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Regulation of Fatty Acid Oxidation by Twist 1 in the Metabolic Adaptation of T Helper Lymphocytes to Chronic Inflammation

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Objective. Inflamed tissue is characterized by low availability of oxygen and nutrients. Yet CD4+ T helper lymphocytes persist over time in such tissue and probably contribute to the chronicity of inflammation. This study was undertaken to analyze the metabolic adaptation of these cells to the inflamed environment.

Methods. Synovial and blood CD4+ T cells isolated ex vivo from patients with juvenile idiopathic arthritis (JIA) and murine CD4+ T cells were either stimulated once or stimulated repeatedly. Their dependency on particular metabolic pathways for survival was then analyzed using pharmacologic inhibitors. The role of the transcription factor Twist 1 was investigated by determining lactate production and oxygen consumption in *Twist1*-sufficient and *Twist1*-deficient murine T cells. The dependency of these murine cells on particular metabolic pathways was analyzed using pharmacologic inhibitors.

Results. Programmed death 1 (PD-1)+ T helper cells in synovial fluid samples from patients with JIA survived via fatty acid oxidation (mean ± SEM survival of 3.4 ± 2.85% in the presence of etomoxir versus 60 ± 7.08% in the absence of etomoxir on day 4 of culture) ($P < 0.0002$; n = 6) and expressed the E-box–binding transcription factor *TWIST1* (2–14-fold increased expression) ($P = 0.0156$ versus PD-1– T helper cells; n = 6). Repeatedly restimulated murine T helper cells, which expressed *Twist1* as well, needed *Twist1* to survive via fatty acid oxidation. In addition, *Twist1* protected the cells against reactive oxygen species.

Conclusion. Our findings indicate that *TWIST1* is a master regulator of metabolic adaptation of T helper cells to chronic inflammation and a target for their selective therapeutic elimination.

INTRODUCTION

CD4+ T lymphocytes are considered a driving force and relevant therapeutic target in chronic inflammatory rheumatic diseases. In the inflamed synovial tissue of patients, CD4+ T lymphocytes persist despite low levels of oxygen and nutrients, and they are refractory to conventional immunosuppressive therapies (1,2). With respect to nutrients qualifying as a metabolic energy source, there is little glucose (3) and glutamine (4) in inflamed tissue, while fatty acids are readily available (5,6). Herein, we describe the metabolic adaptation of CD4+ T lymphocytes to this inflamed environment.

Among the CD4+ T lymphocytes present in inflamed tissues, CD4+ T cells expressing programmed death 1 (PD-1) protein (7) are a subpopulation of potential relevance for pathogenesis (8). In this study, we show that PD-1+ Th1 cells isolated from the synovial

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fluid of patients with juvenile idiopathic arthritis (JIA) are dependent on fatty acid oxidation for survival. Their survival is blocked by the carnitine palmitoyltransferase 1 inhibitor etomoxir (9), which inhibits the transport of fatty acids from the cytoplasm into the mitochondria. We show that CD4+ PD-1+ T cells in inflamed synovia express the E-box–binding transcription factor *TWIST1*, a hallmark of T lymphocytes persisting in chronically inflamed human tissue (2).

Twist1 expression is selective for repeatedly activated murine Th1 cells, as compared to other types of T helper cells and Th1 cells activated only once. *Twist1* expression by murine Th1 cells has been shown to dampen immunopathology in an autoregulatory, cell-intrinsic manner (2). At the same time, *Twist1* supports the persistence of repeatedly activated Th1 cells by inducing expression of microRNA-148a (miR-148a), which in turn regulates expression of the proapoptotic protein Bim (10). We previously demonstrated that selective depletion of *Twist1*-expressing Th1 cells through blockade of miR-148a, a *Twist1*-induced miRNA, in vivo with antagomirs ameliorates inflammation, identifying *Twist1*-expressing T helper cells as those driving inflammation (11). Conditional inactivation of *Twist1* in repeatedly activated Th1 lymphocytes relieves their dependency on fatty acid oxidation and allows them to survive alternatively on glycolysis, demonstrating that *Twist1* forces T helper cells into fatty acid oxidation, and thus regulates their proinflammatory activity (12,13). *Twist1* thus qualifies as an essential regulator of the metabolism of T helper lymphocytes in chronic inflammation, inhibiting glycolysis, and thus limiting immunopathology, while at the same time stimulating fatty acid oxidation, allowing the cells to persist in and contribute to the chronification of inflammation.

MATERIALS AND METHODS

Mice. C57BL/6J mice were purchased from Charles River. OT-II × Twist1$^{fl/fl}$ × CD4Cre$^{+/-}$ and OT-II × Twist1$^{wt/wt}$ × CD4Cre$^{+/-}$, Twist1$^{fl/fl}$ × CD4Cre$^{+/-}$, and Twist1$^{wt/wt}$ × CD4Cre$^{+/-}$ mice were bred in the Deutsches Rheuma-Forschungszentrum animal facility under specific pathogen–free conditions in individually ventilated cages. Mice were handled in accordance with good animal practice as defined by the German animal welfare bodies, and killed by cervical dislocation. All experiments were approved by the State Office for Health and Social Affairs (Berlin, Germany).

Human patient samples. Peripheral blood and synovial fluid samples were collected at the Department of Pediatrics, Pediatric Rheumatology Section of Charité–Universitätsmedizin Berlin as approved by the ethics committee of Charité–Universitätsmedizin Berlin (approval no. EA2/069/15).

Human T cell phenotyping and cultivation. Mononuclear cells from peripheral blood were isolated by Ficoll density-gradient centrifugation. Synovial fluid cells were depleted of CD14+ granulocytes by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec). CD4+ T helper lymphocytes were isolated using CD4 microbeads (Miltenyi Biotec). For analysis of cytokine expression, synovial CD4+ T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) and ionomycin (1 μg/ml) (both from Sigma-Aldrich) for a total of 5–6 hours in medium. After 1 hour, 5 μg/ml brefeldin A (BioLegend) was added to block the secretion of cytokines. Cells were fixed with Cytofix/Cytoperm (BD Biosciences) for 20 minutes at 4°C and stained intracellularly with anti–interferon-γ (anti-IFNγ) (4SB3; BioLegend), anti–interleukin-17a (anti–IL-17a) (BL168; BioLegend), anti–tumor necrosis factor (anti-TNF) (cA2; Miltenyi Biotec), anti–IL-2 (MQ1-17H12; BioLegend), anti–IL-10 (JES3-9D7; Miltenyi Biotec), anti–IL-4 (8D48; BioLegend), and anti–IL-21 (7H20-I19-M3; BioLegend) according to published guidelines (14). For intracellular T-bet staining (4B10; BioLegend), cells were additionally permeabilized with 0.01% Triton X-100 for 10 minutes on ice. PD-1+ and PD-1– CD3+CD4+CD14–CD45RO+ T lymphocytes were sorted by fluorescence-activated cell sorting (FACS) using a FACSAria cell sorter (BD Biosciences) and plated in RPMI medium containing human AB serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Etomoxir was added to the final concentrations as indicated. The numbers of viable CD4+ T cells were monitored over time by flow cytometry, using DAPI to exclude dead cells.

In vitro cultivation and differentiation of murine T helper cells. Murine CD4+CD62L+ (naive) T helper cells were isolated as previously described (15) and cultured at a concentration of 2.5 × 10^6 cells/5 ml in 6-well plates in RPMI medium supplemented with 10% fetal calf serum (Merck), 300 μg/ml glutamine (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 50 μ*M* β-mercaptoethanol (Sigma-Aldrich) at 37°C in 5% CO_2 and 4.2% oxygen. The T helper cells were stimulated polyclonally with plate-bound anti-CD3 antibody (145-2C11; 3 μg/ml) and soluble anti-CD28 antibody (37.51; 1.5 μg/ml). For Th1 polarization, 5 ng/ml recombinant IL-12, 10 ng/ml recombinant IL-2, and 10 μg/ml anti–IL-4 antibody (11B11) was added. To induce Th17 polarization, 10 μg/ml anti-IFNγ (AN18.17.24), 10 μg/ml anti–IL-4 (11B11), 20 ng/ml recombinant IL-6, 20 ng/ml recombinant IL-23, and 1 ng/ml recombinant transforming growth factor β (all from R&D Systems) were added. After 48 hours of stimulation, the cells were removed from the antibody-coated culture dishes and cultured for an additional 3–4 days. T cell receptor–transgenic OT-II lymphocytes were activated with 1 μg/ml of ovalbumin 327–339 peptide in the presence of irradiated (30 Gy) CD90-depleted splenocytes from C57BL/6 mice. For repeated activation, viable T helper cells were isolated using Ficoll density-gradient centrifugation and stimulated again under the original conditions.

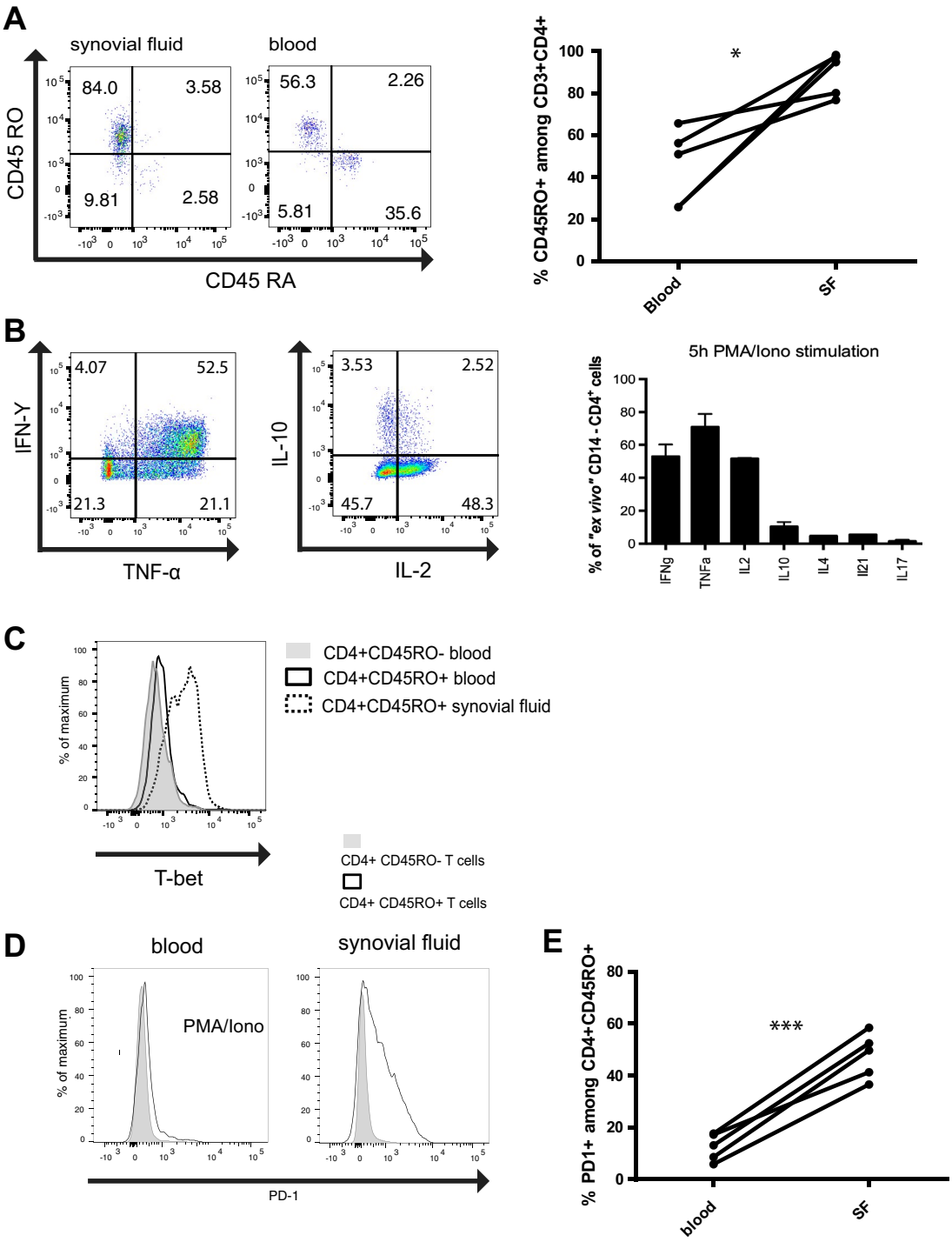

Figure 1. Synovial fluid (SF) T cells from patients with juvenile idiopathic arthritis (JIA) have a Th1 phenotype and express programmed death 1 (PD-1). **A**, Left, Flow cytometric analysis indicating the frequencies of CD45RA+ and CD45RO+ cells among CD3+CD4+ T cells in synovial fluid and blood from patients with JIA. Results are representative of 5 experiments. Right, Percentage of CD3+CD4+ cells expressing CD45RO in blood and synovial fluid from patients with JIA (n = 5). * = $P < 0.05$ by 2-tailed *t*-test. **B**, Left, Expression of interferon-γ (IFNγ), tumor necrosis factor (TNF), interleukin-10 (IL-10), and IL-2 in ex vivo-isolated synovial T cells after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (iono), as analyzed by intracellular cytokine staining. Results are representative of 5 experiments. Right, Frequencies of synovial fluid CD4+ T cells expressing IFNγ, TNF, IL-2, IL-10, IL-4, IL-21, and IL-17A after 5 hours of restimulation with PMA and ionomycin. Bars show the mean ± SEM (n = 5). **C**, T-bet expression in CD4+CD45RO– and CD4+CD45RO+ T cells in blood and CD4+CD45RO+ T cells in synovial fluid from a patient with JIA. Results are representative of 3 experiments. **D**, PD-1 expression in CD4+CD45RO– and CD4+CD45RO+ T cells isolated from blood stimulated with PMA and ionomycin and synovial fluid from a patient with JIA. Results are representative of 5 experiments. **E**, Percentage of CD4+CD45RO+ cells expressing PD-1 in blood and synovial fluid from patients with JIA (n = 5). *** = $P < 0.001$ by 2-tailed *t*-test. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40939/abstract.

Quantitative RNA expression analysis. Total RNA was isolated using an RNeasy kit (Qiagen) or a Direct-zol RNA kit (Zymo Research) according to the manufacturer's instructions. RNA concentration and quality were determined by NanoDrop spectrometry. RNA (200 ng to 1 μg) was reverse transcribed using a TaqMan Reverse Transcription kit (ThermoFisher Scientific) according to the manufacturer's recommendations. Real-time quantitative polymerase chain reaction was used to quantify the messenger RNA of interest with the following primer sets (TIB Molbiol Berlin): for murine hypoxanthine guanine phosphoribosyltransferase (Hprt), forward 5′-TCCTCCTCAGACCGCTTTT-3′ and reverse 5′-CATAA CCTGGTTCATCATCGC-3′; for human HPRT, forward 5′-ACCC TTTCCAAATCCTCAGC-3′ and reverse 5′-GTTATGGCGACC CGCAG-3′; for murine *Twist1*, forward 5′-CGCACGCAGTCGC TGAACG-3′ and reverse 5′-GACGCGGACATGGACCAGG-3′; for human *TWIST1*, forward 5′-GGCACCCAGTCGCTGAACG-3′ and reverse 5′-GACGCGGACATGGACCAGG-3′; for murine *Pdcd1*, forward 5′-CGTCCCTCAGTCAAGAGGAG-3′ and reverse 5′-GTCCCTAGAAGTGCCCAACA-3′. Prior to *Twist1* analysis, cultured cells were restimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 5 hours.

Glucose uptake assay. Murine naive CD4+ T helper cells were stimulated with 3 μg/ml of plate-bound anti-CD3 and 1.5 μg/ml of soluble anti-CD28 antibodies at 3×10^5 cells per well under Th1-inducing conditions for 5 hours. Medium was then aspirated, and 100 μl of glucose-free medium with 1.5 μg/ml of soluble anti-CD28 antibody was added to the cells. Following incubation for 60 minutes, 100 μl of glucose-free RPMI with 300 μ*M* 2-([7-nitro-2,1,3-benzoxadiazol-4-yl]amino)-2 deoxyglucose (2-NBDG) was added to the well, to reach a final concentration of 150 μ*M* 2-NBDG. Cultures with no 2-NBDG added and cultures with 150 μ*M* 2-NBDG and 30 μ*M* cytochalasin B were used as controls. Cells were incubated for 30 minutes at 37°C. After incubation, cells were washed twice in cold phosphate buffered saline (PBS) and maintained at 4°C. Uptake of 2-NBDG was determined by flow cytometry using a MACSQuant analyzer. Dead cells were excluded by propidium iodide staining.

Determination of lactate production and oxygen consumption. Glycolysis, as determined by extracellular acidification, and oxidative phosphorylation, as determined by oxygen consumption, were measured using a Seahorse XP analyzer (Agilent). Prior to the assay, T lymphocytes were starved in glucose-free RPMI assay medium and equilibrated in 5% CO_2 at 37°C, under normoxic conditions.

T cell survival assay. CD4+ T helper cells were seeded in a 96-well plate coated with 3 μg/ml anti-CD3 antibodies and 1.5 μg/ml soluble anti-CD28 antibodies under Th1- or Th17-polarizing conditions. The inhibitors 2-deoxy-D-glucose (2-DG; 2 m*M*), 6-diazo-5-oxo-L-norleucine (50 μ*M*), oligomycin (2 μ*M*), or etomoxir (150 μ*M*) were added. Dead cells were excluded by staining with 100 ng/ml propidium iodide or 100 ng/ml DAPI. Numbers of viable cells were determined by flow cytometry using a MACSQuant analyzer.

Lipid peroxidation. BODIPY 581/591 C11 was added to CD4+ T lymphocytes in a 96-well plate at a final concentration of 3 μ*M* in RPMI without serum and 2-mercaptoethanol. Cells were incubated for 40 minutes at 37°C and then washed with PBS/bovine serum albumin. Viable cells were quantified using a MACSQuant analyzer, and dead cells were excluded by DAPI staining.

RESULTS

Survival of PD-1+CD4+ T helper lymphocytes in inflamed synovia via fatty acid oxidation. CD4+ T helper lymphocytes were isolated from the synovial fluid of patients with JIA. More than 70% of the CD3+CD4+ cells were CD45RO+CD45RA– (Figure 1A). Upon stimulation with PMA and ionomycin ex vivo, ~50% of them expressed IFNγ, 70% expressed TNF, 50% expressed IL-2, and 10% expressed IL-10. IL-4, IL-17, and IL-21 were each expressed by <10% of the cells (Figure 1B). Consistent with the cytokine expression pattern, almost all JIA synovial T helper cells expressed the T-box–binding transcription factor T-bet, identifying them as bona fide Th1 cells (Figure 1C). A significantly higher proportion of synovial T helper cells than peripheral blood T helper cells expressed PD-1 (Figures 1D and E).

PD-1+ and PD-1– synovial T helper cells were separated by FACS (Figure 2A and Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40939/abstract), and their dependency on fatty acid oxidation was analyzed. CD4+CD45RO+ T cells isolated from the blood of healthy donors did not survive when cultured in vitro under hypoxic conditions (4.2% O_2), with >90% of them dying within 2 days. Of the PD-1– T helper cells isolated from synovial fluid, a mean ± SEM of 24.6 ± 2.29% survived until day 2 of culture and 19 ± 1.31% survived until day 4. Their survival was not affected by the addition of etomoxir, an inhibitor of fatty acid oxidation. In the presence of etomoxir, 20% of the cells survived until day 2 and 15.6 ± 4.5% survived until day 4 (Figure 2B). Of the PD-1+ synovial T helper cells, 39.85 ± 7.35% survived until day 2, and these surviving cells expanded again to 60 ± 7.08% of the original number on day 4. Survival and expansion of these cells was entirely dependent on fatty acid oxidation, since they could be blocked with etomoxir, reducing cell numbers to a mean ± SEM of 3.4 ± 2.85% on day 4 (Figure 2B).

We have previously shown that the E-box–binding transcription factor *TWIST1* is a hallmark of T helper lymphocytes isolated from the inflamed joints of patients with rheumatic diseases (2). In this study, we found that PD-1+ synovial T cells isolated from the inflamed joints of patients with JIA expressed significantly higher

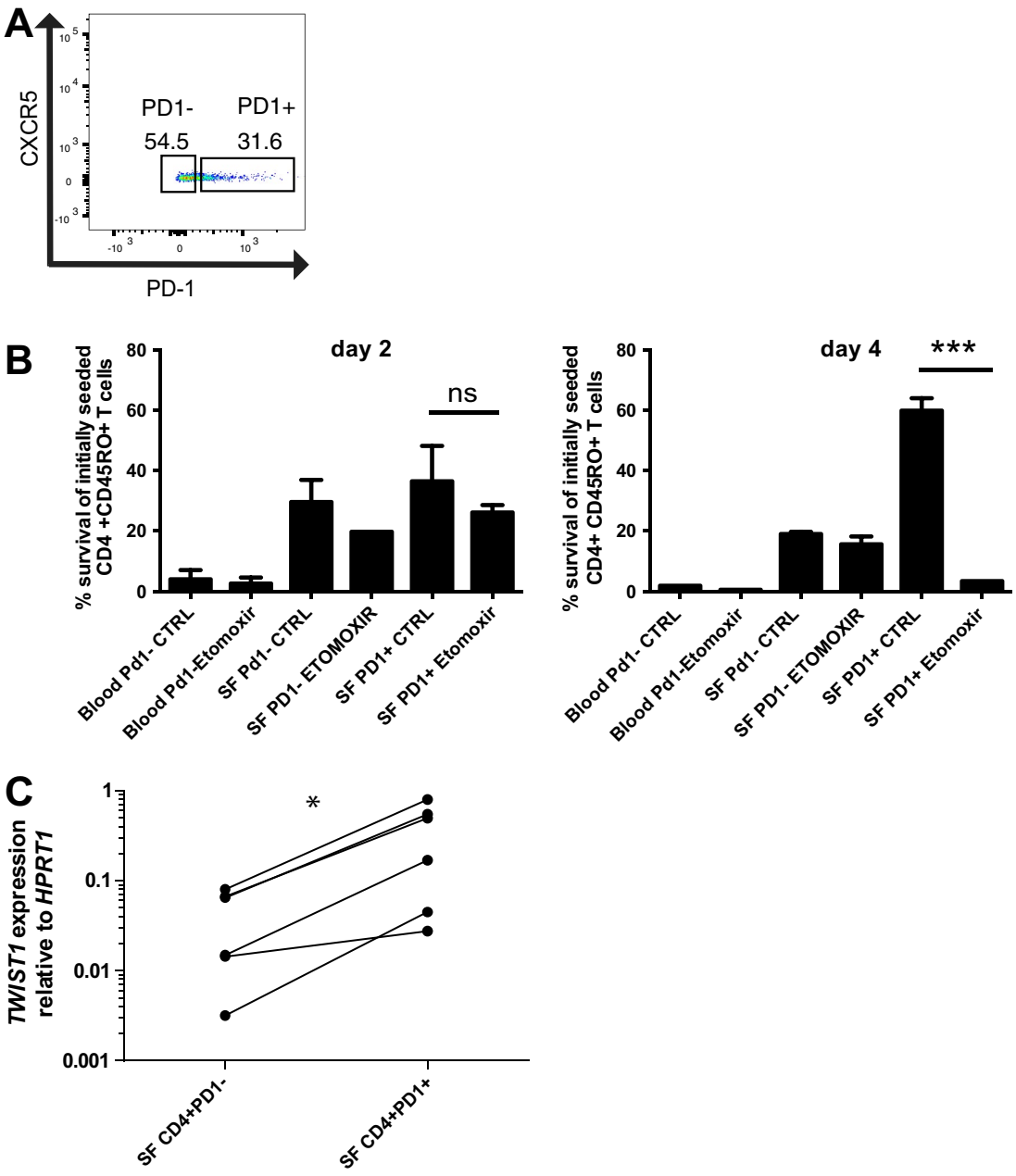

Figure 2. Survival of synovial CD4+CD45RO+PD-1+ T cells in patients with JIA is dependent on fatty acid oxidation. **A**, Representative gating strategy for sorting PD-1+ and PD-1– CD4+CD45RO+ T cells from synovial fluid and blood. **B**, Frequency of viable cells relative to the number of initially seeded cells determined on day 2 and day 4 after stimulation with anti-CD3 and anti-CD28 antibodies in the presence or absence (control) of 200 μ*M* etomoxir. Bars show the mean ± SEM. Data were pooled from 2 experiments (n = 3 blood and synovial fluid samples per experiment). *** = $P < 0.0002$ by unpaired 2-tailed *t*-test. NS = not significant. **C**, *TWIST1* mRNA expression relative to *HPRT* in PD-1+ and PD-1– CD4+CD45RO+ T cells directly isolated from synovial fluid from patients with JIA (n = 6). * = $P = 0.016$ by Wilcoxon's 1-tailed paired rank test. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40939/abstract.

levels of *TWIST1* than PD-1– T helper cells isolated from the same synovia (Figure 2C).

Upon repeated activation, murine Th1 cells shift from glycolysis to fatty acid oxidation. To investigate the role of *Twist1* in the regulation of the metabolism of T helper lymphocytes, we used a murine model of repeatedly activated Th1 lymphocytes, which was previously used as a model to study transcriptional adaptations of chronic activation and in which we had previously demonstrated up-regulated *Twist1* expression (2). Naive CD4+CD62L+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies 4 times at 6-day inter-

vals. We then compared the capacity of these cells to take up glucose and perform glycolysis, the latter reflected by the production of lactate, as outlined in Figure 3A. Uptake of the fluorescent glucose analog 2-NBDG was monitored by flow cytometry. Repeatedly activated mouse Th1 cells took up 2.3-fold less 2-NBDG than mouse Th1 cells that were activated one time (Figure 3B).

Repeatedly activated murine Th1 cells were also less efficient in glycolysis, compared to Th1 cells activated once. Murine Th1 cells activated once produced 1.4-fold more lac-

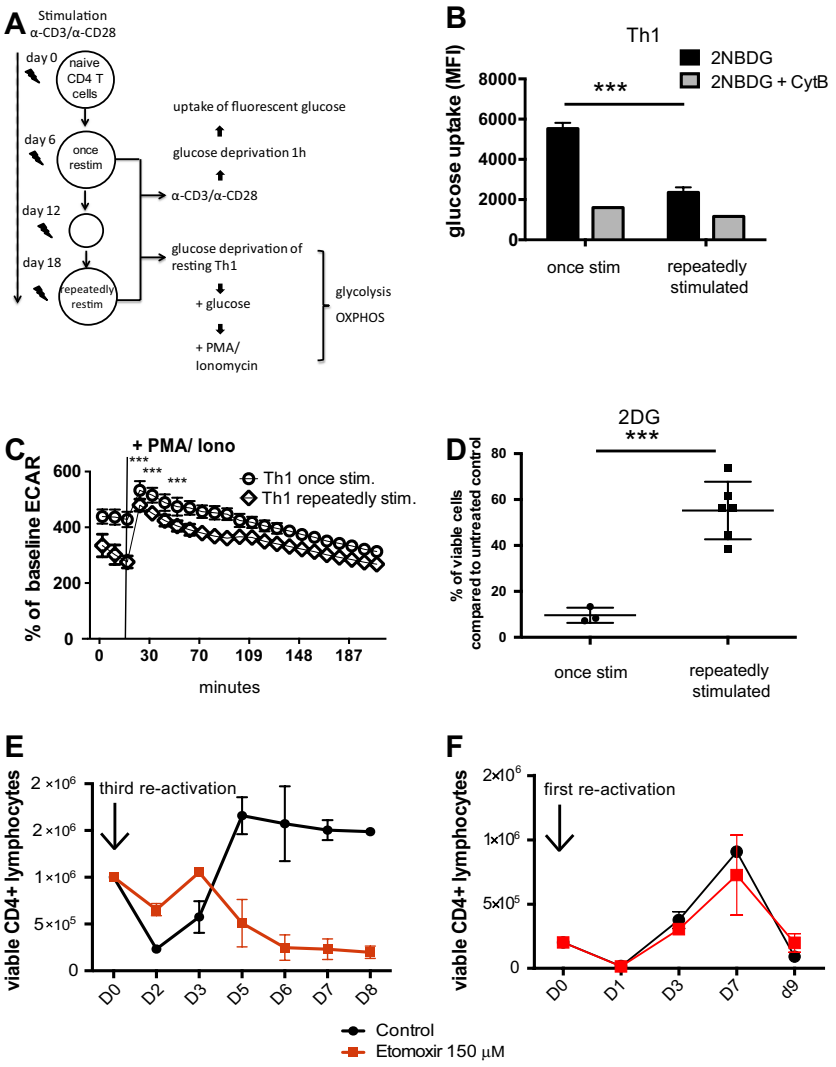

Figure 3. Repeatedly stimulated (stim) murine Th1 cells switch their metabolism from glycolysis to fatty acid oxidation. **A**, Experimental design. Naive CD4+CD62L+ T cells isolated from C57BL/6 mice were stimulated once or stimulated 2 additional times at 6-day intervals with anti-CD3/anti-CD28 antibodies under Th1-differentiating conditions. Cells were analyzed for energy metabolism. OXPHOS = oxidative phosphorylation. **B**, Glucose uptake in murine Th1 cells stimulated once and repeatedly stimulated murine Th1 cells, measured by flow cytometry using the fluorescent glucose analog 2-([7-nitro-2,1,3-benzoxadiazol-4-yl]amino)-2 deoxyglucose (2-NBDG). Th1 cells were restimulated for 6 hours with anti-CD3 and anti-CD28 antibodies prior to analysis. Cytochalasin B (CytB) was used to determine background fluorescence. Bars show the mean ± SEM. Results are representative of 3 independent experiments. *** = $P < 0.0006$ by Mann-Whitney 2-tailed test. **C**, Glycolytic activity, measured by extracellular acidification rate (ECAR), in murine Th1 cells stimulated once and repeatedly stimulated murine Th1 cells before and after restimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (iono). Values are the mean ± SEM (n = 4 samples per group). *** = $P < 0.0001$ at 14 minutes, $P = 0.0027$ at 30 minutes, and $P = 0.0027$ at 50 minutes after glucose addition, by 2-tailed *t*-test. **D**, Frequency of viable murine CD4+ T cells stimulated once and murine CD4+ T cells stimulated repeatedly after restimulation with anti-CD3/anti-CD28 antibodies in the presence of the glycolysis inhibitor 2-deoxy-D-glucose (2-DG; 2 m*M*) for 72 hours, relative to cells cultivated without inhibitor (untreated control). Symbols represent individual samples; horizontal lines and error bars show the mean ± SEM. *** = $P = 0.0005$ by *t*-test. **E** and **F**, Absolute number of repeatedly stimulated murine Th1 cells (**E**) and murine Th1 cells stimulated once (**F**) and restimulated with anti-CD3/anti-CD28 antibodies in the presence or absence of 150 μ*M* etomoxir. Viability and cell count were determined by propidium iodide exclusion using flow cytometry. Values are the mean ± SEM. Results are representative of 3 experiments. MFI = mean fluorescence intensity.

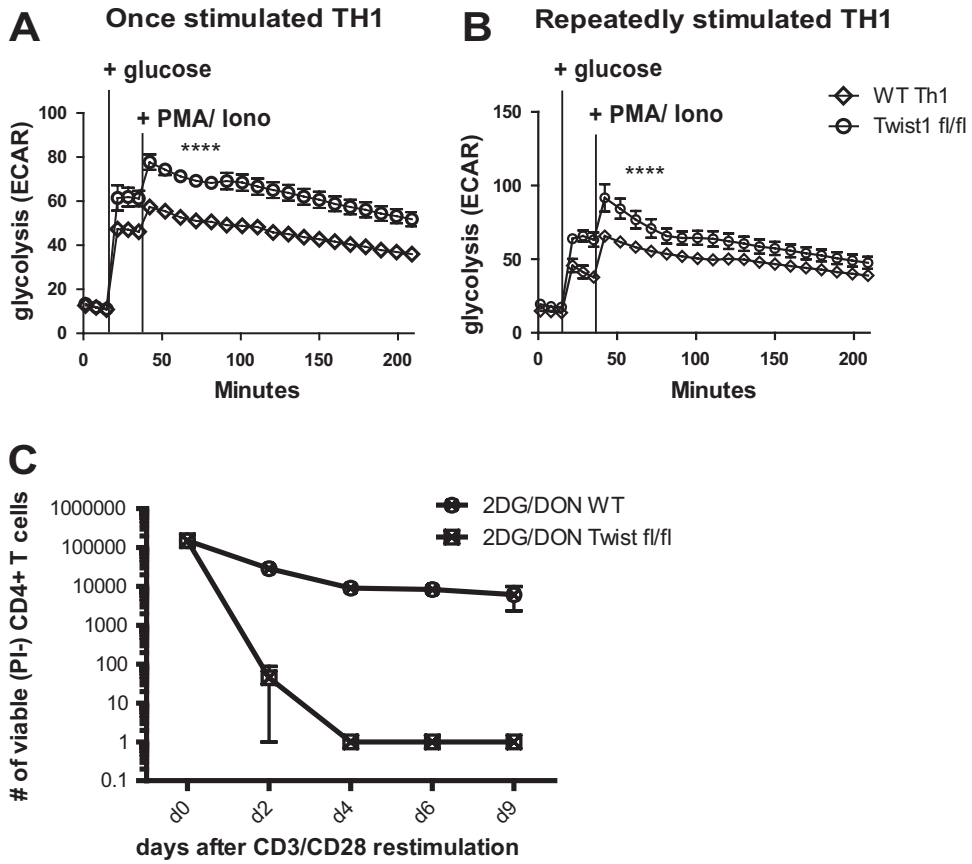

Figure 4. Twist 1 inhibits glycolysis in murine Th1 cells stimulated once and murine Th1 cells stimulated repeatedly. **A** and **B**, Glycolytic activity, measured by extracellular acidification rate (ECAR) of *Twist1*-deficient (Twist1$^{fl/fl}$) and *Twist1*-sufficient (wild-type [WT]) OT-II TCR-transgenic mouse Th1 cells stimulated once (**A**) or stimulated repeatedly (**B**) with ovalbumin peptide before and after the addition of glucose and restimulation with 12-myristate 13-acetate (PMA) and ionomycin (iono). **** = $P < 0.0001$ at all time points following glucose addition in **A** and at all time points from glucose addition to 120 minutes in **B**, by paired *t*-test. **C**, Absolute number of viable repeatedly stimulated *Twist1*-deficient and *Twist1*-sufficient mouse Th1 cells treated with inhibitors of glycolysis (2-deoxy-D-glucose [2-DG]) and glutaminolysis (6-diazo-5-oxo-L-norleucine [DON]). Viable cells were determined every 2 days by propidium iodide (PI) exclusion using flow cytometry. Values are the mean ± SEM. Results are representative of 2 experiments.

tate than repeatedly activated murine Th1 cells in the presence of glucose, before and after stimulation with PMA and ionomycin (Figure 3C). Lactate production remained significantly lower in repeatedly activated murine Th1 cells thereafter. Of the Th1 cells that had been activated once, inhibition of glycolysis with 2-DG killed 85% within 72 hours after activation, suggesting that such Th1 cells were dependent on glycolysis. In contrast, 50% of the repeatedly stimulated murine Th1 cells survived when glycolysis was inhibited by 2-DG (Figure 3D), demonstrating that repeatedly stimulated T helper cells have alternative metabolic options, i.e., fatty acid oxidation. Blocking fatty acid oxidation with etomoxir for up to 8 days after a third reactivation showed that repeatedly stimulated murine Th1 cells use and are dependent on fatty acid oxidation, in particular in the late phase of expansion, between days 3 and 5 (Figure 3E). This was not the case for murine Th1 cells during the first week after the first reactivation (Figure 3F), which survived and proliferated even in the presence of etomoxir. The metabolism of repeatedly restimulated murine Th1 cells thus corresponds to that of PD-1^{high} human T helper cells isolated from inflamed synovia, in that they are dependent on fatty acid oxidation.

Inhibition of glycolysis of Th1 lymphocytes by *Twist1*. To analyze the role of *Twist1* in regulating the metabolism of T helper cells functionally, we used mice with a conditional, cell type–specific knockout of *Twist1* (CD4Cre × *Twist1*$^{fl/fl}$) (10) (Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40939/abstract). Both the *Twist1*-deficient mouse Th1 cells that had been stimulated once and those that had been stimulated repeatedly showed an increased rate of glycolysis, as reflected by an increased production of lactate, when compared to their wild-type counterparts (Figures 4A and B), indicating that *Twist1* inhibits glycolysis. *Twist1* selectively regulated the glycolysis of Th1, but not of repeatedly stimulated Th17 cells (Supplementary Figure 3,

available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40939/abstract).

Requirement of *Twist1* for the survival of Th1 lymphocytes via fatty acid oxidation. *Twist1* is indispensable for the survival of repeatedly stimulated Th1 cells via fatty acid oxidation. When glycolysis and glutaminolysis were blocked by 2-DG and 6-diazo-5-oxo-L-norleucine, respectively, *Twist1*-deficient mouse T helper cells did not survive (Figure 4C). In this situation, when fatty acid oxidation is the only metabolic pathway available, the competence to survive on this pathway depends on *Twist1*.

***Twist1* protects Th1 cells against reactive oxygen species (ROS).** Consistent with fatty acid oxidation being the major energy source for repeatedly stimulated murine Th1 cells, these cells show a higher degree of oxidative phosphorylation than murine Th1 cells stimulated once, both in the resting phase and after reactivation ex vivo (Figure 5A). Oxidative phosphorylation is associated with the generation of ROS. ROS promote peroxidation of lipids in cell membranes, an effect that has been suggested to be involved in the pathogenesis of rheumatoid arthritis (16,17). In T helper lymphocytes, lipid peroxides of the membrane, as detected by BODIPY C11 staining, were more abundant in T helper cells that had been stimulated 3 times, 8 days after reactivation, compared to T helper cells that had been stimulated once (Figure 5B). Compared to *Twist1*-deficient mouse Th1 cells, *Twist1*-sufficient (wild-type) mouse Th1 cells, both those stimulated once and those stimulated repeatedly, showed significantly reduced lipid peroxidation ($P = 0.002$ for cells stimulated once and $P = 0.0323$ for cells stimulated repeatedly) (Figure 5B). These data suggest that in addition to promoting oxidative phosphorylation through fatty acid oxidation, *Twist1* protects murine Th1 cells against harmful lipid peroxides, presumably by enhancing lipid peroxide scavenging.

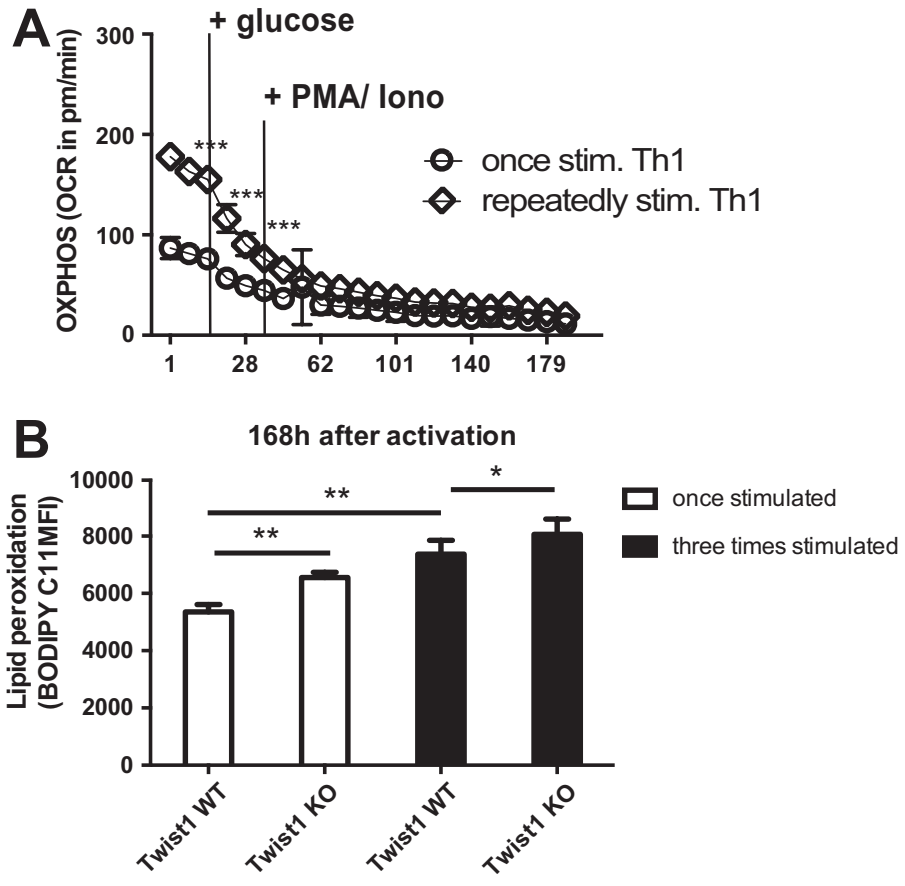

Figure 5. Repeatedly stimulated Th1 cells have increased oxidative phosphorylation. **A**, Oxidative phosphorylation (OXPHOS), measured by oxygen consumption rate (OCR) in Th1 cells stimulated once and Th1 cells stimulated repeatedly, before and after addition of glucose and restimulation with 12-myristate 13-acetate (PMA) and ionomycin (iono). Values are the mean ± SEM. *** = $P < 0.0001$ at 15 minutes; $P = 0.0009$ at 28 minutes; and $P = 0.64$ at 52 minutes, by paired *t*-test. Results are representative of 3 independent experiments. **B**, Levels of lipid peroxides in wild-type (WT) and *Twist1*-deficient (knockout [KO]) mouse Th1 cells stimulated once or stimulated repeatedly. Levels were determined by BODIPY 581/591 C11 (2 μ*M*) staining 7 days after the last reactivation with anti-CD3/anti-CD28 antibodies. Bars show the mean ± SEM. * = $P = 0.0323$; ** = $P = 0.002$, by paired 2-tailed *t*-test. MFI = mean fluorescence intensity.

DISCUSSION

Acute, protective immune reactions are characterized by the apparent elimination of the antigen triggering them. The lymphocytes involved are either eliminated as well, or they develop into memory lymphocytes, resting in terms of activation and proliferation (18–20). Persistent antigens, whether they be pathogens, tumor antigens, or autoantigens, are a challenge to the immune system. Lymphocytes involved in chronic immune responses have to adapt to chronic inflammation and in doing so may become refractory to physiologic and conventional immunosuppression, resulting in chronic inflammatory diseases. CD4+ T helper lymphocytes in chronic inflammation express the E-box–binding transcription factor *TWIST1*, which limits their ability to induce immunopathology (2) and promotes their resistance to apoptosis (10). In this study, we demonstrated that T helper lymphocytes in chronic inflammation also adapt their metabolism to become entirely dependent on fatty acid oxidation. These findings are consistent with the findings of ^{13}C-glucose tracing studies showing that chronically activated splenocytes in mice with lupus, and also chronically activated human T cells, have reduced lactate production, i.e., down-regulate their glycolysis rate (21).

Reasoning that persistent antigen would lead to repeated restimulation of the T lymphocytes recognizing it, we previously compared the transcriptomes of murine T helper lymphocytes activated once with those activated 3 or 4 times. We identified transcription factors like Hopx (22) and Twist 1 (2) and miRNAs like miR-182 and miR-148a (10,23) as selectively expressed in repeatedly activated T helper cells. Of particular interest is the E-box–binding transcription factor *Twist1*, originally identified as an anticachectic gene, with a strong dose dependency (24), since mice that are haploinsufficient for *Twist1* and its isologue *Twist2* are already prone to die young of cachexia (24). Expression of *Twist1* is induced in activated T helper lymphocytes by STAT4 signaling, and thus a characteristic of Th1 lymphocytes, and its expression increases upon subsequent reactivations (2). The comparison of *Twist1*-deficient and -sufficient, repeatedly activated T helper cells shows that *Twist1* controls the ability of the cells to survive on fatty acid oxidation.

We previously showed that up-regulation of the expression of *Twist1*, upon restimulation ex vivo, is a hallmark of CD4+ T lymphocytes isolated from the inflamed joints of patients with inflammatory rheumatic diseases, or from the inflamed intestinal mucosa of patients with inflammatory bowel diseases (2). *Twist1* expression apparently is a hallmark of pathogenic CD4+ T cells, as selective targeting of such cells via the *Twist1*-induced miR-148a ameliorates inflammation without affecting memory CD4+ T cells induced by vaccination (11). In this study we confirm the observation that *TWIST1* is highly expressed in CD4+ T lymphocytes isolated from the synovia of patients with JIA. We compared PD-1^{high} and PD-1^{low} synovial T helper cells, since PD-1 expression by these cells has been invoked as a correlate of their involvement in chronic inflammation (7,25–28). PD-1^{high} cells from the synovial fluid of patients with JIA indeed showed a significant up-regulation of expression of *TWIST1*, directly ex vivo. Like the repeatedly activated murine Th1 cells, the PD-1^{high} synovial human T helper cells were dependent on fatty acid oxidation for their survival, as evidenced by the fact that they could be killed by etomoxir, an inhibitor of fatty acid oxidation, unlike their PD-1^{low} synovial counterparts. This result points to an interesting therapeutic option to ablate PD-1^{high} CD4+ T lymphocytes selectively in chronic inflammatory diseases. Moreover, in preclinical models this would allow the determination of whether these cells indeed are the driving force of chronic inflammation, substantiating evidence that is thus far only correlative.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Chang and Radbruch had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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