Estrogen-related receptor-α is a metabolic regulator of effector T-cell activation and differentiation

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Stimulation of resting CD4$^+$ T lymphocytes leads to rapid proliferation and differentiation into effector (Teff) or inducible regulatory (Treg) subsets with specific functions to promote or suppress immunity. Importantly, Teff and Treg use distinct metabolic programs to support subset specification, survival, and function. Here, we describe that the orphan nuclear receptor estrogen-related receptor-α (ERRα) regulates metabolic pathways critical for Teff. Resting CD4$^+$ T cells expressed low levels of ERRα protein that increased on activation. ERRα deficiency reduced activated T-cell numbers in vivo and cytokine production in vitro but did not seem to modulate immunity through inhibition of activating signals or viability. Rather, ERRα broadly affected metabolic gene expression and glucose metabolism essential for Teff. In particular, up-regulation of Glut1 protein, glucose uptake, and mitochondrial processes were suppressed in activated ERRα$^{-/-}$ T cells and T cells treated with two chemically independent ERRα inhibitors or by shRNAi. Acute ERRα inhibition also blocked T-cell growth and proliferation. This defect appeared as a result of inadequate glucose metabolism, because provision of lipids, but not increased glucose uptake or pyruvate, rescued ATP levels and cell division. Additionally, we have shown that Treg requires lipid oxidation, whereas Teff uses glucose metabolism, and lipid addition selectively restored Treg—but not Teff—generation after acute ERRα inhibition. Furthermore, in vivo inhibition of ERRα reduced T-cell proliferation and Teff generation in both immunization and experimental autoimmune encephalomyelitis models. Thus, ERRα is a selective transcriptional regulator of Teff metabolism that may provide a metabolic means to modulate immunity.

glycolysis | fatty acid | oxidative metabolism | mammalian target of rapamycin | AMPK

Activated CD4$^+$ T cells initiate a program of gene expression that results in the proliferation and generation of effector T-cell subsets (Teff) to promote protective immunity or regulatory T cells (Treg) to suppress inappropriate inflammation (1). These processes require energy expenditure and biosynthesis (2, 3) and T-cell stimulation triggers a rapid increase in cellular metabolism to support activation and differentiation pathways (4, 5). We have shown that specific metabolic demands differ between CD4$^+$ T-cell subsets, as Teff cells (Th1, Th2, and Th17) are dependent upon glycolysis, while Tregs use and require lipid oxidation (6). Contributing to these metabolic phenotypes, the phosphatidyl-inositol 3-kinase (PI3K)/Akt mammalian target of rapamycin (mTOR) and hypoxia-inducible factor 1α (HIF1α) signaling pathways promote glycolysis and cell-surface trafficking of the glucose transporter Glut1, while diminishing lipid oxidation (7–9). Conversely, AMPK suppresses mTOR activity and promotes lipid oxidation (6, 10). The balance between mTOR and AMPK signaling has a proven role in Teff and Treg lineage commitment, with mTOR and HIF1α promoting Teff and AMPK promoting Treg (6, 11–14). Importantly, the mechanisms that coordinate T-cell metabolism of Teff and Treg remain poorly understood.

As activated Teff and cancer cells use a similar metabolic program (4, 15), we hypothesized that these cell types share a common transcriptional regulation of metabolism. The orphan nuclear hormone receptor, estrogen-related receptor-α (ERRα), is associated with poor prognosis (16, 17) and tumor growth in breast cancer (18, 19). ERRα is also known to regulate cell metabolism (20, 21) and *Drosophila* ERR can promote gene expression to drive carbohydrate metabolism associated with proliferating cells in larval development (22). In immunity, ERRα$^{-/-}$ mice exhibit increased susceptibility to *Listeria monocytogenes* infection (23). Although not previously investigated in lymphocytes, these findings suggest ERRα may regulate genes that contribute to lymphocyte metabolism and activation (20, 21).

Metabolic control of T-cell function may provide a novel approach to modulate immune responses and here we describe a key role for ERRα in CD4$^+$ T-cell metabolism and Teff fate. ERRα was induced upon T-cell activation and facilitated gene expression and glucose and mitochondrial metabolism required for Teff growth and proliferation. Importantly, both acute and chronic loss of ERRα decreased Teff metabolism and function and reduced morbidity in experimental autoimmune encephalomyelitis (EAE). Treg, however, were only modestly impacted by ERRα inhibition when provided lipids as an alternate fuel. These results demonstrate that Teff CD4$^+$ T-cell subsets selectively use ERRα as a global metabolic regulator to support specification and function.

Results

ERRα Contributes to T-Cell Homeostasis. Because ERRα regulates the transcription of a broad array of metabolic genes (20, 21), we examined ERRα expression in CD4$^+$ T lymphocyte metabolism and function. Resting CD4$^+$ T cells expressed ERRα, and although ERRα mRNA expression modestly decreased in CD4$^+$ T cells after 1 d of stimulation (Fig. S1*A*), ERRα protein expression significantly increased in a CD28 costimulation-dependent manner (Fig. 1*A* and Fig. S1 *B–D*). Subsequently, *Esrra*-null (ERRα$^{-/-}$) mutant mice, which are lean and exhibit systemic metabolic alterations (23, 24), were examined to assess the

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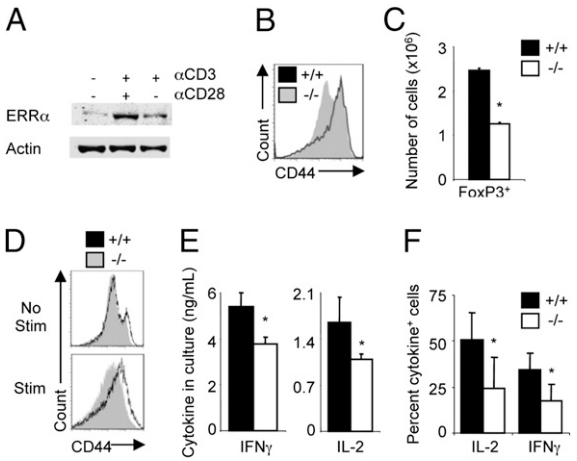

Fig. 1. ERRα expression regulates CD4$^+$ T-cell homeostasis and function. (*A*) Immunoblot of resting or 1-d anti-CD3+/– anti-CD28–stimulated WT CD4$^+$ T cells. (*B* and *C*) Representative histogram and quantitation of CD4$^+$ CD44^{high} and CD4$^+$ Foxp3$^+$ T cells in 1-y-old sex-matched ERRα$^{-/-}$ (–/–) and WT (+/+) littermates. (*D*) CD44 expression was measured flow cytometrically in 6- to 8-wk-old ERRα$^{-/-}$ and WT resting and stimulated CD4$^+$ T cells at 24 h. (*E*) Cytokine levels were measured by ELISA after 3 d of stimulation. (*F*) Frequency by FACS analysis of cytokine expressing CD4$^+$ T cells from ERRα$^{-/-}$ and WT mice after 3 d stimulation and acute restimulation. Results are representative of a minimum of three independent experiments, and graphs are displayed as the average and SD. *$P \leq 0.05$.

contribution of ERRα to T-cell homeostasis. Although young ERRα$^{-/-}$ mice had normal T-cell numbers and phenotypes, aged ERRα$^{-/-}$ mice failed to accumulate effector/memory phenotype T cells compared with littermate controls. Instead, T cells in 1-y-old ERRα$^{-/-}$ mice remained phenotypically naive, with decreased numbers and frequency of CD4$^+$ and CD8$^+$CD44^{high} effector/memory T cells and CD4$^+$ FoxP3$^+$ Treg cells (Fig. 1 *B* and *C* and Fig. S2 *A*–*C*). Additionally, the number and percentage of ERRα$^{-/-}$ T cells that expressed the activation markers CD25 and CD69 was unchanged or decreased compared with WT controls (Fig. S2 *D* and *E*). Consistent with reduced activation and differentiation of ERRα$^{-/-}$ T cells, stimulation of CD4$^+$ T cells isolated from naive 6- to 8-wk-old ERRα$^{-/-}$ mice resulted in decreased CD44 expression after 24 h (Fig. 1*D*) and reduced production of IL-2 and IFN-γ (Fig. 1 *E* and *F*) compared with WT mice.

ERRα Broadly Regulates Glucose and Mitochondrial Metabolism. ERRα associates with the coactivators peroxisome proliferator-activated receptor-γ coactivator-1α/β (PCG1α/β) to regulate multiple metabolic pathways (20, 21) and may link lymphocyte metabolism and function. As CD4$^+$ T cells were also found to express PCG-1β (Fig. S3*A*), gene expression was analyzed by microarray in resting and stimulated ERRα$^{-/-}$ or WT CD4$^+$ T cells after acute ERRα inhibition by the ERRα inverse agonist XCT790, which promotes acute ERRα degradation (25), or the chemically distinct ERRα antagonist compound A (18) (Fig. S3*B*). As predicted based on studies of ERRα function in other tissues (20, 21), gene ontology analysis identified genes broadly involved in metabolic processes (Fig. S3 *C* and *D*).

To examine metabolic pathways specifically, quantitative RT-PCR arrays were examined for mitochondrial and glucose metabolic gene expression in CD4$^+$ T cells stimulated in the presence or absence of XCT790 (Fig. 2 *A* and *B*). Importantly, ERRα inhibition led to a broad decrease in electron transport genes and altered expression of numerous genes involved in glucose metabolism (Fig. 2*A*). In particular, dihydrolipoamide S-acetyltransferase (DLAT), a component of the pyruvate dehydrogenase

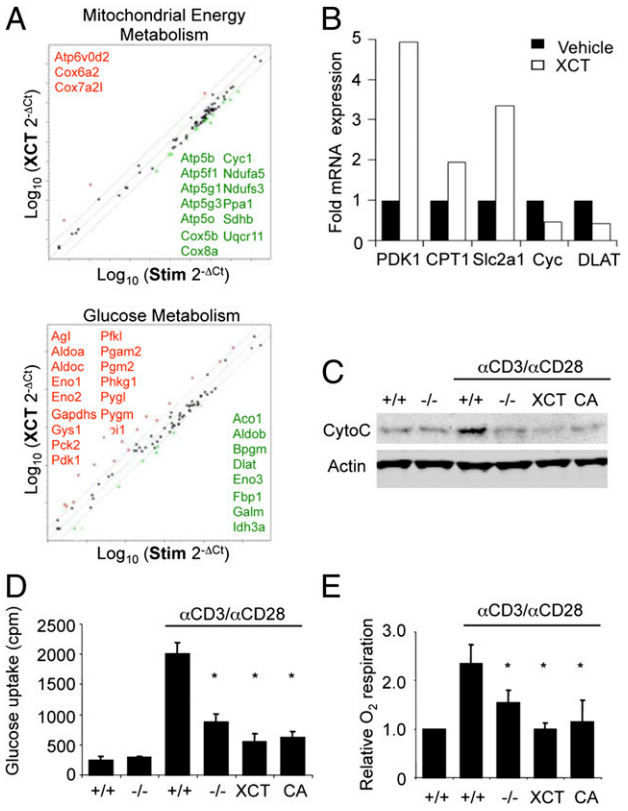

Fig. 2. ERRα contributes to activation-induced changes in CD4$^+$ T-cell metabolism. (*A* and *B*) Gene expression analyses were performed on WT CD4$^+$ T cells stimulated for 12 h in the presence or absence of XCT790. (*A*) Quantitative RT-PCR of mitochondrial energy metabolism and glucose metabolism genes. Probes exhibiting a greater than twofold change compared with stimulated T cells are indicated as increased (red) or decreased (green) with XCT790 treatment. (*B*) Representative graph of genes of interest confirmed by independent quantitative RT-PCR analysis. (*C*–*E*) WT and ERRα$^{-/-}$ CD4$^+$ T cells were examined ex vivo or activated for 24 h, and (*C*) cytochrome *c* expression, (*D*) glucose uptake, and (*E*) O_2 respiration were assessed in the presence or absence of XCT790 (XCT) or compound A (CA). Results are representative of a minimum of three independent experiments, and graphs are displayed as the average and SD. *$P \leq 0.05$.

(PDH) complex, was decreased and pyruvate dehydrogenase kinase 1 (PDK1), which inhibits PDH, was increased with XCT790 treatment. Together, this action would decrease pyruvate flux into acetyl-CoA and the tricarboxylic acid (TCA) cycle, even though the expression of several glycolytic genes, including mRNA for enolase 1 (*Eno1*) and Glut1 (*Slc2a1*), were increased relative to stimulated control cells (Fig. 2 *A* and *B*). In addition, cytochrome *c* (*Cyc1*) was reduced at both the mRNA and protein levels by ERRα inhibition (Fig. 2 *A*–*C*) and carnitine palmitoyltransferase 1a (CPT1a) expression was increased with XCT790 treatment. These data suggested reduced pyruvate flux and electron transport although mitochondrial lipid uptake for β-oxidation may increase (Fig. 2 *A* and *B*).

Metabolic pathways were next directly measured in ERRα$^{-/-}$ and WT CD4$^+$ T cells treated with XCT790 or compound A. Although WT and ERRα$^{-/-}$ resting T cells had similar levels of Glut1 expression and glucose uptake before stimulation, and T cells treated with ERRα inhibitor had elevated Glut1 mRNA, ERRα-deficient T cells failed to up-regulate glucose uptake and Glut1 protein expression after 1 d of stimulation compared with WT cells (Fig. 2*D* and Fig. S4 *A* and *B*). Furthermore, although hexokinase 2 (HK2) mRNA was not significantly changed after ERRα inhibition, HK2 protein was reduced in XCT790-treated

cells (Fig. S4*C*) and hexokinase activity failed to increase in stimulated ERRα-deficient CD4$^+$ T cells (Fig. S4*D*). Similar results were obtained in a growth factor-stimulated lymphoid cell line transfected with ERRα shRNAi (Fig. S4 *E* and *F*). In addition, glucose oxidation through mitochondrial and pentose phosphate pathways, glucose-dependent lipid synthesis, mitochondrial membrane potential, and respiration were all reduced in stimulated ERRα-deficient T cells (Fig. 2*E* and Fig. S4 *G–I*). Importantly, ERRα inhibition did not appear to alter initial antigen receptor or costimulatory signals, or decrease cell viability as receptor proximal signals (Fig. S5*A*) and viability (Fig. S5*B*) in T-cell activation were not detectably affected. Thus, genetic loss, two chemically independent inhibitors, and shRNAi of ERRα each led to widespread failure of stimulated lymphocytes to increase glucose and mitochondrial metabolism.

Acute ERRα Inhibition Results in Growth and Proliferation Defects. The broad array of metabolic pathways altered in the absence of ERRα suggested a role in T-cell growth and proliferation. Therefore, ERRα$^{-/-}$ and WT CD4$^+$ T cells were stimulated in the presence or absence of XCT790 or compound A to examine lymphocyte activation. Similar to reduced expression of CD44 in ERRα$^{-/-}$ cells, acute ERRα-deficiency inhibited maximal upregulation of CD44 and CD25 at 24 h poststimulation (Fig. 3*A*). By 48 h postactivation, CD44 expression was equivalent (Fig. S5*C*). Consistent with normal proximal signaling events (Fig. S5*A*), however, induction of the early activation marker CD69 was not significantly changed by ERRα inhibition (Fig. 3*A* and Fig. S5*C*). Additionally, although vehicle-treated T cells increased twofold in size after 1 d of stimulation, WT T cells failed to efficiently grow when activated in the presence of XCT790 or compound A (Fig. S5 *B* and *D*).

In contrast and despite reduced cytokine production, decreased numbers of effector/memory T cells (Fig. 1), and multiple metabolic defects (Fig. 2), ERRα$^{-/-}$ CD4$^+$ T cells increased in cell size with sustained in vitro T-cell receptor (TCR) and costimulatory signals (Fig. S5 *B* and *D*). It was possible that acute and chronic ERRα-deficiency differed in T-cell responses because of developmental compensation for chronic ERRα-deficiency or because XCT790 and compound A acted independently of ERRα. However, neither XCT790 nor compound A altered the growth of ERRα$^{-/-}$ T cells (Fig. S5 *B* and *D*), suggesting a target-dependent action of these compounds and potential compensation with chronic developmental ERRα deficiency.

Similar to regulation of cell growth, assessment of cell proliferation by carboxyfluorescein succinimidyl ester (CFSE; a fluorescent dye that is diluted upon cell division) revealed that acute inhibition of ERRα strongly suppressed CD4$^+$ T-cell proliferation (Fig. 3*B*). This decrease in T-cell proliferation correlated directly with the degradation or inhibition of ERRα expression (Figs. S3*B* and S6*A*). Although the cell-cycle inhibitor p27 was degraded in T cells activated in the presence of XCT790, both cyclin D1 upregulation and DNA synthesis were prevented (Fig. S6 *B* and *C*). The failure of activated T cells to grow and divide when treated with ERRα inhibitors could have been the result of inadequate metabolism or transcriptional suppression of the T-cell activation program. Decreased IL-2 production, however, did not account for inhibition of T-cell proliferation, as the addition of recombinant IL-2 did not rescue proliferation of activated T cells treated with ERRα inhibitors (Fig. 3*B*). Similarly, stimulated T cells with transgenic expression of Glut1 (2) also failed to divide when treated with ERRα inhibitors, demonstrating that metabolic defects downstream of glucose uptake contribute to the inhibition of T-cell proliferation in the acute absence of ERRα.

In contrast, ERRα$^{-/-}$ CD4$^+$ T cells were able to proliferate under sustained in vitro TCR and costimulatory signals (Fig. 3*B*). Indeed, ERRα$^{-/-}$ CD4$^+$ T cells also increased glycolytic rate and cellular ATP levels upon in vitro stimulation (Fig. 3*C* and Fig.

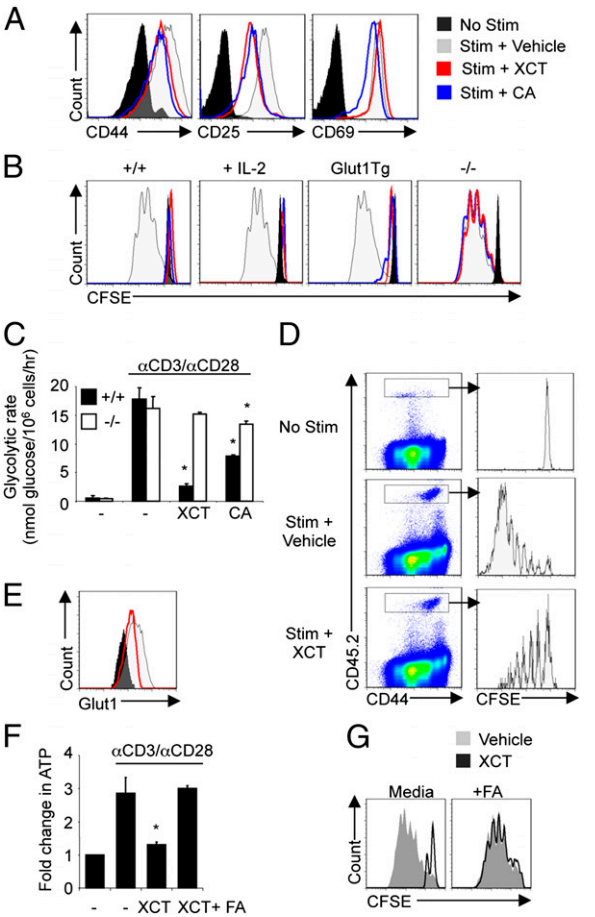

Fig. 3. Reduced CD4$^+$ T-cell growth and proliferation after acute ERRα inhibition. (*A*) Flow cytometric analyses of CD4$^+$ T cells stimulated in the presence or absence of XCT790 or compound A for 24 h. (*B*) Cell proliferation was assessed by CFSE fluorescence in WT, Glut1-transgenic, or ERRα$^{-/-}$ CD4$^+$ T cells stimulated for 72 h in the presence or absence of XCT790 (XCT) or compound A (CA) in the presence or absence of IL-2 (10 ng/mL). (*C*) WT and ERRα$^{-/-}$ CD4$^+$ T cells were stimulated for 1 d in the presence or absence of XCT790 (XCT) or compound A (CA) and assessed for glycolytic flux. (*D* and *E*) Ovalbumin (OVA) -specific CD45.2$^+$ OTII$^+$ T cells were CFSE-labeled and adoptively transferred into congenic CD45.1$^+$ recipient mice that were not immunized (No Stim) or immunized (Stim) with OVA and treated with vehicle or XCT790. Splenocytes were analyzed by flow cytometry on day 3. (*F* and *G*) WT CD4$^+$ T cells were stimulated with XCT790 and lipids as indicated. (*F*) ATP levels were measured after 1 d, and (*G*) proliferation by CFSE dilution was measured by flow cytometry after 3 d. Results are representative of two or more independent experiments, and graphs displayed average and SDs. *$P \leq 0.05$.

S7*A*). Importantly, neither XCT790 nor compound A altered the growth, proliferation, glycolysis, or steady-state ATP levels of ERRα$^{-/-}$ T cells (Fig. 3 *B* and *C*, and Figs. S5 *B* and *D* and S7*A*), suggesting that the observed effects of these drugs were ERRα-specific. It was possible that developmental compensation in ERRα$^{-/-}$ T cells selectively rescued some metabolic and functional components of T-cell activation. Indeed, although the ERRα-related isoforms ERRβ and ERRγ were undetectable, ERRα$^{-/-}$ CD4$^+$ T cells exhibited alterations in the mTOR and AMPK pathways. The downstream mTOR substrate S6 had increased phosphorylation and TGF-β failed to increase phospho-AMPK in ERRα$^{-/-}$ CD4$^+$ T cells (Fig. S7*B*). This imbalance in mTOR activation correlated with diminished TGF-β–dependent Treg generation in ERRα$^{-/-}$ T cells that could be rescued by rapamycin (Fig. S7*C*). In support of the theory that elevated

mTOR activity can render CD4$^+$ T cells independent of ERRα, TSC2$^{-/-}$ T cells that have constitutive mTOR activity were highly glycolytic and could proliferate even in the presence of ERRα inhibitors (Fig. S7 *D* and *E*). Taken together, these data demonstrate an ERRα-based mechanism for XCT790 and compound A on the metabolism and proliferation of CD4$^+$ T cells and suggest that chronic loss of ERRα led to partial compensation through the mTOR and AMPK pathways.

We next sought to determine if acute pharmacologic inhibition of ERRα could alter T-cell metabolism and proliferation in vivo. CD45.2$^+$ marked OT-II ovalbumin-specific CD4$^+$ T cells were CFSE-labeled and adoptively transferred into CD45.1$^+$ WT recipients followed by ovalbumin immunization and administration of vehicle or XCT790. Three days of treatment with XCT790 reduced ERRα expression in CD4$^+$ T cells in vivo (Fig. S8*A*) and diminished Glut1 up-regulation on antigen-specific CD4$^+$ T cells compared with vehicle-treated counterparts (Fig. 3*D* and Fig. S8*B*), suggesting lower levels of glucose metabolism. In addition, although T cells from both treated and untreated mice up-regulated CD44 after 3 d, T-cell proliferation was significantly decreased in mice treated with XCT790 (Fig. 3*E*).

Acute ERRα Deficiency Can Be Rescued by Lipids. The disruption of T-cell proliferation and function upon acute ERRα inhibition may have been because of suppression of the cell cycle or failure to up-regulate metabolic processes necessary to support cell growth. To directly test if cell metabolism was insufficient for growth, exogenous nutrients were provided to determine if they could rescue T-cell activation defects upon acute ERRα inhibition. Consistent with decreased DLAT and increased PDK1 expression leading to reduced mitochondrial capacity to metabolize pyruvate (Fig. 2 *A* and *B*), addition of methyl-pyruvate (MePyr), a cell-permeable form of the glycolytic end-product pyruvate, failed to restore division of CD4$^+$ T cells stimulated in the presence of XCT790 (Fig. S9*A*).

Increased expression of CPT1a (Fig. 2*B*) suggested that ERRα inhibition may promote lipid metabolism. In agreement with this theory, mass spectrometry-based metabolomic analyses of ERRα-deficient T cells showed elevated levels of long-chain acyl-carnitines indicative of CPT1a conjugation of fatty acids to support mitochondrial oxidation (Fig. S9*B*) (26). A fatty-acid mixture of 1:1 oleate and palmitate (FA) was therefore added to cultures in the presence of XCT790 to determine if the provision of excess lipids for mitochondrial metabolism could overcome ERRα inhibition to support cell growth and proliferation. Surprisingly, FA addition rescued mitochondrial membrane potential, ATP levels, and proliferation in XCT790-treated CD4$^+$ T cells (Fig. 3 *F* and *G* and Fig. S9*C*). These effects did not result from FA-mediated inactivation of XCT790, as treatment led to ERRα degradation, regardless of lipid addition (Fig. S9*D*). Instead, the ability of FA to rescue mitochondrial metabolism and proliferation of ERRα-inhibitor–treated CD4$^+$ T cells was dependent on lipid oxidation as the CPT1 inhibitor, etomoxir, could suppress FA-mediated proliferation (Fig. S9*E*). Thus, failure of T-cell proliferation upon acute ERRα inhibition occurs because of insufficient mitochondrial metabolism that lipids, but not pyruvate, can rescue.

ERRα Is Essential for Teff Metabolism. In addition to growth and proliferation, increased T-cell metabolism is critical for the differentiation of Teff and Treg cells, with Teff preferentially using glycolytic metabolism and Treg using lipid oxidation (6). To determine the contribution of ERRα to these T-cell fates, naive T cells were polarized in vitro toward Th1, Th2, and Th17 Teff subtypes or Tregs in optimized recombinant cytokine conditions in the presence of XCT790. XCT790 treatment on its own significantly diminished all CD4$^+$ differentiation (Fig. 4*A*), likely because of the inability to proliferate and perform essential epigenetic modifications (27). Although lipid addition rescued T-cell proliferation in initial activation (Fig. 3*G*), Teff cells were unable to use lipids to optimally restore cell division or effector function (Fig. 4 *A* and *B*), and only FoxP3$^+$ Treg were efficiently generated by lipid rescue of cell proliferation upon ERRα inhibition. ERRα was also required to sustain function of established Teff, as treatment of differentiated WT but not ERRα$^{-/-}$ T helper cells with XCT790 resulted in decreased percentage of Teff but not FoxP3$^+$ Treg cells (Fig. 4*C* and Fig. S10*A*). Taken together, these results support an acute role for ERRα to facilitate metabolic changes selectively necessary for proliferation and the generation and maintenance of Teff cells.

Reduced Severity of EAE After ERRα-Deficiency. To determine if ERRα regulates in vivo differentiation and expansion of Teff and Treg cells, ERRα$^{-/-}$ and WT mice administered vehicle or XCT790 were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide to induce EAE. Consistent with a role for ERRα in Teff generation and maintenance, the percentage (Fig. 5 *A* and *B*) and number (Fig. S10*B*) of MOG-specific IL-17– and IFN-γ–producing CD4$^+$ T cells was decreased in the lymph nodes of ERRα$^{-/-}$ and XCT790-treated mice compared with vehicle-treated WT mice. In contrast, the percentage and number of Treg was similar between all conditions (Fig. 5*C* and Fig. S10*C*). Ultimately, these differences in Teff between the conditions were reflected in the EAE disease severity (Fig. 5*D*). Although ERRα$^{-/-}$ animals did reach a similar maximal clinical score, this was not sustained, suggesting a mild EAE response characterized by reduced generation of Th17 cells (Fig. 5 *A* and *B*). Furthermore, XCT790-treated and ERRα$^{-/-}$ mice exhibited significantly lower mean clinical scores compared with WT mice throughout the course of the EAE response ($P < 0.01$). Thus,

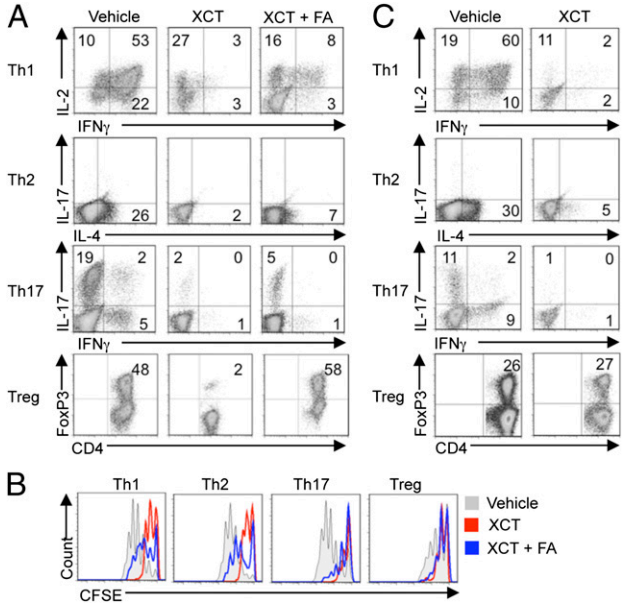

Fig. 4. Exogenous lipids rescue Treg but not Teff differentiation in the absence of ERRα. (*A* and *B*) Teff (Th1, Th2, Th17) and Treg cells were generated in vitro from WT CD4$^+$ T cells in the presence or absence of XCT790 (XCT) and in the presence or absence of fatty acid (FA) on day 0. (*A*) After 5 d, T-cell lineages were assayed by intracellular flow cytometry for cytokine production. (*B*) CFSE-labeled CD4$^+$ T cells were differentiated in the presence or absence of XCT790 or FA and assessed by flow cytometry 3 d post-stimulation. (*C*) On day 5 after T-cell lineages were established, XCT790 was added to cultures, and cytokine production was assessed after an additional 48 h by intracellular flow cytometry for cytokine production. Results are representative of three independent experiments.

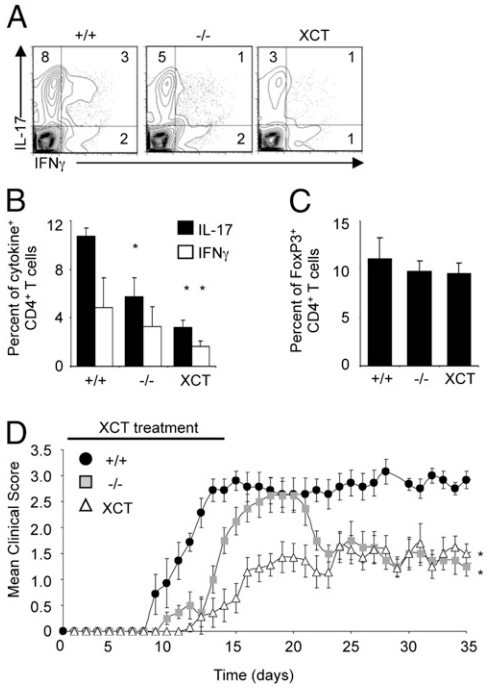

Fig. 5. Reduced Teff function and severity of EAE in the absence of ERRα. (*A–D*) EAE was induced in WT and ERRα$^{-/-}$ mice with or without XCT790 (XCT) treatment as indicated. T cells from draining lymph nodes were analyzed on day 9 by flow cytometry; (*A*) representative plots and (*B*) averages of each group for cytokine production and (*C*) FoxP3^{+} cells are shown. Graphs are displayed as the average and SD (*$P \leq 0.05$). (*D*) Mean clinical score and SEM of EAE disease severity in each population over 35 d (*$P \leq 0.01$). Results are compiled from two independent experiments.

targeting ERRα as a metabolic regulator of T-cell differentiation and function can limit the size and severity of an immune response in vivo and suggests that ERRα is an important regulator of Teff metabolism and differentiation.

Discussion

T-cell activation and differentiation are highly energetic processes that require metabolic reprogramming to support the growth and specific functions of Teff and Treg subsets. Although we have previously shown that Teff require glycolytic metabolism and Treg use lipid oxidation (6), the transcriptional regulation that supports these selective metabolic transitions is poorly understood. In addition to the Akt/mTOR pathway (6, 12), it is essential that cells up-regulate gene expression to allow mitochondria to use glucose as a metabolic fuel to support ATP generation and biosynthesis. Although the transcription factors Myc and HIF1α may play key roles to up-regulate expression of glycolytic genes (14, 28, 29), our data suggest that ERRα broadly impacts T-cell metabolism and is particularly critical in T cells to reprogram mitochondria to use glucose as an anabolic source (Fig. S10*D*). As a gatekeeper between the quiescent oxidative metabolism of resting cells and the highly dynamic process of aerobic glycolysis in activated Teff cells, ERRα is positioned as a transcriptional metabolic regulator of both Teff growth and function.

ERRα has no known endogenous ligands, but is instead regulated by expression, interaction with transcriptional cofactors, and by posttranslational modifications that may be important in T-cell metabolism and activation. ERRα up-regulation was costimulation-dependent and not mediated through increased mRNA. The CD28-dependent increase in ERRα expression correlated with the requirement of costimulation for the transition to a highly glycolytic phenotype (30), and likely occurred through increased protein stability or translation. When expressed, ERRα associates with PGC1α/β to regulate mitochondrial biogenesis and oxidative energy production (31). ERRα has also been described to associate with and enhance HIF1α activity (32) and the homeobox protein prospero-related homeobox 1, which can inhibit the activity of the ERRα/PGC-1 complex (33). Additionally, ERRα can be directly acetylated by Sirt1 to dictate its ability to bind to gene targets (34), further increasing the potential of ERRα-driven metabolic regulation. Ultimately, a key result of ERRα expression and activity appears to be to promote stability or translation of glycolytic proteins and the flux of pyruvate into acetyl-CoA and the TCA cycle, possibly through regulation of DLAT and PDK1 expression. An inability to properly direct glucose to mitochondrial metabolism would lead to decreased lipid synthesis and cell growth, similar to what has been described in ERR-deficient *Drosophila* (22).

Although acute ERRα inhibition and ERRα$^{-/-}$ T cells shared many metabolic and functional defects, both in vitro and in vivo, ERRα$^{-/-}$ CD4^{+} T cells, nevertheless, could up-regulate glycolysis and proliferate when stimulated under optimal conditions in vitro. We cannot fully rule-out contributions of off-target pharmacologic effects of the inhibitors; the inability of either chemically independent inhibitor to alter phenotypes of ERRα$^{-/-}$ T cells provides chemical and genetic evidence for a target-based mechanism for these compounds. However, our data support a compensatory alteration in mTOR and AMPK signaling with chronic ERRα-deficiency that supports glycolysis and proliferation required for development. Although this potential compensation is not understood, the data show a clear role for ERRα in T-cell metabolism in parallel to mTOR, as both acute and genetic ERRα-deficiency leads to profound T-cell metabolic defects and reduced immunity in vivo.

Modulating T-cell metabolism may be a viable approach for regulating an immune response in vivo. Inhibition of ERRα prevented the subsequent metabolic switch required for optimal T-cell activation and resulted in decreased T-cell proliferation, inflammatory cytokine production, and disease morbidity in both immunization and EAE models. The selective sensitivity of Teff to acute ERRα inhibition may be because of the lack of Treg dependence on glucose metabolism (6, 14) and thus provide a means to target Teff and spare Treg. Macrophages also use ERRα (23) and the effects of ERRα inhibition in vivo likely reflect the combined effect of disruption of critical metabolic pathways in multiple cell types. Nevertheless, the altered metabolism of purified T cells upon acute in vitro ERRα inhibition and selective sensitivity of both developing and established Teff also indicates a T-cell–intrinsic role for ERRα.

These data demonstrate a key role for ERRα in the regulation of Teff function and development. In this model, ERRα may play a key role to induce metabolic gene expression to program mitochondria for aerobic glycolysis, while interacting with other transcription factors, such as HIF-1α to regulate glycolytic metabolism (14). As such, ERRα together with other metabolic regulators, such as mTOR or HIF1α, may provide a unique approach to coordinately regulate the balance between Teff proliferation and function and the generation of suppressive Treg cells. Ultimately, the differential metabolic requirements of Teff and Treg cells (6) and the role of ERRα as a key regulator of cell Teff metabolism, may provide new directions to selectively disrupt Teff generation and function in the treatment of immune diseases.

Methods

Mice and Cells. C57BL/6 mice were obtained from Jackson Laboratories, and Glut1 transgenic and ERRα$^{-/-}$ mice were previously described (2, 24). Mice were housed and cared for at Duke University under an Institutional Animal Care and Use Committee approved protocol. Media, T-cell isolation and stimulation, immunoblots, surface and intracellular staining, and metabolic

assays were performed as previously described (6) and are detailed in *SI Methods*.

Quantitative RT-PCR. WT and ERR$\alpha^{-/-}$ CD4$^+$ T cells were stimulated for 12 h in the presence or absence of XCT790 and RNA was harvested (RNeasy Plus; Qiagen). Data are a total of three independent experiments. The mitochondrial energy metabolism and glucose metabolism quantitative RT-PCR arrays were purchased and prepared using the SuperArray RT^2 Profiler PCR array system according to the manufacturer's instructions (Qiagen) and assayed on a ViiA 7 (Applied Biosystems). Data were analyzed using the RT^2 Profiler program supplied by Qiagen and normalized to *Hprt* and *Gapdh* housekeeping genes. A twofold change in gene expression between vehicle- and XCT790-treated stimulated T cells was used as a threshold.

EAE. EAE was induced in WT and ERR$\alpha^{-/-}$ mice by s.c. injection of 100 ng MOG peptide mixed with one part Complete Freund's Adjuvant and one part *Mycobacterium tuberculosis* followed by 1 μg/mL Pertussis Toxin administered by intraperitoneal injection on days 0 and 2. Methyl-cellulose vehicle or 5 mg/kg per d of XCT790 were administered daily for 15 d during the course of the experiment. Clinical signs of EAE were assessed according to the following score: 0, no signs of disease; 1, loss of tone in the tail; 2, hindlimb paresis; 3, hindlimb paralysis; 4, tetraplegia.

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1. Kronenberg M, Rudensky A (2005) Regulation of immunity by self-reactive T cells. *Nature* 435:598–604.
2. Jacobs SR, et al. (2008) Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. *J Immunol* 180: 4476–4486.
3. Cham CM, Gajewski TF (2005) Glucose availability regulates IFN-gamma production and p70S6 kinase activation in CD8+ effector T cells. *J Immunol* 174:4670–4677.
4. Jones RG, Thompson CB (2007) Revving the engine: Signal transduction fuels T cell activation. *Immunity* 27:173–178.
5. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB (2008) The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. *Cell Metab* 7:11–20.
6. Michalek RD, et al. (2011) Cutting edge: Distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J Immunol* 186:3299–3303.
7. Edinger AL, Thompson CB (2002) Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol Biol Cell* 13:2276–2288.
8. Wieman HL, Wofford JA, Rathmell JC (2007) Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. *Mol Biol Cell* 18:1437–1446.
9. Zhong H, et al. (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* 59:5830–5835.
10. Hardie DG, Hawley SA, Scott JW (2006) AMP-activated protein kinase—Development of the energy sensor concept. *J Physiol* 574:7–15.
11. Lee K, et al. (2010) Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. *Immunity* 32:743–753.
12. Delgoffe GM, et al. (2009) The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 30:832–844.
13. Haxhinasto S, Mathis D, Benoist C (2008) The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med* 205:565–574.
14. Shi LZ, et al. (2011) HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med* 208:1367–1376.
15. Frauwirth KA, Thompson CB (2004) Regulation of T lymphocyte metabolism. *J Immunol* 172:4661–4665.
16. Deblois G, et al. (2009) Genome-wide identification of direct target genes implicates estrogen-related receptor alpha as a determinant of breast cancer heterogeneity. *Cancer Res* 69:6149–6157.
17. Jarzabek K, et al. (2009) The significance of the expression of ERRalpha as a potential biomarker in breast cancer. *J Steroid Biochem Mol Biol* 113:127–133.
18. Chisamore MJ, Wilkinson HA, Flores O, Chen JD (2009) Estrogen-related receptor-alpha antagonist inhibits both estrogen receptor-positive and estrogen receptor-negative breast tumor growth in mouse xenografts. *Mol Cancer Ther* 8:672–681.
19. Stein RA, et al. (2008) Estrogen-related receptor alpha is critical for the growth of estrogen receptor-negative breast cancer. *Cancer Res* 68:8805–8812.
20. Giguère V (2008) Transcriptional control of energy homeostasis by the estrogen-related receptors. *Endocr Rev* 29:677–696.
21. Villena JA, Kralli A (2008) ERRalpha: A metabolic function for the oldest orphan. *Trends Endocrinol Metab* 19:269–276.
22. Tennessen JM, Baker KD, Lam G, Evans J, Thummel CS (2011) The *Drosophila* estrogen-related receptor directs a metabolic switch that supports developmental growth. *Cell Metab* 13:139–148.
23. Sonoda J, et al. (2007) Nuclear receptor ERR alpha and coactivator PGC-1 beta are effectors of IFN-gamma-induced host defense. *Genes Dev* 21:1909–1920.
24. Luo J, et al. (2003) Reduced fat mass in mice lacking orphan nuclear receptor estrogen-related receptor alpha. *Mol Cell Biol* 23:7947–7956.
25. Busch BB, et al. (2004) Identification of a selective inverse agonist for the orphan nuclear receptor estrogen-related receptor alpha. *J Med Chem* 47:5593–5596.
26. An J, et al. (2004) Hepatic expression of malonyl-CoA decarboxylase reverses muscle, liver and whole-animal insulin resistance. *Nat Med* 10:268–274.
27. Ansel KM, Lee DU, Rao A (2003) An epigenetic view of helper T cell differentiation. *Nat Immunol* 4:616–623.
28. Morrish F, Isern N, Sadilek M, Jeffrey M, Hockenbery DM (2009) c-Myc activates multiple metabolic networks to generate substrates for cell-cycle entry. *Oncogene* 28: 2485–2491.
29. Lum JJ, et al. (2007) The transcription factor HIF-1alpha plays a critical role in the growth factor-dependent regulation of both aerobic and anaerobic glycolysis. *Genes Dev* 21:1037–1049.
30. Frauwirth KA, et al. (2002) The CD28 signaling pathway regulates glucose metabolism. *Immunity* 16:769–777.
31. Mirebeau-Prunier D, et al. (2010) Estrogen-related receptor alpha and PGC-1-related coactivator constitute a novel complex mediating the biogenesis of functional mitochondria. *FEBS J* 277:713–725.
32. Ao A, Wang H, Kamarajugadda S, Lu J (2008) Involvement of estrogen-related receptors in transcriptional response to hypoxia and growth of solid tumors. *Proc Natl Acad Sci USA* 105:7821–7826.
33. Charest-Marcotte A, et al. (2010) The homeobox protein Prox1 is a negative modulator of ERRalpha/PGC-1alpha bioenergetic functions. *Genes Dev* 24:537–542.
34. Wilson BJ, Tremblay AM, Deblois G, Sylvain-Drolet G, Giguère V (2010) An acetylation switch modulates the transcriptional activity of estrogen-related receptor alpha. *Mol Endocrinol* 24:1349–1358.