21 production in the presence or absence of IL-2 throughout the assay. Interestingly, IL-2 supplement slightly promoted IL-21 production by DUSP6^{+/+} T_{FH} cells but drastically increased IL-21 production by DUSP6^{-/-} T_{FH} cells (Fig. 1C). The increase of IL-21 production by DUSP6 deficiency was only observed in the

Fig. 1. DUSP6^{-/-} T cells produce increased IL-21. (A) ELISAs to detect IL-21 and IL-6 in supernatants of cultures of T cells isolated from OTII-transgenic mice of the indicated genotypes that were immunized with OVA-CFA. At d7 postimmunization, peripheral T cells were left unstimulated (0) or restimulated with OVA^{323–339} for the indicated times. Data are the mean $\pm$ SEM ($n = 5–12$ per group). (B) ELISAs to detect IL-21 and IL-6 in culture supernatants of in vitro-generated T_H0 or T_{FH} cells. On day 5, all cells were restimulated with anti-CD3 for 24 h before supernatants were collected. Data are the mean $\pm$ SEM ($n = 5$ per group) and representative of three independent experiments. (C) ELISA to detect IL-21 production in culture supernatants of in vitro-generated T_{FH} cells in the absence (–) or presence (+) of IL-2. Data are representative for two independent experiments. (D) Flow cytometric analysis of IL-21 versus CD4, IFN- γ versus CD4, and IFN- γ versus IL-21 (from upper to lower) in in vitro-generated T_{FH} cells. Numbers in quadrants are frequencies of IL-21⁺ or IFN- γ ⁺ T_{FH} cells relative to total CD4⁺ T cells. * $P < 0.05$; *** $P < 0.0005$; ns, nonsignificant.

presence of IL-2. Importantly, although IL-2 signaling suppresses Bcl6 expression through STAT5, a low concentration of IL-2 can induce Bcl6 mRNA in T_H1 effector cells via Foxo factors (43). We thus speculated that DUSP6 deficiency accelerated T_{FH} cell differentiation with T_H1 features in the presence of IL-2. To prove this hypothesis, we compared the expression profile of IL-21 versus IFN- γ between in vitro-generated DUSP6^{+/+} and DUSP6^{-/-} T_{FH} cells by intracellular staining upon anti-CD3 restimulation for 24 h. As expected, under T_{FH} conditions with supplement of IL-2, DUSP6^{-/-} T_{FH} cells generated more IL-21⁺ IFN- γ ⁺ cells than WT T_{FH} cells (Fig. 1D). The results reveal that DUSP6 deficiency promotes the differentiation of T_{FH} cells with T_H1 features in the presence of IL-2.

Increased TCR-Mediated JNK and p38 Signaling Contribute to Elevated IL-21 Production by DUSP6^{-/-} T Cells. Engagement of the TCR initiates various signaling cascades and the activation of MAPK pathway components such as ERK, JNK, and p38 (44). In DUSP6^{+/+} T cells, TCR engagement led to maximal phosphorylation of JNK1/2, p38, and ERK1/2 at 5 min poststimulation as expected, with a gradual decrease in signal intensity from 15 to 60 min poststimulation (Fig. 2A). In sharp contrast, TCR engagement in DUSP6^{-/-} T cells dramatically increased p-JNK and p-p38 but not p-ERK (Fig. 2A and B). Indeed, basal levels of p-JNK and p-p38 in resting DUSP6^{-/-} T cells appeared to be higher than those in controls (Fig. 2B). To confirm this result, we treated the Jurkat T cell line with the small molecule drug (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI) to inhibit DUSP6 as previously described (45) and examined TCR-induced JNK, p38, and ERK phosphorylation. In the absence of BCI, maximal TCR-mediated phosphorylation of JNK and p38 occurred in Jurkat cells at 15 min poststimulation, whereas the greatest TCR-induced ERK phosphorylation at 5 min was followed

Fig. 2. The increased IL-21 production in $DUSP6^{-/-}$ T cells is due to the increased TCR-mediated JNK and p38 signaling. (A) Immunoblotting to detect the indicated proteins in purified T cells that were left unstimulated (0) or stimulated with anti-CD3/CD28 for the indicated times. GAPDH and GRP96, loading controls. Images representative of two independent experiments are shown. (B) Kinetics of induction of phospho-JNK1/2 normalized to total JNK1/2 (p-JNK/JNK) and phospho-p38 normalized to total p38 (p-p38/p38) as depicted in A. (C) Immunoblotting to detect the indicated proteins in Jurkat cells that were pretreated with DMSO (control) or 2.4 μ M or 12 μ M BCI ($DUSP6$ inhibitor) at 37 $^{\circ}$ C for 60 min. Treated cells were subsequently left unstimulated (0) or stimulated with anti-CD3/CD28 for 5, 15, and 30 min, and lysates were subjected to standard immunoblotting. Images are representative of two independent experiments. (D) Cell counts and ELISA to detect IL-21 production in culture supernatants of in vitro-generated T_{FH} cells, which were restimulated for 24 h with anti-CD3 in the presence/absence of 5 μ M SP600125 or 5 μ M SB203580 on day 5. Data are the mean $\pm$ SEM ($n = 6$ per group). * $P < 0.05$ (Student's t test); *** $P < 0.0005$; ns, nonsignificant.

by a gradual decrease (Fig. 2C, lanes 1–4). After treatment with 2.4 μ M BCI, a drastic induction in basal levels of p-JNK and p-38 was detected, and TCR-mediated p-JNK and p-p38 levels were much higher and reached much faster than in DMSO-treated controls. Consistent with our earlier observation, the effect of BCI on TCR-mediated ERK phosphorylation was minor (Fig. 2C, lanes 5–12). These results reveal that genetic loss of $DUSP6$ and BCI-induced inhibition of $DUSP6$ lead to stronger p-JNK and p-p38 signals in T cells, both at steady state and following TCR stimulation.

To determine whether the increased IL-21 production by $DUSP6^{-/-}$ T cells was due to their elevated TCR-mediated JNK and/or p38 signaling, we performed T_{FH} differentiation assays in vitro in the presence of the JNK inhibitor SP600125 or the p38 inhibitor SB203580 on day 5 for 24 h. To exclude drug effects on T cell proliferation, T cell counts were performed on day 6 and the amounts of IL-21 produced were normalized to the cellularity. Treatment with SB203580 (but not DMSO or SP600125) led to a significant reduction in $DUSP6^{-/-}$ T cell number (Fig. 2D, Left). Strikingly, $DUSP6^{-/-}$ T cells treated with either SP600125 or SB203580 showed profound suppression of IL-21 production (Fig. 2D, Right). These data suggest that the increased IL-21 production by $DUSP6^{-/-}$ T cells is due to abnormally strong TCR-triggered p-JNK and p-p38 signaling. It is noteworthy that SP600125 or SB203580 suppresses IL-21 production in WT T cells as well, suggesting that TCR-mediated JNK and p38 signaling is required for IL-21 production under physiological status.

To address how $DUSP6$ deficiency affected T_{FH} cell program, we conducted RNA sequencing (RNA-seq) analysis to study Bcl6-targeted genes in those in vitro-generated T_{FH} cells as described previously (46). The expression profile of 16 T_{FH} -associated genes directly induced by Bcl6 was comparable between $DUSP6^{+/+}$ and

$DUSP6^{-/-}$ T_{FH} cells, except the gene encoding Nav2, which was particularly down-regulated in $DUSP6^{-/-}$ T_{FH} cells (SI Appendix, Fig. S3A). Within the 44 genes directly suppressed by Bcl6, the mRNA levels of *Lif* and *Il7r* in $DUSP6^{-/-}$ T_{FH} cells were slightly more than those in $DUSP6^{+/+}$ T_{FH} cells (SI Appendix, Fig. S3B). In general, the results suggested that Bcl6 activity in $DUSP6^{-/-}$ T_{FH} cells was comparable to control T_{FH} cells. Kyoto Encyclopedia of Genes and Genomes pathway analysis of 84 genes significantly affected in $DUSP6^{-/-}$ T_{FH} cells (including 32 genes up-regulated and 52 genes down-regulated) revealed that $DUSP6$ deficiency was strongly associated with the activation of JAK-STAT signal transduction (Stat3 and Socs3), cytokine/cytokine receptors interaction (*Lif*, *Il12rb2*, *Il21*, and *Il7r*), and the inhibition of ECM-receptor interaction, cell communication, and cell adhesion (SI Appendix, Fig. S3C). Taken together, the results suggest that the accelerated T_{FH} cell differentiation by $DUSP6$ deficiency revealed by the increased IL-21 production is governed by stronger IL-12 receptor family and Stat3-associated signaling pathway(s).

$DUSP6^{-/-}$ Mice Show Increased T_{FH} Cells. T_{FH} cells, which are characterized by their surface expression of ICOS, PD-1, and CXCR5, are present at only a low level in unimmunized mice but are greatly induced by immunization. To determine whether $DUSP6$ deficiency affects T_{FH} cells in vivo, we analyzed this subset in mice before (d0) and on day 7 (d7) postimmunization with trinitrophenyl keyhole limpet hemocyanin (TNP-KLH) emulsified in CFA (TNP-KLH-CFA). In resting $DUSP6^{+/+}$ animals, 0.5–0.9% of total T cells were CXCR5⁺PD-1⁺ or CXCR5⁺ICOS⁺, respectively, indicating that less than 1% of total T cells were T_{FH} cells in control mice (Fig. 3A and C). Remarkably, the corresponding frequencies of CXCR5⁺PD-1⁺ or CXCR5⁺ICOS⁺ T_{FH} cells in resting $DUSP6^{-/-}$ mice were 1.6%, significantly higher than in controls (Fig. 3A and C). In both mice, the expression levels of Bcl6 in CXCR5⁺PD-1⁺ CD4⁺ T cells were higher than those in total CD4⁺ T cells, confirming the subsets as T_{FH} lineage (Fig. 3B). Postimmunization, CXCR5⁺PD-1⁺ and CXCR5⁺ICOS⁺ T_{FH} cells were markedly induced in $DUSP6^{+/+}$ and $DUSP6^{-/-}$ mice (Fig. 3A and C). However, immunized $DUSP6^{-/-}$ mice comprised more CXCR5⁺PD-1⁺ or CXCR5⁺ICOS⁺ T_{FH} cells than immunized $DUSP6^{+/+}$ mice, suggesting that $DUSP6$ deficiency promoted T_{FH} cell generation at steady state and after immunization.

The presence of T_{FH} cells is known to stabilize the formation of GCs populated by B cells (20). We next examined whether the increased T_{FH} population in $DUSP6^{-/-}$ mice was associated with an increase in GC B cells. In WT spleen, GL7⁺FAS⁺ GC B cells turned from 0.5% at steady state to 2.5% after immunization, while those GC B cells increased from 0.9% in the resting status to ~5% after immunization in $DUSP6^{-/-}$ spleen (SI Appendix, Fig. S4A and B), suggesting that there are almost two times more GC B cells in resting and immunized mutant mice than in controls. The increased GC B cells in unimmunized $DUSP6^{-/-}$ mice were also found in the LNs (SI Appendix, Fig. S4C and D). To address whether the affected T_{FH} and B cell populations by $DUSP6$ deficiency was conducted by CD4⁺ T cells, we immunized OTII-mice with OVA-CFA and analyzed T_{FH} cell and GC B cell numbers before immunization (d0) and on d7 postimmunization. As expected, T_{FH} cells in OTII- $DUSP6^{+/+}$ mice increased from 1.7 to 2.7% postimmunization (Fig. 3D and F). Consistent with our earlier data, T_{FH} cells in OTII- $DUSP6^{-/-}$ mice increased from 2.8 to 5.6% after immunization, which was more than the proportion in control mice (Fig. 3D and F). In OTII- $DUSP6^{-/-}$ and control mice, the expression levels of Bcl6 in CXCR5⁺PD-1⁺ CD4⁺ T cells were much higher than in CD4⁺ T cells, confirming the subset as T_{FH} lineage (Fig. 3E). Furthermore, immunized OTII- $DUSP6^{-/-}$ mice contained more than twice as many PNA⁺FAS⁺ GC B cells as did immunized OTII- $DUSP6^{+/+}$ mice (SI Appendix, Fig. S4E and F). To preclude an intrinsic role of $DUSP6$ in B cell function, we examined B cell proliferation, activation, and the production of T cell-independent

Fig. 3. DUSP6^{-/-} mice show increased frequencies of T_{FH} cells and GC B cells. (A) Flow cytometric analysis of CXCR5 versus PD-1 and CXCR5 versus ICOS in splenic CD4⁺ T cells in mice before (d0) and on d7 after TNP-KLH-CFA-immunization. Numbers in quadrants are frequencies of CXCR5⁺PD-1⁺ or CXCR5⁺ICOS⁺ T_{FH} cells relative to total CD4⁺ T cells. (B) Flow cytometric analysis of Bcl6 expression in CXCR5⁺PD-1⁺ T_{FH} cells (red line) overlaid CD4⁺ T cells (gray filled color) in unimmunized mice shown in A. (C) Statistical analysis of CXCR5⁺PD-1⁺ T_{FH} cell frequencies in A. Data points are values for individual mice ($n = 6-12$ per group). Horizontal lines are mean $\pm$ SEM and representative of three independent experiments. (D) Flow cytometric analysis of CXCR5 versus PD-1 in splenic CD4⁺ T cells in mice on d0 and d7 after OVA-CFA-immunization. Numbers in quadrants are frequencies of CXCR5⁺PD-1⁺ T_{FH} cells relative to total CD4⁺ T cells. (E) Flow cytometric analysis of Bcl6 expression in CXCR5⁺PD-1⁺ T_{FH} cells (red line) overlaid CD4⁺ T cells (gray filled color) in unimmunized OTII-mice shown in D. (F) Statistical analysis of the data in D. Data points are values for individual mice ($n = 6-8$ per group) and representative of two independent experiments. Horizontal lines are mean $\pm$ SEM. (G) Flow cytometric analysis of CXCR5 versus PD-1 in CD4⁺ T cells from ALN plus ILN, MLN, spleen, and Peyer's patches in chimeric RAG1/2-KO recipient mice on d0 and d7 postimmunization with TNP-KLH-CFA. Numbers in quadrants are frequencies of CXCR5⁺PD-1⁺ T_{FH} cells relative to total CD4⁺ T cells. (H) Flow cytometric analysis of Bcl6 expression in CXCR5⁺PD-1⁺ T_{FH} cells (red line) overlaid CD4⁺ T cells (gray filled color) in unimmunized mice shown in G. (I) Statistical analysis of the data in G. Data points are values for individual mice ($n = 3$ per group) and representative for two independent experiments. Horizontal lines are mean $\pm$ SEM. * $P < 0.05$; *** $P < 0.0005$; ns, nonsignificant.

antibodies in DUSP6^{-/-} mice. DUSP6^{-/-} B cell proliferation stimulated by anti-IgM, CD40 ligand, LPS, or CpG was comparable to WT B cells (SI Appendix, Fig. S5A). Upon those stimuli, the induced expression of CD86, CD25, CD40, and MHCII in DUSP6^{-/-} B cells was similar to control cells (SI Appendix, Fig. S5B). Before (d0) and after immunization (d7–d28) with soluble TNP-Ficoll, DUSP6^{-/-} mice generated equal amounts of TNP-specific IgM, IgG1, and IgG3 (SI Appendix, Fig. S5C). It suggested that B cell-intrinsic DUSP6 deficiency did not affect antigen-mediated B cell proliferation, activation, and antibody production. Taken together, these data establish that DUSP6 deficiency promotes the spontaneous differentiation of T_{FH} cells at steady state and upon immunization, which stabilizes GC B cell formation.

T_{FH} cell differentiation can be extrinsically regulated by dendritic cells (DCs) or antigen-presenting cells (APCs) (47). To address whether the increased T_{FH} cell differentiation in DUSP6^{-/-} mice was mediated by non-T, non-B cells, we performed an adoptive transfer experiment by reconstituting bone marrow cells from DUSP6^{+/+} or DUSP6^{-/-} mice to irradiated RAG1/2-KO mice. Eight weeks after bone marrow transfer, those recipient mice were immunized with TNP-KLH-CFA and the restored T cells were analyzed on d7 postimmunization. In RAG1/2-KO mice that received DUSP6^{-/-} cells (DUSP6^{-/-} → RAG1/2-KO mice) before immunization, T_{FH} populations with Bcl6 expression in axillary LNs (ALN), inguinal LNs (ILN), and mesenteric LNs (MLN), spleens, and Peyer's patches were slightly but significantly more than in control recipient mice (Fig. 3 G–I).

Consistent with the findings, the increased GC B cells were identified in the spleens and LNs of unimmunized DUSP6^{-/-} → RAG1/2-KO mice (SI Appendix, Fig. S4 G–J). After immunization, the proportions of T_{FH} cells in the LNs and Peyer's patches of DUSP6^{-/-} → RAG1/2-KO mice remained higher than in control mice (Fig. 3 G and I). The results suggest that DUSP6^{-/-} T cells sustain a better ability to undergo T_{FH} cell differentiation in vivo in the presence of WT APCs. In addition, the increased T_{FH} cells by DUSP6 deficiency appear not only in the spleens but also in the draining LNs or Peyer's patches.

DUSP6^{-/-} Mice Show Increased Antigen-Specific IgG2. We next examined whether the elevated numbers of GC B cells and T_{FH} cells in DUSP6^{-/-} mice could lead to an increase in antigen-specific Ig production. Before immunization, DUSP6^{+/+} and DUSP6^{-/-} mice showed comparable levels of TNP-specific Ig(s) (Fig. 4A). After immunization with TNP-KLH-CFA, however, DUSP6^{-/-} mice produced significantly more TNP-specific IgG2a and IgG2b than DUSP6^{+/+} mice. In OTII-DUSP6^{-/-} mice immunized with OVA-CFA, an increase in OVA-specific IgG1, IgG2a, and IgG2b was observed (Fig. 4B). The increase in IgG2 was consistent with an enhanced generation of CD138⁺IgG2⁺ plasma cells in immunized OTII-DUSP6^{-/-} mice (SI Appendix, Fig. S4 K and L). Based on a role of IL-21 in promoting Ig class switch to IgG1 and IgG2b (48), our results suggest that the increased T_{FH} cells and GC B cells by DUSP6 deficiency promote antigen-specific Ig class switch toward T_H1-biased IgG2a and IL-21-mediated switching to IgG1 and IgG2b.

DUSP6 Is Required for TCR-Mediated Glycolysis. The metabolic requirements of T_{FH} cell differentiation are not well understood. To examine the effect of DUSP6 deficiency on the metabolic reprogramming to glycolysis that occurs in activated T cells, we determined T cell glycolysis using the Seahorse Extracellular Flux Analyzer. In this assay, the extracellular acidification rate (ECAR) of the culture medium, which reflects the amount of proton efflux, represents the glycolytic rate. By this measure, the addition of glucose to cultures of anti-CD3-stimulated DUSP6^{+/+} T cells resulted in increased glycolysis, as expected (Fig. 5 A and B). In contrast, the addition of glucose barely stimulated glycolysis in DUSP6^{-/-} T cells, whether the cells were

at steady state or stimulated with anti-CD3. In addition, while anti-CD3-stimulated DUSP6^{+/+} T cells showed enhanced glycolytic capacity following treatment with oligomycin (Oli), and an increased glycolytic reserve (which is equal to the difference between glycolytic capacity and glycolysis), anti-CD3-stimulated DUSP6^{-/-} T cells exhibited severe deficits in both of these parameters (Fig. 5 A and B). This defect in glycolysis was confirmed when DUSP6^{-/-} T cells were costimulated in vitro with anti-CD28 (Fig. 5 C and D). These findings suggest that DUSP6 function promotes TCR-triggered glycolysis. In the meantime, we determined if DUSP6 deficiency also affected B cell receptor (BCR)-mediated glycolysis and found that B cells activated through the BCR had high levels of glycolysis, glycolytic capacity, and glycolytic reserve, with no significant differences between DUSP6^{+/+} and DUSP6^{-/-} B cells (SI Appendix, Fig. S6A). We further observed that the induction of glycolysis in LPS/IFN- γ -activated DUSP6^{-/-} bone-marrow-derived macrophages (BMMs) or bone-marrow-derived DCs (BMDCs) was comparable to that of the corresponding DUSP6^{+/+} myeloid cells (SI Appendix, Fig. S6 B and C). We examined DUSP6 expression by immunoblotting and observed that DUSP6 protein was detectable in T cells and B cells, not in BMDCs and BMMs (SI Appendix, Fig. S6D). It suggests that DUSP6 is optional for the glycolysis engagement in myeloid cells due to the absent expression.

The PI3K/Akt/mTOR complex 1 (mTORC1) pathway is essential for the metabolic reprogramming and the expression of GLUT1 on the T cell surface (36, 49). Engagement of TCR leads to the activation and phosphorylation of Akt at S473 (50, 51), and the subsequent phosphorylation of mTOR at S2448, which correlates with increased mTORC1 activity (52). To address whether the PI3K/Akt/mTOR pathway was affected by DUSP6 deficiency, we examined the activation of Akt and mTOR by p-Akt S473 and p-mTOR S2448 immunoblots in T cells. CD28 costimulation led to an undistinguishable and increased phosphorylation of Akt S473 at 5 min poststimulation with a decrease in signal intensity from 15 min poststimulation in DUSP6^{-/-} and control cells (SI Appendix, Fig. S7A). However, DUSP6^{-/-} T cells showed a stronger p-mTOR S2448 signal than control cells at 5 and 15 min poststimulation (SI Appendix, Fig. S7 A and B), suggesting that DUSP6 deficiency causes a stronger TCR-mediated mTORC1 activation.

To investigate whether the impaired glycolysis in DUSP6^{-/-} T cells was due to defective GLUT1 expression or an impaired glucose uptake, we stimulated T cells with anti-CD3 or with anti-CD3/CD28 for 20 h, determined GLUT1 expression by intracellular staining, or monitored glucose uptake by 2-NBDG-pulse in viable cells by flow cytometry. In the absence of any stimulus (culture in medium alone) or with the supplement of IL-2, the frequency of GLUT1⁺ DUSP6^{-/-} T cells was more than that of control cells (SI Appendix, Fig. S7 C and D). Stimulation through anti-CD3 or anti-CD3/CD28 significantly induced more GLUT1 expression in DUSP6^{+/+} T cells than in unstimulated controls, while only CD28 costimulation led to more GLUT1 expression in DUSP6^{-/-} T cells. Nevertheless, DUSP6^{-/-} T cells took up a similar amount of 2-NBDG comparable to DUSP6^{+/+} controls whether in medium alone, IL-2 alone, or upon stimulation with anti-CD3 or anti-CD3/CD28 (SI Appendix, Fig. S7 E and F). Thus, treatment with anti-CD3 or anti-CD3/CD28 significantly increased glucose uptake by both T cells. However, DUSP6^{+/+} and DUSP6^{-/-} T cells were not significantly different in this respect. The results reveal that DUSP6 deficiency exerts a stimulatory effect on TCR-mediated mTORC1 activation, leading to more GLUT1 expression in the steady state and an unchanged glucose uptake upon TCR stimulation. The finding suggests that the impaired glycolysis in DUSP6^{-/-} T cells is not due to an aberrant GLUT1 expression or glucose uptake.

To determine if the glycolytic defect in DUSP6^{-/-} T cells was due to aberrant TCR-mediated JNK or p38 signaling, we treated anti-CD3-activated T cells in vitro with DMSO (vehicle), JNK inhibitor SP600125, or p38 inhibitor SB230580 for 30 min before

Fig. 4. DUSP6^{-/-} mice show increased serum antigen-specific IgG2 upon immunization. (A) ELISAs of TNP-specific serum IgM, IgG1, IgG2a, IgG2b, and IgG3 (from left to right) in mice before immunization (d0) and at d7 postimmunization with TNP-KLH-CFA. Data points are values for individual mice ($n = 4$ –8 per group). (B) ELISAs of OVA-specific serum IgM, IgG1, IgG2a, IgG2b, and IgG3 (from left to right) in OTII-mice before immunization (d0) and at d7 postimmunization with OVA-CFA. Data points are values for individual mice ($n = 4$ –12 per group). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$; ns, nonsignificant.

Fig. 5. DUSP6^{-/-} T cells exhibit impaired glycolysis upon TCR stimulation. (A) Real-time determination of ECAR values in milli-pH units per minute (mpH/min) at the indicated time points for T cells cultured in medium alone (control) or with anti-CD3. The timing to add glucose (Glu), Oli, and 2-DG was as indicated. Data are the mean $\pm$ SEM ($n = 3$ per treatment) and representative of two independent experiments. (B) Statistical analyses of ECAR values associated with nonglycolytic acidification, glycolysis, glycolytic capacity, and glycolytic reserve in the T cells in A. (C and D) Real-time determination and statistical analyses of ECAR values in mpH/min at the indicated time points for T cells cultured with anti-CD3/CD28. Data are the mean $\pm$ SD and representative of two independent experiments. (E) Real-time determination of ECAR values in mpH/min for T cells that were cultured with anti-CD3 for 20 h and treated for the indicated times with DMSO (control), SP600125, or SB230580. Data are the mean $\pm$ SD ($n = 3$ per treatment). (F) Statistical analysis of ECAR values associated with nonglycolytic acidification and glycolysis of the cells in E. (G) Real-time determination of ECAR values in mpH/min for T cells cultured with anti-CD3 for 20 h and treated for the indicated times with DMSO (control) or 2.4 μ M BCI. Data are the mean $\pm$ SD ($n = 3$ per treatment). (H) Statistical analysis of ECAR values associated with nonglycolytic acidification and glycolysis of the cells in G. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

induction of glycolysis by addition of glucose. DUSP6^{+/+} T cells pretreated with SB230580 significantly increased glycolysis in response to anti-CD3 treatment, whereas pretreatment with SP600125 had no effect (Fig. 5 E and F). However, treatment of DUSP6^{-/-} T cells with neither SP600125 nor SB230580 restored anti-CD3-induced glycolysis to the control level. To determine if inhibition of DUSP6 directly led to the glycolysis defect, we treated anti-CD3-activated DUSP6^{+/+} T cells in vitro with DMSO or DUSP6 inhibitor BCI for 30 min before induction of glycolysis by addition of glucose and found that DUSP6^{+/+} T cells pretreated with BCI significantly reduced glycolysis by addition of glucose (Fig. 5 G and H). Thus, the impaired glycolysis in DUSP6^{-/-} T cells is not due to their heightened TCR-mediated JNK or p38 signaling. Further, inhibition of DUSP6 in WT T cells is directly linked with a glycolysis defect.

PFK Activity Is Impaired in Activated DUSP6^{-/-} T Cells. Glycolysis is regulated by three rate-limiting enzymes, namely HK, PFK, and pyruvate kinase (PKM). Among these enzymes, PFK is the most important because its activity makes glycolysis irreversible. We first used immunoblotting to examine these three enzymes in resting T cells and found that the protein levels of HK, PFK, and PKM were comparable between the mutant and control cells (Fig. 6 A and B). In addition, the expression level of PFK was strongly induced in anti-CD3-stimulated DUSP6^{+/+} T cells. The induced level of PFK in anti-CD3-stimulated DUSP6^{-/-} T cells was comparable to that in DUSP6^{+/+} T cells (Fig. 6C). We next determined the enzymatic activities of HK, PFK, and PKM in T cells either at rest or after anti-CD3 stimulation. Although anti-CD3-stimulated DUSP6^{+/+} and DUSP6^{-/-} T cells both up-regulated HK activity, the degree of the increase was much less in DUSP6^{-/-} T cells (Fig. 6D, Left). PFK activity was significantly

induced in anti-CD3-stimulated DUSP6^{+/+} T cells but, surprisingly, not in activated DUSP6^{-/-} T cells (Fig. 6D, Middle). PKM activity was undetectable in resting DUSP6^{+/+} T cells but unexpectedly elevated in resting DUSP6^{-/-} T cells (Fig. 6D, Right). However, upon anti-CD3 stimulation, PKM activity was up-regulated to a similar extent in DUSP6^{+/+} and DUSP6^{-/-} T cells (Fig. 6D, Right). To further address whether the impaired HK and PFK activities in DUSP6^{-/-} T cells led to a reduction of the metabolites G-6-P and F-1,6-BP, we determined the level of intracellular G-6-P and F-1,6-BP in resting and anti-CD3-stimulated T cells. In both conditions, the levels of G-6-P and F-1,6-BP in DUSP6^{-/-} T cells were significantly increased over those in DUSP6^{+/+} T cells (SI Appendix, Fig. S8 A and B), suggesting an abnormal homeostasis of glycolytic metabolites in DUSP6^{-/-} T cells. To address whether an addition of exogenous G-6-P or F-1,6-BP rescued the glycolysis defect in DUSP6^{-/-} T cells, we preincubated anti-CD3-stimulated T cells with G-6-P or F-1,6-BP for 30 min, respectively, before the glycolysis assay. Whether in DUSP6^{+/+} or DUSP6^{-/-} T cells, an exogenous supplement of G-6-P or F-1,6-BP did not promote glycolysis nor glycolytic capacity (SI Appendix, Fig. S8C).

The activity of PFK is activated by high ADP and low ATP and inhibited by lactate and citrate (53). To understand whether the impaired PFK activity in DUSP6^{-/-} T cells was affected by these parameters, we examined ADP/ATP ratios and intracellular lactate and citrate in resting and anti-CD3-activated T cells. ADP/ATP ratios and lactate amounts were comparable between DUSP6^{+/+} and DUSP6^{-/-} T cells, suggesting that the impaired PFK activity in DUSP6^{-/-} T cells was not due to an unbalanced ADP/ATP or an aberrant induction of lactate (Fig. 6 E and F). However, the concentration of intracellular citrate in DUSP6^{-/-} T cells were nearly twice as much as those in WT T cells in the resting status

Fig. 6. DUSP6^{-/-} T cells fail to up-regulate PFK activity upon TCR stimulation. (A) Immunoblotting to detect protein levels of HK, PFK, and PKM in T cells of steady state from two individual mice (1 and 2). GP96, loading control. Data are representative of two independent trials. (B) Statistical analysis of densitometry determinations for the blot in A. Data are the mean fold increase in the mutant over the control $\pm$ SD ($n = 2$ per treatment). (C) Immunoblotting to detect protein levels of HK, PFK, and PKM in T cells cultured in medium alone (control) or with anti-CD3/CD28 for 20 h. (D) Colorimetric assay to detect the enzymatic activities of HK, PFK, and PKM in T cells that were cultured in medium alone or with anti-CD3 for 20 h. Data are the mean $\pm$ SEM ($n = 3$ per treatment) and representative of two independent experiments. (E) Colorimetric assay to detect ADP/ATP ratios in T cells that were cultured in medium alone or with anti-CD3 for 20 h. (F) Intracellular levels of lactate and citrate in T cells that were cultured in medium alone or with anti-CD3 for 20 h. ns, nonsignificant; un, undetectable; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

or upon anti-CD3 stimulation (Fig. 6F). These results indicate that the impaired glycolysis in TCR-stimulated DUSP6^{-/-} T cells is associated with a partial reduction in HK activity and a severe loss of PFK activity, presumably due to an excess of intracellular citrate.

DUSP6^{-/-} T Cells Show Greater Dependence on Glutamine and Fatty Acids to Fuel Mitochondrial Respiration. To examine whether DUSP6^{-/-} T cells increased their cellular respiration to compensate for their glycolytic defect, we determined mitochondrial respiration by measuring the oxygen consumption rate (OCR) in cultures of purified T cells, with or without anti-CD3 stimulation. During this assay, glutaminase inhibitor BPTES (54) and carnitine palmitoyltransferase 1A (CPT1A) inhibitor ETOMOXIR (55) were added to block glutaminolysis and lipolysis. We also added UK5099 (56) to some T cell cultures to inhibit the function of the mitochondrial pyruvate translocator and thus prevent the utilization of pyruvate by the TCA cycle. We found that the basal level of OCR was slightly higher in cultures of anti-CD3-stimulated DUSP6^{-/-} T cells than in DUSP6^{+/+} controls, suggesting that mitochondrial respiration is more active in the mutant T cells (SI Appendix, Fig. S9A). BPTES and ETOMOXIR administered at 28 min during the OCR assay had no differential effect on OCR values in cultures of anti-CD3-stimulated DUSP6^{+/+} and DUSP6^{-/-} T cells (SI Appendix, Fig. S9A and B). However, UK5099 administered at 80 min during the assay decreased the OCR in anti-CD3-stimulated DUSP6^{-/-} T cells but not in anti-CD3-stimulated DUSP6^{+/+} T cells. These data suggest that, in the absence of energy supplied by glutamine and/or fatty acids, anti-CD3-stimulated

DUSP6^{-/-} T cells rely heavily on mitochondrial respiration driven by pyruvate as their main energy source.

To confirm this dependence on pyruvate, we treated anti-CD3-stimulated T cells with UK5099 at 28 min during the OCR assay and observed that the OCR dropped immediately in the mutant T cells but not in the controls (SI Appendix, Fig. S9C). Addition of BPTES plus ETOMOXIR at 80 min (i.e., after UK5099 treatment) further reduced the OCR in anti-CD3-stimulated DUSP6^{-/-} T cells but had no effect on control cells (SI Appendix, Fig. S9C and D). Because mitochondrial respiration is a major source of intracellular reactive oxygen species (ROS), we determined if the enhanced mitochondrial respiration in TCR-stimulated DUSP6^{-/-} T cells was associated with an increase in ROS production. As expected, DUSP6^{-/-} T cells generated more ROS than control T cells both at steady state and shortly after anti-CD3 stimulation (15 min) (SI Appendix, Fig. S9E and F). Taken together, these real-time measurement data indicate that, due to their glycolytic defect, DUSP6^{-/-} T cells rely on pyruvate derived from glutamine or fatty acids to maintain mitochondrial respiration. The mutant T cells therefore possess less energy flexibility than WT cells to adapt to culture conditions deficient in pyruvate, glutamine, or fatty acids.

DUSP6 Deficiency Increases T Cell Sensitivity to Apoptosis Induced by Glycolysis Inhibition. The impaired glycolysis in DUSP6^{-/-} T cells raised the question of whether homeostasis was affected in these cells. We used [³H]thymidine incorporation in vitro to examine the proliferation of DUSP6^{-/-} T cells stimulated for 24, 48, or 72 h with either anti-CD3, anti-CD3/CD28, anti-CD3/CD28 plus IL-2, or phorbol 12-myristate 13-acetate plus ionomycin. In all cases, DUSP6^{+/+} and DUSP6^{-/-} T cells exhibited comparable proliferation (SI Appendix, Fig. S10A and B), indicating that DUSP6 deficiency does not affect the T cell proliferation machinery. To address the effect of DUSP6 deficiency in antigen-induced CD4⁺ T cell proliferation in vivo, we injected OVA³²³⁻³³⁹ i.p. into OTII-DUSP6^{+/+} and OTII-DUSP6^{-/-} mice followed by BrdU pulse for three consecutive days at 12 h-intervals. The analysis of OVA-specific CD4⁺ T cells in G1/S, S, and G2/M phases revealed that DUSP6 deficiency did not affect antigen-specific CD4⁺ T cell proliferation in vivo (SI Appendix, Fig. S10C and D). In SI Appendix, Fig. S9C we show that DUSP6^{-/-} T cells depend on pyruvate in the culture medium to sustain their basal OCR. To determine whether DUSP6^{-/-} T cells require glutamine-derived pyruvate to bypass their glycolytic defect and support TCR-triggered proliferation, we assayed the proliferation of T cells in cultures that were stimulated with anti-CD3/CD28/IL-2 for 24 or 48 h in the presence of UK5099, BPTES, or ETOMOXIR or various combinations of these three inhibitors. We found that inhibition of glutaminase by BPTES strongly blocked T cell proliferation regardless of DUSP6 function (SI Appendix, Fig. S10E and F). In contrast, inhibition of the pyruvate translocator by UK5099 did not affect the proliferation of activated cells of either genotype, suggesting that glutaminolysis, but not mitochondrial pyruvate, is indispensable for T cell proliferation in vitro.

The sustained and higher level of p-JNK signaling promotes an apoptosis and the unresponsiveness of DUSP6^{-/-} T cells upon TCR-stimulation (57). Consistent with this finding, a slight but significant increase in spontaneous apoptosis was observed in freshly isolated CD4⁺ T cells as well as CXCR5⁺PD-1⁺ T_{FH} cells from DUSP6^{-/-} mice (SI Appendix, Fig. S10G and H). We could not observe increased apoptotic CD8⁺ T cells from DUSP6^{-/-} mice. To explore how metabolic pathways affected the survival of activated DUSP6-deficient T cells, we stimulated T cells for 24 h with anti-CD3 plus IL-2 or anti-CD3/CD28 (CD28 costimulation) plus IL-2 in the presence of either DMSO (control), 2-deoxyglucose (2-DG), UK5099, BPTES, or ETOMOXIR and determined viability by 7AAD staining. After normalization of the data to the DMSO control, we found that 2-DG-mediated

inhibition of glycolysis reduced the survival of anti-CD3-stimulated DUSP6^{+/+} T cells by 18.7% ($\pm 5.02\%$) (Fig. 7A, a). Furthermore, inhibition of either pyruvate entry into the mitochondria by UK5099, glutaminolysis by BPTES, or fatty acid degradation by ETOMOXIR decreased the viability of anti-CD3-stimulated DUSP6^{+/+} T cells by 4–8%. In contrast, 2-DG treatment of anti-CD3-stimulated DUSP6^{-/-} T cells reduced cell survival by 23.5% ($\pm 2.37\%$), whereas UK5099, BPTES, or ETOMOXIR treatment of these cells had no significant effect on their viability (Fig. 7A, b). When the cells were costimulated with anti-CD28, the inhibitory effect of 2-DG on the viability of DUSP6^{+/+} T cells was similar to that of the same cells stimulated with anti-CD3 alone, and treatment with UK5099, BPTES, or ETOMOXIR did not diminish their viability at all (Fig. 7A, c). Interestingly, however, 2-DG treatment of CD28-costimulated DUSP6^{-/-} T cells decreased their survival by 36.3% ($\pm 0.64\%$), a significantly more severe effect than observed in controls (Fig. 7A, d). Moreover, treatment of CD28-costimulated DUSP6^{-/-} T cells with UK5099, BPTES, or ETOMOXIR led to 11–18% reductions in survival. The results suggest that the limited glycolysis in TCR-stimulated DUSP6^{-/-} T cells led to higher levels of cell death. However, the survival of WT T cells that are fully activated through CD28 costimulation depends solely on glycolysis. Stimulation in WT T cells by anti-CD3 alone is not sufficient to induce metabolic commitment to glycolysis. Strikingly, activated DUSP6^{-/-} T cells with CD28 costimulation required mitochondrial pyruvate, glutaminolysis, and β -oxidation pathways to maintain cell survival. Our findings suggest that the survival of activated WT T cells through CD28 costimulation depends solely on glycolysis, while CD28 costimulated DUSP6^{-/-} T cells of glycolytic defects require alternative energy fuels to maintain viability.

We next addressed whether a complete block of glycolysis in differentiated mature DUSP6^{-/-} T_{FH} cells affected IL-21 production. We performed T_{FH} differentiation assays in vitro and on day 5 during the differentiation assay. T_{FH} cells were restimulated with anti-CD3 with DMSO or inhibitors including 2-DG, UK5099, BPTES, or ETOMOXIR for 24 h. On day 6, the levels of IL-21 in the supernatant were determined. Treatment with 2-DG or BPTES led to a significant reduction of IL-21 produced by DUSP6^{-/-} T_{FH} cells to control levels (Fig. 7B, Left). Remarkably, DUSP6^{-/-} T cells treated with ETOMOXIR showed profound suppression of IL-21 production. These data suggest that the aberrantly increased IL-21 production by DUSP6^{-/-} T_{FH} cells mostly relies on lipolysis as the energetic source. By contrast, none of the treatments affected IL-21 production by mature WT T_{FH} cells, indicating a metabolic flexibility for IL-21 production by T_{FH} cells with DUSP6 expression. Meanwhile, the in vitro-differentiated DUSP6^{-/-} T_{FH} cells produced more IFN- γ than controls. Treatment with 2-DG drastically inhibited IFN- γ production close to background levels in WT and DUSP6^{-/-} T_{FH} cells (Fig. 7B, Right), which was consistent with earlier reports describing the indispensable role of glycolysis for IFN- γ production (36, 58, 59). In contrast, treatment in DUSP6^{-/-} T_{FH} cells with UK5099, BPTES, or ETOMOXIR only reduced IFN- γ production to control levels, while none of the treatments influenced the level of IFN- γ produced by WT T_{FH} cells. The results suggest that mature WT T_{FH} cells do not have energetic preference of certain metabolic pathway to produce IL-21, whereas inhibition of fatty acid β -oxidation by ETOMOXIR particularly suppresses IL-21 production by DUSP6^{-/-} T_{FH} cells.

Taken together, the results show that DUSP6 deficiency leads to a partial defect in glycolysis that is associated with reduced survival upon TCR/CD28 costimulation.

Discussion

It is well-documented that strong TCR-mediated JNK or p38 signaling favors a T_H1-biased immune response and IFN- γ production (60, 61). However, whether this also drives T_{FH} cell

Fig. 7. Impaired homeostasis of DUSP6^{-/-} T cells. (A) Statistical analysis of viable cells (7AAD⁻) in cultures of CD4⁺ T cells that were stimulated for 24 h with anti-CD3 plus IL-2 (anti-CD3/IL-2) or anti-CD3/CD28/IL-2 with or without treatment with 2-DG, UK5099, BPTES, or ETOMOXIR, as indicated. Viability values were normalized to the DMSO control. Data are the mean $\pm$ SD ($n = 2$ per treatment). (B) ELISAs to detect IL-21 and IFN- γ in culture supernatants of in vitro-generated T_{FH} cells, which were restimulated with anti-CD3 in the absence/presence of 2-DG, UK5099, BPTES, and ETOMOXIR on day 5 for 24 h. Data are the mean $\pm$ SEM ($n = 6$ per group). * $P < 0.05$ (Student's t test); ** $P < 0.005$; ns, nonsignificant.

generation has never been reported. DUSP6 has been shown to regulate MAPK activities, including those of ERK, JNK, and p38 (10, 62, 63). In our model system, we find that DUSP6 deficiency mainly affects the phosphorylation of p38 and JNK in peripheral T cells. Consistent with this observation, treatment of Jurkat cells with the DUSP6 inhibitor BCI induces greater phosphorylation of JNK and p38. Moreover, the increased IL-21 production by DUSP6^{-/-} T cells induced to undergo T_{FH} differentiation in vitro is partially blocked by treatment with the JNK inhibitor SP600125 or the p38 inhibitor SB203580. These results suggest that the TCR-JNK/p38-IL-21 axis contributes to the heightened ability of DUSP6^{-/-} T cells to generate T_{FH} effectors.

The increased p-JNK/p38 signaling in DUSP6^{-/-} T cells most likely affects the basal activities of several transcription factors, including AP-1 and NFAT. A recent study has revealed that NFAT promotes T_{FH} differentiation (64), making it plausible that the vigorous TCR-mediated JNK/p38 signaling in DUSP6^{-/-} T cells drives the formation of the NFAT/AP-1 complex and thus activation of the T_{FH} differentiation program. The adoptive transfer experiment reveals that the increased T_{FH} cells in unimmunized DUSP6^{-/-} mice exit in the spleens, LNs, and Peyer's patches. Noteworthy, T_{FH} pools in the Peyer's patches are diminished in WT and DUSP6^{-/-} mice in response to TNP-KLH-CFA by i.p. injection, suggesting that the antigen character, including the glycosylation, the entry route, or the antigen uptake are not sufficient to induce T_{FH} cells in the Peyer's patches (65). Despite that, the T_{FH} subset remains greater in the LNs and Peyer's patches of DUSP6^{-/-} $\rightarrow$ RAG1/2-KO recipient mice postimmunization with TNP-KLH-CFA. Most importantly, DUSP6 deficiency promotes the differentiation of T_{FH} cells with T_H1 features in the presence of IL-2. Supportive of our findings, a low concentration of IL-2 has been reported to induce Bcl6 mRNA in T_H1 effector cells (43). The T_{FH} cells in DUSP6^{-/-} mice characterized by CXCR5⁺, ICOS⁺, and PD-1⁺ with Bcl6 expression support the production of an antigen-specific IgG2 response upon

immunization in vivo. Of in vitro level, DUSP6^{-/-} T_{FH} cells are more potent than WT T_{FH} cells to coexpress IL-21 and IFN- γ . The findings suggest that DUSP6 deficiency favors the induction of T_{FH} cells and GC response with T_H1 features and imply that targeting DUSP6 would be beneficial for the induction of a protective antiviral or antitumor adaptive immunity.

DUSP6 promotes hepatic gluconeogenesis and influences glucose output by liver cells (6, 7). In our study, we have shown that the glycolysis defect associated with DUSP6 deficiency is a T cell-specific phenomenon that is not observed in B cells, DCs, or macrophages. Our finding that the increased IL-21 production and T_{FH} differentiation of activated DUSP6-deficient T cells is associated with a profound block in glycolysis is unexpected. This impairment of glycolysis is not due to the elevated TCR-mediated JNK/p38 signaling in activated DUSP6^{-/-} T cells because treatment with JNK or p38 inhibitors did not restore glycolysis as reflected by ECAR measurement. It has been reported that TCR-mediated ERK and p38 signaling are necessary for the expression of HK and induction of its activity (34). Strikingly, however, we find that while HK activity is modestly reduced in anti-CD3-stimulated DUSP6^{-/-} T cells, PFK activity is severely decreased in these cells. Phosphorylation of PFK on its serine, threonine, or tyrosine residues by diverse protein kinases influences PFK enzymatic activity, presumably by altering the allosteric affinity and intracellular localization of the protein (66, 67). In addition to a potential direct effect on PFK, it is highly possible that DUSP6 is implicated in the regulation of intracellular citrate balance in activated T cells. Thus, loss of DUSP6 leads to an increase of citrate level, resulting in the impaired PFK activation. It remains mysterious why DUSP6 deficiency specifically affects TCR-induced glycolysis, not BCR-induced glycolysis. It is possible that an unknown DUSP family member plays a redundant role to regulate B cell glycolysis, as the role of DUSP6 in T cells. In addition, compared with T cells, the metabolic reprogramming from naive to activated B cells is obscure, as activated B cells through LPS or BCR engage both glycolysis and oxidative phosphorylation without a metabolic preference (68, 69). Thereby, BCR signaling differs from TCR signaling in delivering signals for the metabolic reprogramming, as activated B cells do not commit to glycolysis and do not develop the regulatory loop to adjust the oxidative phosphorylation activity. Furthermore, the comparable TCR-mediated Akt phosphorylation on S473 and the increase of mTOR phosphorylation on S2448 by DUSP6 deficiency suggest that DUSP6 is implicated in TCR signaling between Akt and mTORC1 activation. It is possible that the stronger TCR-mediated JNK/p38-signaling by DUSP6 deficiency exerts a cross-talk effect on Akt-mTOR, which impacts T cell metabolism and effector T cell differentiation. Whether DUSP6 directly dephosphorylates PFK or it targets substrates involved in citrate balance or mTORC1 pathway in primary T cells requires further investigation.

To overcome the glycolytic defect, DUSP6-deficient T cells utilize pyruvates derived from glucose-independent energetic fuels, including glutamine or fatty acids, to maintain mitochondrial respiration. For WT T cells, only combined stimulation by anti-CD3 plus anti-CD28 is sufficient to drive the metabolic switch to glycolysis and dependence on this pathway for survival. It is noteworthy that anti-CD3 stimulation alone of WT T cells does not result in their reliance on their limited capacity for glycolysis for survival. In contrast, the mutant T cells with CD28 costimulation turn to engage mitochondrial pyruvate, glutaminolysis, and β -oxidation to maintain survival. These data suggest that the glycolytic defect induced by DUSP6 deficiency reduces T cell survival and inhibits metabolic reprogramming during T cell activation.

We have also shown that engagement of the glutaminolysis pathway, but not utilization of mitochondrial pyruvate, is indispensable for T cell proliferation in vitro. This might explain why mutant T cells use glutamine as energy fuels to fulfill proliferation

demands. It implies that, even in the absence of DUSP6, the glutaminolysis pathway and residual HK activity can support sufficient T cell proliferation to allow T_{FH} cell generation.

It is well-established that, during the clonal contraction stage of a T cell response, some remaining effector T cells differentiate into memory T cells. How TCR signaling modulates metabolic reprogramming at this final stage is unknown. Our study suggests that down-regulation of DUSP6 might be one mechanism by which TCR-mediated glycolysis might be switched off, promoting clonal contraction and setting the stage for memory T cell generation. Interestingly, IL-21 production by the in vitro-differentiated DUSP6-deficient T_{FH} cells is slightly reduced by glycolysis or glutaminolysis inhibition. However, blocking of fatty acid oxidation drastically reduces IL-21 level, suggesting that DUSP6-deficient T_{FH} cells rely on fatty acid oxidation more than other metabolic pathways for the effector function. It requires further investigation to address whether DUSP6-deficient T_{FH} cells are prone to differentiate to memory T_{FH} cells through the stronger metabolic dependence on lipolysis.

In summary, our study sheds light on the molecular mechanisms by which DUSP6 restrains the TCR-JNK/p38-IL-21 pathway leading to T_{FH} differentiation, and how DUSP6 connects TCR signaling to metabolic switching to glycolysis in fully activated T cells. DUSP6 thus emerges as a player whose regulation appears to fine-tune aspects of TCR signaling that control T_{FH} differentiation and metabolic reprogramming in activated T cells.

Materials and Methods

DUSP6^{-/-} mice generated as previously described were purchased from the Jackson Laboratory (stock no. 009069) and maintained in the Laboratory Animal Center of the National Health Research Institutes (NHRI) (5). The OTII-mice (stock no. 004194, OVA³²³⁻³³⁹ peptide-specific CD4⁺ TCR transgenic; Jackson Laboratory) used in all experiments were heterozygous for the transgenes. Age-matched (8–12 wk old) DUSP6^{+/+} or OTII-DUSP6^{+/+} littermate mice were used as controls in the appropriate experiments. The number of mice used in each experiment is specified in each figure legend. All animal studies were reviewed and approved by NHRI's Institutional Animal Care and Use Committee (permit nos. 099111-A, 102006, and 103086-A). Every effort was made to minimize mouse suffering, and killing of mice was performed by carbon dioxide inhalation. To study T_{FH} cell differentiation in vitro, purified T cells were stimulated with plate-bound anti-CD3, soluble anti-CD28, and IL-2 for 48 h. On day 2, the mixture including IL-2, anti-IFN- γ , anti-IL-4, and anti-TGF- β were added to cell culture to induce T_H0 differentiation. To induce T_{FH} differentiation, IL-21 was added to cell culture in addition to the mixture for T_H0 differentiation on day 2. To determine ECAR, purified T cells were stimulated with anti-CD3 plus anti-CD28 for 20 h, seeded in glucose-free DMEM into XF24 cell culture microplates, and incubated at 37 °C without CO₂ for 1 h before ECAR assay. The definition of non-glycolytic acidification (equal to the ECAR after 2-DG treatment), glycolysis (equal to the ECAR after addition of glucose minus ECAR after 2-DG treatment), glycolytic capacity (equal to the ECAR after oligomycin treatment minus ECAR after 2-DG treatment), and glycolytic reserve (equal to the ECAR after oligomycin treatment minus ECAR after addition of glucose) was as described previously (70).

Methods for T cell purification and cell culture; T_{FH} cell differentiation in vitro details; BMs and BMDCs; T_H1-, T_H2-, T_H17-, and Treg-cell differentiation; flow cytometry (FACS); immunoblotting and BCI treatment; immune response, adoptive transfer, ELISA, RNA-seq, and bioinformatic analyses; HK, PFK, and PMK enzymatic activities; ECAR and OCR assay details; determination of ADP/ATP and intracellular metabolites; ROS detection; and statistical analysis are described in *SI Appendix, Supplementary Material and Methods*.

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