Metabolic control of the scaffold protein TKS5 in tissue-invasive, proinflammatory T cells

Yi Shen 1, Zhenke Wen 1, Yinyin Li 1, Eric L Matteson 2, Jison Hong 1, Jörg J Goronzy 1 & Cornelia M Weyand 1

Pathogenic T cells in individuals with rheumatoid arthritis (RA) infiltrate non-lymphoid tissue sites, maneuver through extracellular matrix and form lasting inflammatory microstructures. Here we found that RA T cells abundantly express the podosome scaffolding protein TKS5, which enables them to form tissue-invasive membrane structures. TKS5 overexpression was regulated by the intracellular metabolic environment of RA T cells—specifically, by reduced glycolytic flux that led to deficiencies in ATP and pyruvate. ATP\textsuperscript{\textsubscript{pyruvate}} conditions triggered fatty acid biosynthesis and the formation of cytoplasmic lipid droplets. Restoration of pyruvate production or inhibition of fatty acid synthesis corrected the tissue-invasiveness of RA T cells in vivo and reversed their proarthritogenic behavior. Thus, metabolic control of T cell locomotion provides new opportunities to interfere with T cell invasion into specific tissue sites.

The autoimmune syndrome RA causes relentless joint inflammation that eventually damages cartilage and bone. T cells are key pathogenic drivers that sustain synoviocyte proliferation, tissue inflammation, neoangiogenesis, bony erosion and autoantibody formation. RA T cells age at a faster rate compared with T cells of healthy individuals, acquiring aged phenotypes 20 years prematurely. Functionally, they are prone to differentiation into proinflammatory effector cells that produce excess inflammatory cytokines and amplify the inflammatory activity of non-T cells in the synovial tissue.

The bias of RA T cells toward commitment to proinflammatory effector functions is mechanistically linked to defective metabolic regulation. Specifically, these cells do not sufficiently upregulate the glycolytic enzyme PKFB3, which curbs lactate and ATP generation. Instead, they shunt glucose into the pentose–phosphate pathway (PPP), hyperproduce NADPH, and accumulate reduced glutathione, thereby causing insufficient activation of the redox-sensing kinase ATM, a cell-cycle regulator and DNA-repair molecule. RA T cells with low amounts of ATM and reactive oxygen species (ROS) bypass the G2/M cell-cycle checkpoint, hyperproliferate and accelerate naïve-to-memory T cell conversion. ATM deficiency causes RA T cells to deviate toward differentiation into type 1 helper T cells (T\textsubscript{H}1 cells) and IL-17-producing helper T cells (T\textsubscript{H}17 cells), a phenotype corrected by ROS replenishment.

The PPP enables cells to build chemical constituents for the synthesis of macromolecules such as DNA, RNA, proteins and membranes—a critical prerequisite for biomass generation during massive T cell expansion. The PPP product NADPH functions as a reducing agent for anabolic reactions, such as lipid and cholesterol biosynthesis. Glucose delivers carbon for various lipid classes, but most of the de novo synthesized fatty acids (FAs) are incorporated into phospholipids for membrane biogenesis and localize to lipid rafts to participate in crucial membrane-based processes. The rate-limiting step of FA biosynthesis generates malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase 1 (encoded by ACACA). Subsequent synthetic steps and FA elongation require fatty acid synthase (EASN), stearoyl-CoA desaturase (SCD), and the fatty acid–coenzyme A ligase family to generate diacetylgllycerols, triacylglycerols and long-chain FAs. Through mitochondrial β-oxidation, FA catabolism feeds NADH and FADH\textsubscript{2} into the electron-transport chain and generates acetyl-CoA to fuel the citric acid cycle. T cells depend on glucose, amino acids and lipids as energy sources, but their metabolic preferences are tightly linked to particular life cycle stages.

Pathogenic T cell functions in RA include trafficking to the joint, diapedesis into the matrix, transit within tissue and the construction of compact microarchitectures. T cell diapedesis from blood vessels involves the formation of ‘invasive podosomes’ to build transcellular pores. Once in the matrix, T cells resort to nonproteolytic migration, maneuvering through extracellular space with an amoeba-like movement, adaptively changing shape to crawl along collagen fibrils and squeezing through pre-existing matrix gaps. Such T cell mobility requires the dynamic formation of cell protrusions, which are regulated by the cellular cytoskeleton to accomplish cellular spreading, polarization and ultimately cell motion. Small Rho family GTPases activate actin filament assembly factors to form sheet-like lamellipodia and ruffles, and finger-like filopodia. The adaptor molecule TKS5 is critically involved in determining the localization of membrane protrusions. Clustering of TKS5 at the plasma membrane facilitates F-actin assembly and enhances the interaction of multiple proteins assembled in podosomes, including the nucleation promoting factor N-WASP, the SH3/SH2 adaptor Grb2 and ADAM-family metalloproteases, and the adhesion receptor dystroglycan.

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1 Division of Immunology and Rheumatology, Department of Medicine, Stanford University School of Medicine, Stanford, California, USA. 2 Division of Rheumatology, Mayo Clinic College of Medicine, Rochester, Minnesota, USA. Correspondence should be addressed to C.M.W. (cweyand@stanford.edu).

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RA T cells serve as an excellent model system to define the molecular underpinning of pathogenic T cell behavior. Previous studies have defined a state of metabolic reprogramming in RA T cells: as PFKFB3\textsuperscript{hi}G6PD\textsuperscript{hi} cells, they divert glucose away from energy production toward biosynthetic fitness\textsuperscript{9,11}. Here we show that the rewiring of cellular metabolism renders RA T cells tissue invasive, directly promoting disease-inducing effector functions. The ATP\textsuperscript{lo}-pyruvate\textsuperscript{lo}-NADPH\textsuperscript{hi} metabolic state induced an energy-storage mode with excess synthesis of FAs and cytoplasmic lipid droplet (LD) deposition. This resulted in an upregulated T cell locomotion program, high production of the podosome scaffolding protein TKS5, and ultimately a functional phenotype of hypermotility. TKS5\textsuperscript{hi} RA T cells spontaneously formed actin- and cortactin-rich membrane protrusions, which empowered them to penetrate into non-lymphoid tissue and establish lasting inflammatory infiltrates. Two metabolic interferences corrected the tissue-invasiveness of RA T cells: enhanced activity of pyruvate kinase, and disruption of the downstream effect of excessive lipid storage by FA-synthase targeting. Both interventions amended TKS5 overproduction, prevented T cell entry into synovial tissue and provided effective protection from tissue inflammation \textit{in vivo}.

RESULTS

RA T cells upregulate the locomotion program

A key pathogenic property of RA T cells is their ability to rapidly invade the synovial membrane, maneuver through extravascular space and polarize into proinflammatory effector cells\textsuperscript{11,32}. The tissue infrastructure and chemokine milieu contribute to the attraction and retention of T cells\textsuperscript{32,33}. We explored whether RA T cells have intrinsic defects that increase their tissue-invasiveness.

To understand the T cell locomotion program, we first probed the migratory capacity of RA and healthy human activated naive CD4\textsuperscript{+} T cells in a chemokine-free system. Almost twice as many RA CD4\textsuperscript{+} T cells as healthy control cells migrated through porous membranes, even in the absence of a chemotactic gradient (Fig. 1a). To identify potential factors influencing CD4\textsuperscript{+} T cell motility, we examined the cells’ amoeba-like motion through extracellular matrix in a 3D collagen gel system that was also chemokine free. We loaded stimulated CD4\textsuperscript{+} T cells onto the gel surface, marked matrix-infiltrating cells with the nuclear stain DAPI, and quantified the invasion depth by optical sectioning every 100 µm (Fig. 1b–d). Compared with healthy T cells, RA CD4\textsuperscript{+} T cells entered the collagenous matrix in higher numbers and moved farther within the matrix (700 µm, compared with 500 µm for healthy cells (Fig. 1b–d)).

After an early period of random walk, T cells form organized membrane extensions for directed motion\textsuperscript{32}. We thus analyzed cell shape and membrane protrusions in proliferating RA and control CD4\textsuperscript{+} T cells (Fig. 1e,f). Given cortactin’s involvement in the formation of lamellipodia and invadopodia\textsuperscript{29,34}, we quantified the expression of this core element of the locomotion program. Protein expression of cortactin and of the cytoskeletal protein F-actin was more than doubled in patient-derived cells (Fig. 1f). Healthy CD4\textsuperscript{+} T cells had a thin peripheral actin cortex, whereas F-actin and cortactin clustered in broad-based membrane ruffles in RA T cells (Fig. 1e). Merged F-actin–cortactin expression was sevenfold higher in patient-derived cells compared with control cells (Fig. 1f).

We subsequently compared healthy and RA CD4\textsuperscript{+} T cells for the expression of a core group of genes associated with the cell’s locomotion machinery. We stimulated T cells and then analyzed gene expression by RT-PCR after 72 h. In a cohort of six patient–control pairs, ten motility-related genes had consistently higher expression in patient-derived T cells compared with cells from healthy controls (Fig. 1g). Specifically, RA T cells expressed higher transcript levels of SH3PD2A, which encodes the TKS5 adaptor protein\textsuperscript{35}, a molecule that facilitates the formation and stabilization of cellular projections. Flow cytometry confirmed overexpression of TKS5 (Fig. 1h,i).

To examine the pathogenic relevance of aberrant TKS5 expression in patient-derived T cells, we investigated the cells’ propensity for tissue invasion in a chimeric mouse model. We implanted human synovium into NSG mice and subjected the chimeras to adoptive transfer of human T cells\textsuperscript{9,33,36}. We tracked synovium-invasive T cells by immunohistochemistry and gene expression profiling of the explanted tissue grafts (Fig. 1j–l). Few healthy T cells infiltrated the synovial tissue (Fig. 1j–l). Transfer of RA peripheral blood mononuclear cells (PBMCs) produced a dense T cell infiltrate. Knockdown of SH3PD2A (Supplementary Fig. 1) before adoptive transfer disrupted the invasive capabilities of RA T cells (Fig. 1m–o). Conversely, TKS5 overexpression (Supplementary Fig. 1) in healthy T cells resulted in enhanced T cell invasion, such that TKS5\textsuperscript{hi} control T cells mimicked patient-derived T cells (Fig. 1p–r). We then tested whether TKS5 expression in activated CD4\textsuperscript{+}CD45RA\textsuperscript{+} T cells collected from subjects with RA correlated with the clinical activity of their joint disease. Subjects with higher disease activity, as measured through clinical disease-activity indices, transcribed progressively higher levels of SH3PD2A mRNA in their T cells compared with those in healthy controls (Fig. 1s; R = 0.63).

Thus, RA T cells were equipped to dynamically form membrane ruffles, spread and move, and with spontaneous upregulation of their locomotion gene module they rapidly invaded non-lymphoid tissue sites. The scaffolding protein TKS5 seemed to be nonredundant for this invasive behavior.

TKS5 expression is metabolically regulated

A key characteristic of RA T cells is their metabolic reprogramming. Specifically, RA T cells shunt glucose away from glycolysis toward the PPP and thus produce less ATP but more NADPH than their healthy counterparts\textsuperscript{9,11,17}. We therefore explored whether the T cell locomotion module is under metabolic control. We mimicked the slowed glycolytic breakdown in RA T cells by treating healthy CD4\textsuperscript{+} T cells with the PFKFB3 inhibitor 3PO (200 nM, 72 h). Conversely, we treated patient-derived T cells with the FA synthase (FAS) inhibitor C75 (20 µM, 72 h) to inhibit increased NADPH-dependent FA synthesis. We chose the concentrations of the inhibitors on the basis of dose–response experiments (Supplementary Fig. 2). Expression of the TKS5-encoding gene SH3PD2A was highly sensitive to metabolic interference. PFKFB3 blockade increased SH3PD2A transcript levels, whereas FAS inhibition decreased them (Fig. 2a,b). Expression of five additional genes from the locomotion module normalized in C75-treated RA T cells. PFKFB3 inhibition effectively increased motility gene expression by >40% for eight of the nine genes tested (Fig. 2c).

We used flow cytometric analysis to assess TKS5 protein expression. PFKFB3 inhibition increased TKS5 expression, thus mimicking the phenotype of RA T cells (Fig. 2d,e,h–j). Reduced FA synthesis had the opposite effect, correcting the TKS5 overexpression in RA T cells and restoring a normal phenotype (Fig. 2f,g,k–m). These data directly implicated glycolytic activity and FA synthesis as regulatory steps in locomotion function, thus suggesting a broader effect of metabolic control on normal and pathogenic T cell effector functions.

Aberrant lipid biosynthesis in RA T cells

To further characterize the lipid-biosynthesis program of RA T cells, which may be altered owing to the increased generation of NADPH

1026

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Figure 1  Hypermotility and tissue-invasiveness of RA T cells. (a) The migration of activated CD4+CD45RA+ T cells in Transwells without chemokines for 48 h (n = 10). (b-d) CD4+ T cell invasiveness in 3D collagen matrices, as measured by confocal imaging of DAPI-stained nuclei. (b) Representative confocal images at the indicated depths after 48 h. Scale bars, 200 µm. (c) DAPI indices (signal at defined depth/signal at surface) (n = 5 control–RA pairs). (d) Maximum invasion distances of the indicated cells (n = 5). (e,f) Confocal microscopy images and analysis of cytoskeletal organization and membrane protrusions. Membrane ruffles and podosomes were identified on the basis of F-actin–cortactin colocalization. (e) Representative micrographs showing colocalization of actin mesh with cortactin in lamellipodia and membrane ruffles. Scale bars, 20 µm. (f) Quantification of F-actin, cortactin, and F-actin+cortactin+ membrane protrusions (n = 5 control–RA pairs). (g) Gene expression profiling (RT-PCR) of genes involved in actin nucleation and cell motility. The data shown are from six RA–control pairs. Scaled z-score: red, high transcript levels; blue, low transcript levels. (h,i) Representative histogram (h) and bar graph (i) showing T cell TKS5 protein expression (n = 11 control–RA pairs). MFI, mean fluorescence intensity; FMO, fluorescence-minus-one control. (j-l) Data from human synovium–NSG mice reconstituted with CD4+CD45RO− PBMCs from healthy individuals or RA patients. (j) Immunohistochemistry of synovial CD3+ T cells. Scale bars, 20 µm. (k) The percentage of tissue-invading T cells among total cells. (l) Transcript concentrations for TRB (encoding a T cell receptor). (m-o) Data from human synovium–NSG mouse chimeras reconstituted with RA CD4+CD45RO+ PBMCs transfected with small interfering RNA (siRNA) targeting SH3PXD2A or control siRNA. (m) Immunohistochemistry of synovial CD3+ T cells. Scale bars, 20 µm. (n) The percentage of tissue-invading T cells among total cells. (o) TRB transcript concentrations. (p–r) Data from human synovium–NSG mouse chimeras reconstituted with healthy CD4+CD45RO− PBMCs transfected with SH3PXD2A or control plasmid. (p) Immunohistochemistry of synovial CD3+ T cells. Scale bars, 20 µm. (q) The percentage of tissue-invading T cells among total cells. (r) TRB transcript concentrations. (s) RA disease activity as assessed by the Clinical Disease Activity Index (CDAI), correlated with TKS5 transcripts in activated CD4+CD45RA+ T cells. Data from 29 patients. a.u., arbitrary units. Data in a,c,d,f,i,k,l,n,q,r are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 (Mann–Whitney U-test). Data are pooled from three experiments (a,l), two experiments (b,g,k,l,n,q,r) or four experiments (s), or are from one experiment representative of three experiments (h), or are from one experiment representative of two experiments with 14 synovial grafts for each experimental group (j,m,p).
in the PPP, we activated naive CD4+CD45RA+ T cells from subjects with RA and from age-matched healthy controls by T cell antigen receptor cross-linking for 96 h and quantified the cytosolic lipid stores. Subjects with psoriatic arthritis (PsA) served as disease controls.

BODIPY staining showed accumulation of cytosolic neutral lipids in RA T cells, with BODIPY intensity eightfold higher in RA T cells than in control and PsA T cells (Fig. 3a,b). Naive as well as memory CD4+ T cells from RA subjects accumulated cytosolic LDs (Fig. 3c). To test whether LD formation occurs in individuals with RA in vivo, we stained tissue sections from RA synovial biopsies. Synovial T cells resembled ex vivo–stimulated T cells, harboring densely packed lipid bodies in their cytoplasm (Fig. 3d). As an additional readout of lipid biosynthesis, we quantified palmitic acid (C16) in stimulated T cells from healthy individuals and from subjects with either RA or PsA. Significantly elevated concentrations of palmitic acid were a distinguishing characteristic of RA T cells (Fig. 3e). Finally, we analyzed the gene module relevant for the process of droplet formation, including BSCL2, PLIN1 and CIDEC (FSP27). Expression for all three genes was selectively higher in RA T cells compared with that in healthy and PsA T cells (Fig. 3f). Kinetic studies showed no differences in ACACA, FASN and SCD transcripts in resting cells but higher induction in RA T cells compared with that in control cells 2–6 d after activation (Fig. 4c). Immunoblotting confirmed markedly higher expression of the corresponding proteins in RA T cells compared with controls (Fig. 4d).

To better understand the process of LD accumulation, we analyzed the expression of genes encoding key lipid biosynthesis molecules in a discovery cohort of ten subjects with RA and age-matched controls. We stimulated purified CD4+CD45RA+ T cells with anti-CD3/CD28-coated beads for 72 h. RT-PCR analysis showed markedly higher expression of 20 lipogenesis genes in patient-derived T cells compared with that in controls (Fig. 4a), which we confirmed in a validation cohort of six RA subjects and six matched controls (Fig. 4b). Most significant changes were seen in genes encoding enzymes that facilitate de novo FA synthesis (ACACA, FASN and SCD) or esterification (ACSL3/4/5/6 and DGAT1/2) (Fig. 4b). Three of five genes relevant for mitochondrial β-oxidation (HADHA, HADHB and ACDM) were expressed at significantly higher levels in RA T cells compared with controls (Fig. 4b).

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To induce anabolic lipid-biosynthesis program, which led to cytoplasmic storage of excess FAs. The shift toward anabolic metabolism occurred in the setting of reduced glycolytic activity and overall energy starvation.

The process of LD formation involved changes in lipid metabolic pathways. Reduced glycolytic activity and overall energy starvation led to a decrease in glucose uptake and the availability of ATP for energy metabolism. This was accompanied by an increased reliance on alternative energy substrates, such as fatty acids, which were imported into the cell and converted to lipids for storage.

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Not all genes related to intracellular lipid homeostasis were affected by the shunting toward lipid anabolism in RA T cells. CPT1 regulates the carnitine-dependent cytoplasm-to-mitochondria transport of long-chain fatty acyl-CoAs. Expression levels of CPT1A and CPT1B were indistinguishable between control and RA T cells (Fig. 4e). Also, genes involved in cholesterol biosynthesis, previously reported to be critical in the early expansion of CD8+ effector T cells, were not part of the metabolic signature distinguishing RA T cells from those of healthy controls (Fig. 4f). The lipid storage in RA T cells was highly sensitive to inhibition of FAS with 20 µM C75, which cleared cytoplasmic LDs (Fig. 4g–j), placing this enzyme at the pinnacle of the altered lipid-biosynthesis program.
In summary, after activation, RA T cells induced a broad, yet selective, lipogenic gene program focused on FA and LD formation, but sparing cholesterol production.

**Relationship of lipid droplet formation to glycolytic activity**

Next we aimed to connect the processes of glycolysis, LD formation and T cell locomotion. Quantification of core metabolites at the interface of glycolysis and FA synthesis demonstrated that RA T cells were low in pyruvate and ATP but had elevated levels of acetyl-CoA (Fig. 5a).

To mimic these conditions and generate an ATP-pyruvate state, we slowed glycolytic flux in healthy CD4+ T cells by treating them with the PFKFB3 inhibitor 3PO (200 nM), which forces pyruvate kinase into a high-activity tetrameric form (38,39). PFKFB3 silencing thus mimicked RA T cells. We also activated lipid-synthesizing RA T cells in the presence of ML265 (10 µM), which forces pyruvate kinase into a high-activity tetrameric form (38,39), or we treated the RA T cells with supplemental pyruvate (1 mM) (Supplementary Fig. 3). Pyruvate replenishment from extracellular or intracellular sources significantly reduced the LD load (Fig. 5f–i).

Enhanced glycolytic flux through ML265 or exogenous pyruvate reduced transcript concentrations for FASN, which encodes a key enzyme in FA synthesis (Fig. 5j), mechanistically connecting pyruvate availability to the lipogenic machinery. We considered whether the bias toward lipogenesis in RA T cells might be related to activators of peroxisome proliferator-activated receptor (PPAR) signaling, although treatment of human T cells with well-established PPAR-α or PPAR-γ activators has been reported to induce a potent anti-inflammatory program, with effective suppression of proinflammatory cytokines (40). PPAR-γ protein expression was similar in control and LD-containing RA T cells, and expression levels were independent of enhanced or reduced glycolytic flux (Supplementary Fig. 4), which suggests that it is unlikely that PPAR-γ signaling is involved in shifting the metabolic homeostasis of RA T cells.

These results delineated a direct effect of glycolysis on lipid anabolism and indicated that pyruvate and ATP deficiency promoted de novo FA synthesis and cytoplasmic deposition.

**PFKFB3 silencing induces tissue invasiveness**

To test whether the pinnacle defect in T cell hypermotility arises from insufficient glycolytic breakdown, we determined whether pharmacologic and genetic PFKFB3 impairment is sufficient to impose the RA phenotype on healthy T cells. We assigned synovium-engrafted NSF mice to one of four treatment arms: reconstitution with healthy CD4+ T cells transplanted with or without 3PO (200 nM). We quantified synovitis intensity by enumerating synovial CD3+ T cells and by analyzing the gene expression of ten key inflammatory mediators. PFKFB3 knockdown reduced tissue invasion by RA T cells transfected with control or PFKFB3-specific siRNA, or treated with or without 3PO (200 nM). PFKFB3 knockdown reduced amounts of PFKFB3-specific transcripts by 50% (Supplementary Fig. 5), thereby mimicking RA T cells (50,51). The reduction of PFKFB3 transcript levels was durable over >96 h (Supplementary Fig. 5), which allowed us to carry out in vivo testing of functional consequences. Suppression of PFKFB3 function promoted aggressive T cell infiltration followed by strong innate and adaptive inflammation (Fig. 6), including expression of TNFSF11, thus suggesting the recruitment and retention of RANKL+ T cells and tissue production of TNF, IL-6 and IL-1β (Fig. 6f).

Quantification of IFN-γ-producing effector T cells in the tissue lesions (Fig. 6d,e,j,k) showed that reduced PFKFB3 function resulted in a two- to threefold increase in the frequency of synovial T cells producing this effector cytokine.

Together, these data indicated a critical role for pyruvate generation in controlling T cells’ bioenergetics, tissue-invasiveness and proinflammatory potential.

**Metabolic interference corrects T cell tissue-invasiveness**

To investigate whether the locomotion program of RA T cells is amenable to metabolic interference in vivo, we used the human synovium–NSG model system. We transferred RA-patient–derived T cells into NSG mice engrafted with human synovial tissue (39,33,36). The RA T cells demonstrated high tissue invasion, which resulted in robust synovitis with tissue expression of TNFSF11 (encoding RANKL), TNF, IL-6 and IL-1β (Fig. 7). Tissue-resident T cells were characterized by expression of RANKL, strong IFN-γ and IL-17 upregulation (Fig. 7c,d,f,g), and dominance of the lineage-determining transcription factors.
T-bet and RORγt (Fig. 7c). Lipogenesis retardation by inhibition of FA synthesis with C75 (5 mg/kg body weight per mouse) efficiently suppressed tissue inflammation (Fig. 7), reduced the infiltration of RANKL+ T cells (Fig. 7a,b), diminished the overall density of the T cell infiltrate (Fig. 7c) and resulted in the depletion of IFN-γ+ T cell populations (Fig. 7f,g). Expression of Foxp3, a marker of anti-inflammatory regulatory T (Treg) cells, was unaffected (Fig. 7e). Chimeras tolerated C75 treatment, with no obvious toxic effects on transferred human cells (Supplementary Fig. 6). We then restored pyruvate synthesis with the PKM2 activator ML265 (10 mg/kg per mouse), which

**Figure 4** RA CD4+ T cells favor the anabolic lipogenesis program. Data are from CD4+CD45RA+ T cells from age-matched healthy individuals and RA patients, stimulated for 72 h. (a) Gene expression for 20 lipogenic genes, as profiled by RT-PCR. The heat map shows data from a discovery cohort of ten healthy–RA pairs. Scaled z-score: red, high transcript levels; blue, low transcript levels. (b) Gene sets for de novo FA synthesis, esterification, and FA β-oxidation pathways compared in a validation cohort of six healthy–RA pairs. Relative gene expression was measured by RT-PCR. (c) Expression kinetics of lipid-biosynthesis genes after T cell activation (n = 6 independent samples per time point). (d) Immunoblot analysis of key enzymes involved in FA synthesis (ACC, FAS and SCD1) in four control–RA pairs. Data are representative of eight experiments. (e) Expression kinetics of genes encoding carnitine acyltransferases, involved in lipid transport into mitochondria, after T cell activation (n = 5 independent samples per time point). (f) Expression levels of genes involved in cholesterol synthesis (n = 8 healthy–RA pairs). (g,h) Representative images (g) and quantification (h) of LDs deposited in the cytoplasm. The fluorescence signal of neutral lipids was quantified in C75 treatment groups (n = 5 patients per group). Scale bar, 20 µm. (i,j) Flow cytometry analysis of BODIPY-stained T cells. (i) Representative histogram. (j) Mean fluorescence intensities. n = 5 experiments. Data in b,c,e,f,h,i are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 (Mann–Whitney U-test). ns, nonsignificant. Data are pooled from one (b,f), two (c–e,g–j) or three (a) experiments.
corrected the metabolic disbalance in RA T cells (Fig. 5). Increased pyruvate availability resulted in striking beneficial effects in vivo (Fig. 7). Pyruvate kinase activation attenuated T cell accumulation, reduced the production of TNF and RANKL, and minimized amounts of T cell cytokines (IFN-γ and IL-17). In contrast to blocking of FA synthesis, ML265 treatment left IL6 and IL1B gene expression unaffected (Fig. 7e). ML265 was also less potent than C75 in preventing IFN-γ induced IL6 production (Fig. 5f). Intracellular lipid stores were quantified in BODIPY-stained cells by confocal microscopy. (f) BODIPY accumulation. Scale bar, 20 µm. The plots show fluorescence signal (c), flow cytometry results (d) and mean fluorescence intensity (e) for inhibitor-treated cells versus controls. (f–i) Glycolytic flux in RA CD4*CD45RA* T cells in which PKM2 was activated with ML265 (10 µM) and glycolysis-related energy production was augmented by supplementary pyruvate (PY; 1 mM). Intracellular lipid stores were quantified in BODIPY-stained cells by confocal microscopy. (f) BODIPY accumulation. Scale bar, 20 µm. The plots show fluorescence signal (g), flow cytometry results (h) and mean fluorescence intensity (i) for treated cells versus controls. Representative images and/or summary results from six (b, f, c, g) or eight (d, h, e, i) samples. (j) FASN transcripts measured by RT-PCR in CD4*CD45RA* T cells treated with vehicle, ML265 or pyruvate. Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 (Mann–Whitney U-test (a, c, g, j) or paired t-test (e, i)). Data are pooled from two experiments (a (left and middle), b–i) or one experiment with six (a, right) or five (j) individual experiments.

**DISCUSSION**

T cell pathogenicity depends on several functional domains, including trafficking to peripheral tissues and maneuvering in the tissue environment. Here we define glycolytic flux and FA biosynthesis as critical checkpoints in the inflammatory activity of T cells that ultimately determine membrane configuration and tissue invasiveness. T cells from individuals with RA reshape their membranes and are hypermotile and tissue-penetrating, which enables their proinflammatory effector properties in the joint. The energy-consuming process of tissue invasion by RA T cells is linked to an ATP*pyruvate* state, and pyruvate replenishment is sufficient to abrogate the cellular motility, tissue infiltration and inflammatory potential of these cells. The pinnacle defect appears to be malfunctioning glycolytic flux and shunting of glucose to the PPP, which diverts energy use to a synthetic pattern. Accordingly, impaired glycolytic breakdown in healthy T cells suffices to convert them into tissue-invasive and arthritogenic effectors. Shifting the balance between catabolic and anabolic glucose utilization might be an effective strategy to set the threshold for T-cell-dependent tissue inflammation.

To minimize heterogeneity within T cell populations, we focused on naive CD4*CD45RA* T cells, which exemplify massive biomass generation demands after activation, combined with the need for invasive capabilities to reach relevant tissue sites. RA T cells are distinguished from their healthy counterparts by dampened glycolytic breakdown, reduced pyruvate production, and accelerated PPP shunting. Instead of adopting a primarily catabolic program, they utilize anabolic biosynthesis. FA overproduction leads to cytoplasmic LD deposition commensurate with biosynthetic prerequisites for massive growth, cell cycle passage and accelerated naive-to-memory T cell conversion. The price is the exhaustion of the naive T cell compartment and the accumulation of end-differentiated effector memory T cells. After bypassing the G2/M
cell cycle checkpoint, RA T cells more rapidly commit to IFN-γ and IL-17 production, thus aborting the development of anti-inflammatory $T_{reg}$ cells.

An excess amount of NADPH predicts sufficient reductive elements to drive anabolic reactions, specifically lipid-anabolic pathways. Unexpectedly, the bias toward lipid biosynthesis was selective for saponifiable lipids, whereas genetic modules that support cholesterol homeostasis seem to be SREBP independent. Newly activated CD8$^+$ T cells rapidly upregulate lipid biosynthetic pathways, synthesizing both cholesterol and FAs. Sterol regulatory element–binding proteins (SREBPs) control the cholesterol content in such CD8$^+$ T cells, acting within hours of T cell activation and affecting T cell blasting. Interestingly, homeostatic T cell proliferation and T cell pool maintenance seem to be SREBP independent. We propose that cellular cholesterol signals mainly during very early T cell activation, whereas later stages of T cell expansion depend on FAs.

Figure 6 Reduced glycolytic flux renders CD4$^+$ T cells tissue-invasive and proinflammatory. Data are from CD4$^+$CD45RO$^+$ PBMCs isolated from healthy individuals, transfected with PFKFB3 siRNA or treated with the PFKFB3 inhibitor 3PO (200 nM), and adoptively transferred into human synovium-engrafted NSG mice. Data are presented as mean and s.e.m. from 20 different synovial tissues harvested 7 d after adoptive transfer and processed for immunohistochemical analysis or RT-PCR analysis of cytokine and transcription factor gene expression. (a,g) Immunohistochemical staining for tissue-resident CD3$^+$ T cells after treatment with PFKFB3 siRNA (a) or 3PO (g). (b,h) Quantification of TRB transcripts and of tissue-invading CD3$^+$ T cells, expressed as a percentage of the total number of cells, after treatment with PFKFB3 siRNA (b) or 3PO (h). (c,i) IFNγ and IL17 expression after treatment with PFKFB3 siRNA (c) or 3PO (i). (d,j) Coimmunofluorescence staining of tissue sections for IFN-γ and CD3$^+$ T cells after treatment with PFKFB3 siRNA (d) or 3PO (j). (e,k) Frequencies of IFN-γ$^+$CD3$^+$ T cells in tissue after treatment with PFKFB3 siRNA (e) or 3PO (k). (f,l) Expression of genes encoding lineage-determining transcription factors, key inflammatory markers and TKS5, as determined by RT-PCR, after treatment with PFKFB3 siRNA (f) or 3PO (l). Scale bars, 20 μm. *P < 0.05, **P < 0.01, ***P < 0.001 (Mann–Whitney U-test). ns, nonsignificant; a.u., arbitrary units. Data are representative images (a,d,g,j) and summary results (b,c,e,f,h,i,k,l) pooled from three experiments with 20 synovial grafts for each treatment arm.
Tissue penetration is an absolute requirement for joint-invading T cells. Beyond chemokine-guided trafficking, T cells must form protrusive membrane structures (lamellipodia, ruffles and/or filopodia) to maneuver through the matrix\(^4^2\) while remaining protease independent\(^2^7\). Membrane protrusions and amoeba-like crawling ultimately require cytoskeletal support. RA T cells are highly migratory\(^9\), and low LSP1 expression promotes migration to tissues and lymph nodes, which exacerbates disease severity\(^4^1\). Overall, motility relies on actin nucleation in the membrane, such that an actin mesh can propel the cell across a substrate. Accordingly, we examined a core module of motility genes covering key pathways in actin polymerization, cytoskeletal rearrangement and membrane reshaping. Higher expression of the motility gene module corresponded to hypermotility and tissue-invasiveness. Crawling through extracellular space is a membrane-centered process; hence the coupling of high mobility with anabolic metabolism. Cellular ATP content seems to be of less relevance, as the highly motile T cells are pyruvate and ATP deprived\(^9,1^1\) yet switch to an anabolic program. Pyruvate supplementation and enhanced glycolytic flux efficiently cleared cytoplasmic lipid deposits and corrected the hypermigratory and hyperinflammatory phenotype of RA T cells. Thus, excess lipid storage occurred in the setting of ATP deficiency. Mitochondrial function remained intact in RA T cells, and they generate sufficient citrate to support FA synthesis. However, the AMPK-controlled loop of the coordination of cellular metabolism with functional behavior seems to be defective. Here chronic stimulation and microenvironmental abnormalities may alter the cells’ threshold settings and response patterns to metabolic cues. Experiments that reduced PFKFB3 function in healthy T cells demonstrated that glycolytic slowdown is a pinnacle defect in RA T cells. Genetic or pharmacologic PFKFB3 inhibition converted healthy T cells into arthritogenic T cells.

The current data assign metabolic checkpoint functions to intracellular ATP, pyruvate and FAs in controlling disease-relevant properties. By favoring anabolic G6PD over catabolic PFKFB3, RA T cells become primed for cellular growth and differentiation. Excess amounts of FAs convey fitness for immune-surveillance functions, such as migration from secondary lymphoid tissues and invasion of peripheral tissues to establish long-lasting, highly efficient tertiary lymphoid structures\(^4^6\). The combination of biosynthetic precursor and NADPH ensures active membrane biogenesis and, with sufficient ATP for survival, advances proliferation-prone and tissue-invasive T cells. Such T cells share several features with cancer cells, in which PPP shuttling meets anabolic demands and rapid division occurs, allowing the cells to bypass cell cycle

**Figure 7** Inhibition of FA synthesis or acceleration of pyruvate generation corrects the tissue-invasive and arthritogenic behavior of RA T cells. Data are from CD4+CD45RO+ PBMCs isolated from RA patients with active disease and adoptively transferred into human synovium–NSG chimeric mice. Mice were randomly assigned to one of three treatment arms (n = 14 synovial grafts per treatment arm): vehicle arm (vehicle injection), C75 treatment (5 mg/kg i.p. every other day) or ML265 treatment (10 mg/kg i.p. daily). (a) Representative tissue sections stained with anti-human CD3 (brown) and anti-RANKL (pink), showing infiltration of synovial tissue by CD3+RANKL+ T cells (red arrows). (b) Frequencies of CD3+RANKL+ T cells in tissue as a percentage of the total number of cells. (c) RT-PCR-based quantification of TRB transcripts. (d) IFNG and IL17 expression. (e) Expression of genes encoding transcription factors, key cytokines and TSK5, as determined by RT-PCR. (f) Immunofluorescence staining for IFN-γ and CD3. (g) Frequencies of tissue-resident IFN-γ+CD3+ T cells in the different treatment arms. Scale bars, 20 μm. Data are shown as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 (Mann–Whitney U-test). ns, nonsignificant. Data are representative images (a,f) and summary results (b–e,g) pooled from three experiments with 14 synovial grafts for each treatment arm.
checkpoints and invadopodia formation to seed peripheral tissues with metastases\(^1^,2\). AMPK regulates invadopodia formation in cancer lines, which reemphasizes the mechanistic link between metabolic homeostasis and tissue invasion\(^47\). Lessons learned from the current data should extend therapeutic strategies aimed at weakening the spread of proinflammatory T cells in RA and of malignant cells in cancer patients. Surplus FAs facilitate tissue invasiveness and chronic inflammation in RA. The metabolic constellation of low ATP, low pyruvate and high FA could be exploited to design highly efficient, tissue-penetrating antitumor T cells. Conversely, PPP dampening, strengthening of glycolytic flux and blockade of FA biosynthesis should all restrict the intrusion of T cells into susceptible tissue sites, and may provide a therapeutic option for many chronic inflammatory conditions.

### METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

**Note:** Any Supplementary Information and Source data files are available in the online version of the paper.

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### AUTHOR CONTRIBUTIONS

C.M.W., Y.S. and J.J.G. designed the study and analyzed the data. Y.S., Z.W. and J.J.G. wrote the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Patients and controls. Demographic and clinical characteristics of 162 RA patients and 20 patients with PsA enrolled at Stanford University Clinics, Stanford Hospital and the Mayo Clinic (Rochester, Minnesota) are presented in Supplementary Table 1. All RA subjects fulfilled the diagnostic criteria for RA and were positive for rheumatoid factor and/or anti-CCP. Disease activity was quantified through the Clinical Disease Activity Index (CDAI), a composite score of inflammatory load in RA patients. Individuals with cancer, uncontrolled medical disease or any other inflammatory syndrome were excluded. Healthy individuals, recruited through the Healthy Aging Cohort or the Stanford Blood Center, did not have a personal or family history of autoimmune disease. The Stanford University Institutional Review Board approved the study, and written informed consent was obtained from all participants. Numbers of independent experiments and/or of individual patients and control donors are stated in each figure legend.

T cell preparation and culture. CD4+CD45RO+T cells were purified by negative selection with the EasySep Human Naive CD4+ T Cell Enrichment Kit (STEMCELL Technologies). CD45RO+ cells were targeted for removal with anti-CD45RO, and labeled cells were separated with an EasySep magnet. Subset purity monitored by FACS routinely exceeded 95% (Supplementary Fig. 7). CD4+CD45RO+ T cells (2.0 × 106 cells per well) were stimulated with anti-CD3/CD28-coated beads (Life Technologies AS, Oslo, Norway; ratio, 1:1) and subsequently cultured for 72 or 96 h. To inhibit RA synthesis or glycolytic breakdown, we kept cells in C75 (20 µM; 10005276; Cayman Chemical) or 3PO (200 nM; 525320-25MG; EMD Millipore).

Transwell migration assay. CD4+CD45RA+ T cells were isolated and stimulated with anti-CD3/CD28-coated beads for 24 h. 1 × 105 cells per well were cultured in cell culture inserts (P18P01250; Millicell) with 400 µl of RPMI 1640 medium (11875153; Thermo Fisher Scientific) placed in 12-well plates that contained 600 µl of RPMI 1640 medium. After 48 h, cells in the inserts or the bottom wells were counted, and migration rates were calculated.

3D-collagen invasion assay. CD4+CD45RA T cells were stimulated with anti-CD3/CD28-coated beads for 24 h. 5 × 106 cells were seeded on top of a 3D collagen matrix (1.5 mg/ml PureCol bovine collagen type I (5005-100ML); Advanced Biomatrix; approximately 5 mm in thickness). Invasion depths were measured after 48 h of culture (37 °C, 5% CO2). The collagen matrix was fixed in 4% (vol/vol) paraformaldehyde and stained with DAPI (1:1,000; D21490; Thermo Fisher Scientific) for 1 h at room temperature. Individual wells filled with the collagen matrix were imaged with an LSM710 microscope with an EC Plan-Neofluar 10×/0.3-NA (numerical aperture) DICI objective lens (Carl Zeiss). Z-stacks were set to begin on the surface of the 3D collagen matrix and reach to the bottom of each well. Five random spots per well were measured, and the difference between the z-position of non-invading, surface-positioned cells and that of the deepest invading cells in the data set was recorded. We prepared optical sections by beginning at 0 µm and cutting progressively at 100-µm intervals. The DAPI signal of each image was quantified after import into ImageJ software. The relative ratios of invading/surface cells were calculated for each measurement.

Analysis of membrane protrusive structures. 5 × 105 CD4+CD45RA+ T cells were placed into 96-well plates (Corning) coated with fibronectin (10 µg/ml; 3420-001-01; Trevigen). After 30 min at 37 °C, nonadherent cells were removed and adherent cells were fixed, permeabilized and stained with anti-cortactin (PA5-29799; Thermo Fisher) and rhodamine phalloidin (R415; Thermo Fisher Scientific). Lysates were visualized with DAPI. The LSM710 system (Carl Zeiss) with a Plan Apochromat 63×/1.40-NA oil DICIlll objective lens (Carl Zeiss) was used to acquire images. Podosome-like structures and membrane ruffles were quantified on the basis of F-actin–cortactin colocalization.

Immunohistochemical staining. Cryosections (5 μm) of explanted synovial tissues were stained with H&E and examined for the density and arrangement of inflammatory infiltrates. Synovial T cells were identified by immunohistochemical staining with mouse or rabbit anti-human CD3 (1:50) alone or by costaining with anti-RANKL/CD254 (1:100) or anti-IFN-γ (1:100) as described7,36,40. Sections were analyzed with an Olympus BX41 microscope and CellSense software.

Lipid-staining fluorochromes and analysis. CD4+CD45RO+ T cells were stimulated and cultured for 96 h under standard conditions before being stained with BODIPY 493/503 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) (D3922; Thermo Fisher Scientific). Cell suspensions were incubated with 3 µM BODIPY for 1 h at 37 °C, washed with 1× PBS twice, and fixed with paraformaldehyde solution (4% (vol/vol) in PBS) (19934 1 LT; Affymetrix) for 15 min at room temperature. Cells were cytopsoted onto slides and mounted with ProLong Diamond Antifade Mountant with DAPI (P36962; Thermo Fisher Scientific). Sections were imaged with an LSM710 system (Carl Zeiss); an EC Plan-Neofluar 40×/1.30-NA oil DICIII objective lens (Carl Zeiss) and a Plan Apochromat 63×/1.40-NA oil DICIII objective lens (Carl Zeiss) were used to acquire images. The BODIPY intensity was quantified via the LSM710 colocalization function.

Fatty acid quantification. The free FA palmitic acid was measured in CD4+CD45RO+ T cells after 96 h of activation with Free Fatty Acid Quantification Colorimetric/Fluorometric kits (K612-100; BioVision).

Pyruvate measurements. CD4+CD45RO+T cells were stimulated with anti-CD3/CD28 beads for 72 h, and pyruvate levels were quantified with pyruvate assay kits (MAK071-1KT; Sigma-Aldrich).

Acetyl-CoA quantification. Acetyl-CoA concentrations were determined with a fluorometric kit (MAK039-1KT; Sigma-Aldrich). CD4+CD45RO−T cells were stimulated and cultured for 72 h before measurement.

qPCR. RNA extraction and RT-PCR were done as described39,48. Primer sequences are listed in Supplementary Table 2. Gene expression was normalized to 18S rRNA.

Immunoblotting. Cellular proteins were extracted with cOmplete Lysis-M EDTA-free (04719964001; Sigma-Aldrich). Expression levels were examined after standard western blotting as described39,48.

Plasmids and transfections. To manipulate SH3PXD2A gene expression, we transfected 20 nM ON-TARGETplus human SH3PXD2A siRNA-SMART pool (9646; GE Healthcare Dharmacon Inc.), control siRNA (Life Technologies, pEC2 M2-SH3PXD2A WT (90813; Addgene), or control vector into CD4+CD45RO− T cells that had previously been stimulated with anti-CD3/CD28 beads for 48 h. For silencing of PFKFB3, CD4+CD45RO− T cells were stimulated with anti-CD3/CD28 beads for 48 h and then transfected with 20 nM ON-TARGETplus PFKFB3 siRNA-SMART pool (5209; GE Healthcare Dharmacon Inc.) or control vector with the Amaxa Nucleofector system and the Human T cell Nucleofector kit (LONZA). Cells were cultured for 24 h under standard conditions before further experiments.

Human synovial tissue—NSG chimeras. NOD.Cg-Prkd+cd44+Ifgr1tm1Wfl/Sze (NSG) mice (Jackson Laboratory, Bar Harbor, ME) of both sexes were kept in pathogen-free facilities and used at the age of 10–12 weeks as described36,48. Pieces of human synovial tissue free of inflammatory infiltrates were placed into a subcutaneous pocket. After engraftment, mice were reconstituted with 10 million CD45RO− PBMCs as described48. Mice carrying the same synovial tissue and reconstituted with the same population of CD45RO− PBMCs were assigned to parallel treatment arms. The CD45RO− PBMCs were derived from (1) RA patients with highly active disease, (2) age-matched healthy controls, (3) CD45RO− PBMCs from RA patients transfected with SH3PXD2A siRNA or control siRNA, or (4) CD45RO− PBMCs from healthy donors transfected with SH3PXD2A overexpression plasmids or control vector.

For studies targeting glycolytic flux, chimeric mice were injected with 10 million CD45RO− PBMCs pretreated for 72 h as follows: (A) standard medium (control), (B) PFKFB3 inhibitor 3PO (200 nM), and (C) transfection with a PFKFB3-specific siRNA. For experiments targeting anabolic lipogenesis, chimeric mice carrying the same synovial tissue were randomly assigned to one...
of three treatment arms: (A) control (vehicle), (B) treatment with FAS inhibitor C75 (5 mg/kg/mouse every other day; 191282-48-1; Cayman Chemical), or (C) ML265 (10 mg/kg/mouse per day; 1221186-53-3; Cayman Chemical). Small-molecule reagents were delivered by i.p. injection over a period of 7 d.

At completion, synovial tissues were explanted and OCT-embedded (4583; Sakura Finetek USA) for histology or shock-frozen for RNA extraction.

All experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee at Stanford University.

**Antibodies.** The following primary antibodies were used: allophycocyanin/Cy7-conjugated anti-human CD3 (clone SK7; BioLegend), fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (clone RPA-T4; BioLegend), phycoerythrin (PE)-Cy5-conjugated anti-human CD8a (clone RPA-T8; eBioscience), PE-Cy7-conjugated mouse anti-human CD45RA (clone HI100; BD Biosciences), allophycocyanin-conjugated anti-human CD45RA (clone HI100; BioLegend), allophycocyanin-conjugated mouse anti-human CD45RO (clone UCHL1; BD Biosciences), Brilliant Violet 711-conjugated anti-human CD45 (HI30; BioLegend), anti-cortactin (PA5-29799; Thermo Fisher Scientific), anti-FAS mouse monoclonal antibody (mAb) (M01) (clone 3F2-1F3; Abnova), acetyl-CoA carboxylase (C83B10) rabbit mAb (Cell Signaling Technology), anti-SCD1 (C12H5) rabbit mAb (Cell Signaling Technology), anti-RANKL/CD254 antibody (12A668; Thermo Fisher Scientific), mouse anti-human CD3 mAb (clone F7.2.38; Dako), polyclonal rabbit anti-human CD3 (A045201-2; Dako), β-actin (8H10D10) mouse mAb (3700; Cell Signaling), HRP-linked anti-rabbit IgG (7074; Cell Signaling), WesternSure HRP-conjugated goat anti-mouse IgG (H+L) (LI-COR), anti-IFN-γ (ab25101; Abcam), anti-PPAR-γ (ab178860; Abcam), anti-PPAR-γ (81B8) rabbit mAb (Cell Signaling), and anti-Flag–HRP mAb (A8592-2MG; Sigma-Aldrich).

**Statistical analysis.** All data are presented as mean ± s.e.m. Data were analyzed with GraphPad Prism software. Statistical significance was assessed by paired t-test or Mann–Whitney test, as appropriate. A P value of <0.05 was considered significant.

**Data availability.** All data that support the findings of this study are available from the corresponding author upon request.

48. Li, Y. et al. Deficient activity of the nuclease MRE11A induces T cell aging and promotes arthritogenic effector functions in patients with rheumatoid arthritis. *Immunity* **45**, 903–916 (2016).