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Reduction of Proinflammatory Effector Functions Through Remodeling of Fatty Acid Metabolism in CD8+ T Cells From Rheumatoid Arthritis Patients

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Objective. Rheumatoid arthritis (RA) CD8+ T cells maintain their effector proinflammatory phenotype by changing their metabolism toward aerobic glycolysis. However, their massive energy and biosynthesis needs may require additional substrates other than glucose. Since systemic alterations in lipid metabolism have been reported in RA patients, we explored the role of fatty acid (FA) metabolism in CD8+ T cells to identify potential targets to curb their proinflammatory potential.

Methods. The expression of FA metabolism–related genes was analyzed for total CD8+ T cells and CD8+ T cell subsets in the data of RA patients and healthy controls retrieved from the GEO database. Functional assays were performed using peripheral blood CD8+ T cells isolated from RA (n = 31), psoriatic arthritis (n = 26), and spondyloarthritis (n = 21) patients receiving different therapies (disease-modifying antirheumatic drugs, biologics, and JAK inhibitors) and from healthy controls (n = 14). We quantified the expression of FA transporters, lipid uptake, intracellular FA content, cytokine production, activation, proliferation, and capacity to inhibit tumor cell growth, either with or without FA metabolism inhibitors.

Results. The CD8+ T cell gene expression profile of FA metabolism–related genes was significantly different between untreated RA patients and healthy controls. RA patients who had a good clinical response after 6 months of methotrexate therapy had significantly increased expression of FA metabolism–related genes. Cell surface expression of the FA transporters FA binding protein 4 (FABP4) and G protein–coupled receptor 84 (GPR84) and FA uptake were higher in effector and memory CD8+ T cells from RA patients compared to those from healthy controls. In vitro blockade of FA metabolism significantly impaired CD8+ T cell effector functions.

Conclusion. RA CD8+ T cells present an altered FA metabolism, which could provide potential therapeutic targets to control their proinflammatory profile, particularly therapies directed against the transport and oxidation of free FA.

INTRODUCTION

The treatment of chronic inflammatory autoimmunopathies such as rheumatoid arthritis (RA) has greatly improved with the introduction of cytokine blockers, cell-targeted therapeutic antibodies, and JAK inhibitors. However, in contrast to treatment of psoriasis, complete remission of arthritis in most patients over a prolonged treatment period has still not been achieved, indicating that we might be missing an important molecular component in the initiation and/or chronic propagation of the inflammatory response leading to chronic arthritis as exemplified by RA.

The interest in fatty acid (FA) metabolism involvement in RA dates back to the 1960s when initial studies revealed 40–60% more phospholipids and cholesterol in the synovial fluid of RA

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patients compared to the respective serum samples from healthy controls, and the increase of synovial FA was correlated with the increase of synovial protein concentration (1). Later, it was found that cholesterol-regulating therapies and low-fat diets could improve the symptoms of autoimmune diseases and the T cell–dependent autoantibody response (reviewed in ref. 2). This further underscored the role of lipid metabolism in chronic inflammation and autoimmunity. On this basis, we wanted to characterize lipid metabolism in the CD8+ T lymphocytes of RA patients and to investigate how lipid metabolism is influenced by immunosuppressive therapy.

Upon activation, T cells up-regulate glycolysis and FA and cholesterol synthesis to provide enough energy to meet biosynthetic demands and clonal expansion (3). Then they return to oxidative phosphorylation and FA oxidation when the effector functions are no longer required. However, in chronic autoinflammation, T cells maintain a permanent activated profile that is sustained by an aberrant metabolic profile (4). Studies have shown that RA CD4+ T cells have reduced mitochondrial activity, which prevents the oxidative usage of FA. This leads to an accumulation of intracellular lipid droplets and changes in cellular FA metabolism that are linked to increased proinflammatory effector functions but that can be abrogated by the FA synthase inhibitor C75 (5). While CD4+ T cell FA metabolism has proven to be a promising research target in RA, CD8+ T cell lipid metabolism in RA remains largely unexplored. In a previous study, we presented evidence that CD8+ T cells in RA reveal an altered, hyperglycolytic metabolic profile, which is accompanied by an increased glucose-derived synthesis of sphingophospholipids (4). Thus, it is likely that further studies of FA metabolism—not only of CD4+ T cells but also of CD8+ T cells—will reveal new insights into the immunometabolic mechanisms that maintain chronic inflammation in RA.

Upon CD8+ T cell receptor (TCR) activation, metabolic reprogramming is initiated and mechanistic target of rapamycin complex 1–mediated sterol regulatory element binding protein signaling up-regulates cholesterol and FA synthesis–related enzymes like acetyl-coenzyme A (acetyl-CoA) carboxylase, FA synthase, and hydroxymethylglutaryl-coenzyme A reductase, which have been proven to be necessary processes for clonal expansion and the acquisition of effector functions (3,6–8). Moreover, it has been shown that the synthesis of carnitines as well as the degradation of palmitate, which are needed for FA oxidation, are decreased in stimulated T cells, and de novo FA synthesis is initiated to increase cellular biomass in a Myc-dependent manner (9). Indeed, human RA CD8+ T cells contain elevated amounts of neutral lipids when compared to healthy CD8+ T cells (4). While tissue-resident memory CD8+ T cells cover their FA metabolism requirements by increasing FA import and FA oxidation, central memory CD8+ T cells fuel FA oxidation with de novo synthesized FA and triglycerides (10,11).

In RA CD8+ T cells, these differentiation-related demands for FA availability seem not only to be mediated by cell-intrinsic alterations in FA synthesis or FA oxidation pathway usage but also by differential FA transporter expression (7). The FA uptake in CD8+ T cells is mainly regulated by 3 families of cell surface transporters as follows: CD36 (12,13), FA binding proteins (FABPs)/FA transporting proteins, especially FABP4 and FABP5 (10), and G protein–coupled receptors (GPRs), especially GPR84 (14). Accordingly, recent findings have shown augmented palmitate and long-chain FA uptake rates by effector CD8+ T cells (4,11).

The present study explores the importance of transporter-controlled FA uptake in CD8+ T cells in RA patients receiving different therapeutic regimens as well as the impact of FA metabolism modulation on CD8+ T cell cytotoxic capacities (Figure 1A).

PATIENTS AND METHODS

Patients. Patient recruitment and blood sample collection took place at the Division of Rheumatology outpatient clinic at the Heidelberg University Hospital. Heparinized peripheral blood (80 ml per study participant) was collected from 31 patients with RA, 21 patients with spondyloarthritis (SpA), 26 patients with psoriatic arthritis (PsA), and 14 healthy donors as controls. RA and PsA disease activity was defined as in remission (Disease Activity Score in 28 joints using the C-reactive protein level [DAS28-CRP] <2.6), low disease activity (DAS28-CRP ≥2.6 and <3.2), or high disease activity (DAS28-CRP >3.2). SpA disease activity was defined by the clinical Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score as inactive (BASDAI <2.0) or active (BASDAI >3.5). Therapy responders after 6 months were defined by a drop in DAS28-CRP score of >0.6 points or when they retained a DAS28-CRP score <2.6. Therapy nonresponders after 6 months were defined by an increase in DAS28-CRP of >0.6 points or when their DAS28-CRP score remained >3.2.

Prospective patients and healthy controls were excluded from the study if any of the following applied: 1) diagnosis of malignant neoplastic disease or treatment thereof at any time point, 2) pregnancy, 3) active viral or bacterial infection, 4) vaccination within the past 4 weeks, 5) decline of consent for study inclusion and blood collection. All participants gave their informed, written consent, and the study was approved by the Institutional Ethics Committee of Heidelberg University (approval nos. S-096/2016 and S-969/2020). The clinical and demographic characteristics of the study cohort are summarized in Table 1.

CD8+ T cell isolation, in vitro stimulation, and cell culture. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation, and CD8+ T cells were purified by magnetic bead negative selection and cultured in vitro as previously described (4,15). A detailed description can be found in the Supplementary Methods, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42456.

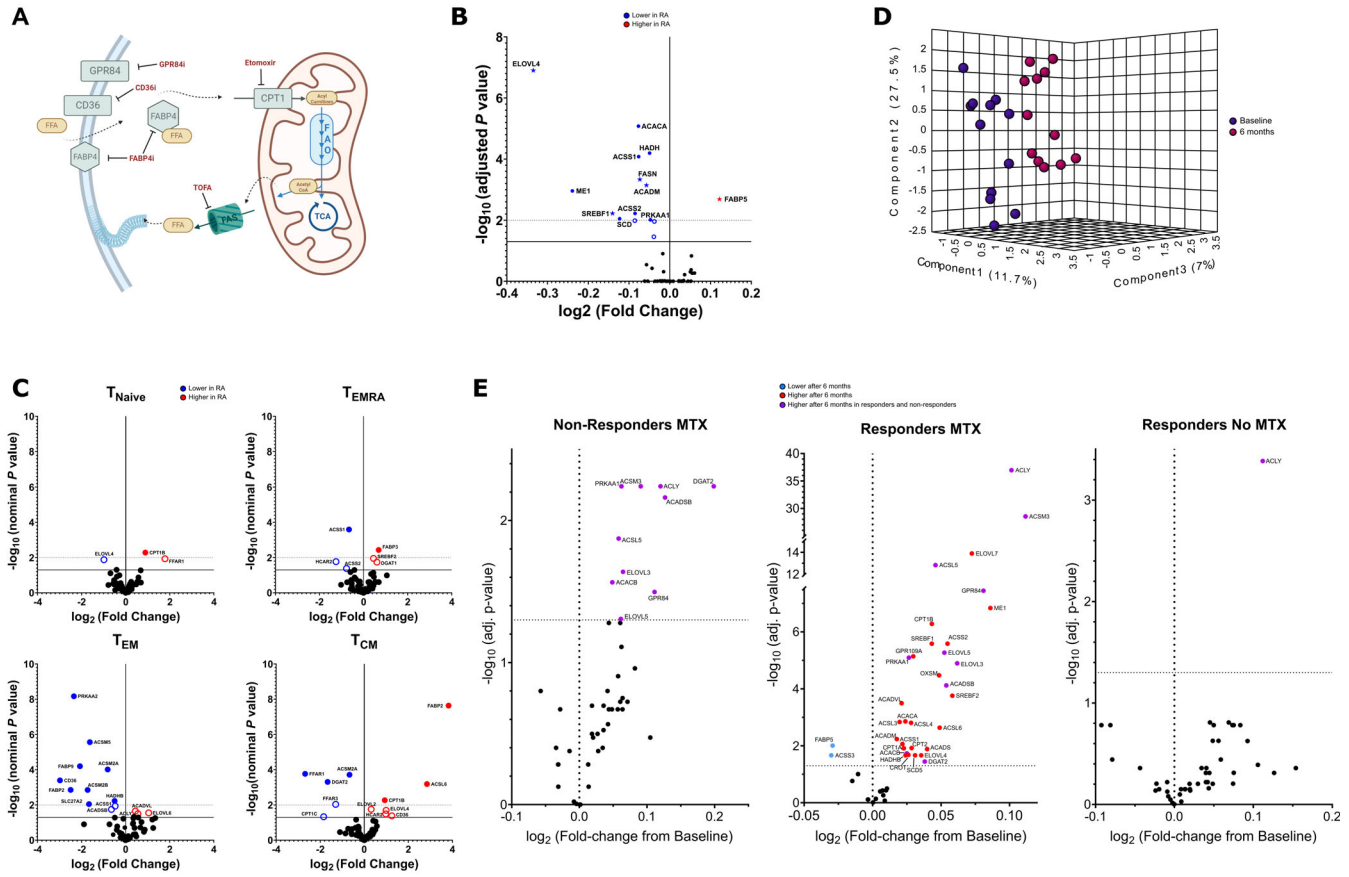

Figure 1. Gene expression of lipid metabolism–related genes in healthy control versus rheumatoid arthritis (RA) CD8+ T cells. **A**, Schematic representation of free fatty acid (FFA) metabolic pathways in CD8+ T cells, their major cell surface transporters, and the points of action for the studied inhibitors (created with BioRender.com). **B**, Volcano-plot showing gene expression differences for total CD8+ T cells between 32 controls and 18 therapy-naive RA patients. **C**, Volcano-plots showing gene expression differences in naive T, EMRA T, effector memory T (Tem), and central memory T (Tcm) cell functional subsets between controls and therapy-naive RA patients (n = 7–10 individuals per subset per group). **D**, Paired partial least-squares discriminant analysis with 3 principal components comparing CD8+ T cells from RA patients at baseline and after 6 months of therapy. **E**, Volcano-plots showing gene expression differences for total CD8+ T cells from RA patients at baseline and after 6 months of therapy with either methotrexate (MTX; in combination with other disease-modifying antirheumatic drug [DMARD] or alone) or other DMARD without MTX in 13 MTX nonresponders, 100 MTX responders, and 10 non-MTX responders. In volcano-plots, dotted horizontal lines indicate the false discovery rate threshold. Gene expression data was retrieved from the GEO database, accession nos. GSE97475, GSE97948, and GSE97810 (**B**,**D**,**E**), and GSE118829 (**C**). GPR = G protein–coupled receptor; FABP = fatty acid binding protein; TOFA = 5-tetradecyloxy-2 furoic acid; FAS = fatty acid synthesis; CPT = carnitine palmitoyltransferase; FAO = fatty acid oxidation; TCA = tricarboxylic acid cycle.

To modulate CD8+ T cell lipid metabolism, cells from 6 RA patients and 5 healthy controls were cultured with the following inhibitors: 75 μ*M* etomoxir (CAS registry no. 828934-41-4), an inhibitor of FA oxidation; 1 μ*M* 5-tetradecyloxy-2-furonic acid (TOFA), an inhibitor of FA synthesis; 100 μ*M* sulfo-succinimidyl oleate sodium, an inhibitor of FA transporter CD36; 1 μ*M* CAS 300657-03-8, an inhibitor of FA transporter FABP4; and 10 μ*M* GPR84 antagonist 8, an inhibitor of FA transporter GPR84.

Flow cytometry analysis of FA transporters in total CD8+ T cells. CD8+ T cells were stained with monoclonal antibodies against CD3, CD8, CCR7 (CD197), and CD45RA. FA transporters were stained with primary antibodies against CD36 (BioLegend), GPR84 (Bioss), and FABP4 (R&D Systems). For palmitate uptake analysis, CD8+ T cells were stained with BODIPY FL C_{16} (Thermo Fisher Scientific) according to the manufacturer's instructions. For the assays with FA metabolism inhibitors, cells were stained with either the viability dye Zombie Violet (BioLegend), BODIPY 493/503, anti-CD3, and anti-CD8 to determine intracellular total neutral lipid content, or with Zombie Violet, anti-CD69, anti–Ki-67, anti–granzyme B, anti-CD3, and anti-CD8 to quantify proliferation, activation, and cytotoxic mediator production. Further information on the flow cytometer calibration and analysis is available in the Supplementary Methods at https://onlinelibrary.wiley.com/doi/10.1002/art.42456.

Nuclear magnetic resonance (NMR) analysis of media. Hydrogen-1–NMR spectra acquisition of cell culture

Table 1. Clinical and demographic data of the patients and healthy controls providing samples for a functional assay study of CD8+ T cell fatty acid metabolism*

	Healthy controls (n = 14)	RA patients (n = 31)	PsA patients (n = 26)	SpA patients (n = 21)
Female	6	18	15	6
Male	8	13	11	15
Age, mean (range) years	36.7 (23–58)	59.5 (35–83)	54.7 (36–71)	50.9 (22–68)
Disease duration, mean (range) years	NA	14.3 (0.5–44)	9.6 (0.5–30)	16.7 (1–45)
DAS28-CRP score, mean (range)	NA	2.2 (0.96–4.04)	2.6 (0.97–6.48)	NA
Active (DAS28-CRP >3.2), % of patients	–	19.4	23.1	–
Remission (DAS28-CRP <2.6), % of patients	–	51.6	53.8	–
BASDAI score, mean (range)	NA	NA	NA	3.2 (0.6–6)
Active (BASDAI >3.5), % of patients	–	–	–	33.3
Remission (BASDAI <2.0), % of patients	–	–	–	28.6
CRP, mean ± SEM mg/liter	NA	4.8 ± 0.9	6.6 ± 1.7	5.1 ± 1.1
Antibodies				
Rheumatoid factor positive	NA	24	0	0
Rheumatoid factor negative	NA	7	26	21
Anti-CCP positive	NA	28	0	0
Anti-CCP negative	NA	3	25	21
HLA–B27 positive	NA	NA	0	16
HLA–B27 negative	NA	NA	26	5
Body mass index, mean kg/m^2	24.2	26.1	28.9	26.4
Therapies received				
MTX	NA	19	13	2
MTX dose, mean ± SEM mg†	–	13.3 ± 0.8	14.0 ± 1.3	15
Glucocorticoid	NA	10	9	2
Glucocorticoid dose, mean ± SEM mg/day†	–	3.7 ± 0.6	6.4 ± 1.2	5
Glucocorticoid plus MTX	NA	6	5	1
Leflunomide	NA	8	2	2
Leflunomide dose, mean ± SEM mg†	–	16.9 ± 1.3	17.5 ± 2.5	10
COX-2 inhibitor	NA	1	1	4
COX-2 inhibitor dose, mean ± SEM mg†	–	60 ± 0	100 ± 0	40.7 ± 16.9
Biologic DMARDs				
Anti–tumor necrosis factor	NA	9	7	8
Anti–IL-6R	NA	2	0	0
Anti–IL-17A	NA	0	1	1
Anti–IL-12/IL-23	NA	0	2	2
JAK inhibitor	NA	5	5	0

* Except where otherwise indicated, values are the no. of individuals. RA = rheumatoid arthritis; PsA = psoriatic arthritis; SpA = spondyloarthritis; NA = not available; DAS28-CRP = Disease Activity Score in 28 joints using the C-reactive protein level; BASDAI = Bath Ankylosing Spondylitis Disease Activity Index; anti-CCP = anti–cyclic citrullinated peptide; DMARD = disease-modifying antirheumatic drug; IL = interleukin; IL-6R = IL-6 receptor.
† The mean dose of each medication (methotrexate [MTX], glucocorticoid, leflunomide, cyclooxygenase 2 [COX-2] inhibitor) was calculated only for the patients taking that medication.

media was performed using a 600 MHz Bruker spectrometer equipped with a 5-mm indirect detection probe. Metabolite levels were quantified by deconvolution of the ^{1}H-NMR spectra using the line-fitting subroutine of NutsPro NMR software (Acorn NMR) as previously described (4,15). A detailed description can be found in the Supplementary Methods, https://onlinelibrary.wiley.com/doi/10.1002/art.42456.

Gene expression data analysis. Sixty-seven FA metabolism–related genes were identified using the Reactome database (16). Gene expression data generated from RNA microarrays for total blood CD8+ T cells from RA patients at baseline and after 6 months and from vaccinated (hepatitis B) controls were retrieved already normalized from the GEO (www.ncbi.nlm.nih.gov/geo/) data repository, accession no. GSE97476 (17). Raw gene expression data generated from RNA microarrays for blood CD8+ T cell subsets from RA patients under different therapeutic regimens and from controls were retrieved from GEO accession no. GSE118829 (18,19). Clinical and demographic data for each GEO data set are summarized in Supplementary Table 1, https://onlinelibrary.wiley.com/doi/10.1002/art.42456.

Prior to analysis, the data sets were manually curated to select only the 67 genes related to FA metabolism. Gene expression analysis was conducted using the NASQAR software (20) packages "GeneCountMerger" for conversion of gene ID numbers to gene names, duplicate removal, and addition of pseudocounts; "Create MetaTable" to merge clinical/demographic data to the gene expression data; and "DESeq2 Shiny" for normalization and differential gene expression analysis between groups. In the case of multiple probe sets for a single specific gene, the

probe showing the maximum average signal across the samples was selected. The heatmaps and the partial least-squares discriminant analysis were performed and generated using the statistical analysis package of MetaboAnalyst version 5.0 (21). The Benjamini-Hochberg method with a false discovery rate threshold of 5% was applied to account for multiple comparisons.

H838 proliferation assay using an Incucyte system. The human adenocarcinoma (non–small-cell lung cancer) epithelial cell line H838 was used to test the capacity of CD8+ T cells to inhibit tumor cell proliferation after culture with FA metabolism inhibitors. H838 culture and proliferation were carried out in an Incucyte S3 instrument as previously described (15). Details on the culture conditions and image data analysis are described in the Supplementary Methods at https://onlinelibrary.wiley.com/doi/10.1002/art.42456.

Cytometric bead array for cytokines. The titers of cytokines and cytotoxic molecules present in the media of CD8+ T cell cultures were determined using a LEGENDplex CD8/NK assay panel (BioLegend) according to the manufacturer's instructions. Cytometric bead array raw data analysis was performed using the cloud-based LEGENDplex Data Analysis Software Suite (BioLegend), and concentrations were normalized and reported per 1 million cells.

Mass spectrometry analysis of intracellular FA. To quantify the intracellular FA content, we followed a previously described procedure (4). We began by preparing FA methyl ester standards. Then we extracted the lipids from the cultured CD8+ T cells and prepared the FA methyl esters. Finally, we analyzed and quantified the FA content by gas chromatography mass spectrometry. A thorough description of the methodology can be found in the Supplementary Methods at https://onlinelibrary.wiley.com/doi/10.1002/art.42456.

Statistical analysis. Continuous data variables were tested for normal distribution using D'Agostino-Pearson omnibus normality test. Significant outliers were identified for each data set using Grubb's test/extreme studentized deviate test with an alpha value of 0.05, and values above the critical Z score were removed from the subsequent statistical analysis. Normally distributed, unpaired data sets were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test to determine individual *P* values and Brown-Forsythe test to determine the homogeneity of variances between groups. Normally distributed, paired data sets were compared using one-way paired ANOVA followed by Geisser-Greenhouse correction for multiple comparisons between single groups. Unpaired data sets not following a normal distribution were compared between groups using Mann-Whitney test followed by Dunn's multiple comparison test to determine the adjusted *P* value. Within each group, the differences between unstimulated versus stimulated versus inhibitor-treated cells were assessed by Wilcoxon's matched pairs signed rank test. Correlations between normally distributed data sets were determined using the parametric Pearson correlation coefficient. The Benjamini-Hochberg method with a false discovery rate threshold of 5% was applied to account for multiple comparisons. Differences were considered statistically significant for *P* values less than 0.05.

RESULTS

Expression analysis of lipid metabolism genes in RA CD8+ T cells. To investigate the expression of genes related to FA and cholesterol metabolism and transport in CD8+ T cells, we retrieved previously published data from 2 independent cohorts available through the GEO database, accession nos. GSE97475, GSE97948, and GSE97810 (17), and accession no. GSE118829 (19). Clinical and demographic characteristics of the data series are summarized in Supplementary Table 1 at https://onlinelibrary.wiley.com/doi/10.1002/art.42456.

Using the data set GSE97475, GSE97948, and GSE97810, we analyzed the differential gene expression before and after therapy initiation on total CD8+ T cells from 212 RA patients and 32 healthy controls and assessed the correlation of these changes to clinical and demographic data. To understand whether these differences could be reproduced in another independent cohort and, additionally, to determine whether they were subset dependent, we analyzed the data set GSE118829. In this cohort, the total CD8+ T cell pool of 10 healthy donors and RA patients receiving different therapy regimens (no therapy, methotrexate [MTX], interleukin-6 receptor [IL-6R], or tumor necrosis factor [TNF] blocker; n = 10 RA patients per group) were separated into functional subsets according to their CD45RA and CCR7 expression as follows: naive T cells (CD45RA+CCR7+), effector (EMRA) T cells (CD45RA+CCR7–), effector memory T (Tem) cells (CD45RA–CCR7–), and central memory T (Tcm) cells (CD45RA–CCR7+). We also investigated how different therapies influenced lipid metabolism–related gene expression.

Compared to healthy controls, CD8+ T cells from untreated RA patients had significantly decreased expression of several genes linked to lipid metabolism, whereas only *FABP5* was significantly overexpressed in RA (Figure 1B). Compared to untreated RA patients, we did not observe significant changes in the expression of several genes involved in FA metabolism in RA patients receiving treatment, irrespective of whether the patients were treated with conventional synthetic disease-modifying antirheumatic drugs (DMARDs) alone or in combination with glucocorticoids (Supplementary Figure 1A, https://onlinelibrary.wiley.com/doi/10.1002/art.42456).

The gene expression profiles were unique for the respective RA CD8+ T cell subsets (Figure 1C). RA naive CD8+ T cells showed a higher expression of FA transport–related genes (*CPT1B*

and *FFAR1*), while CD8+ T cells from controls had increased *ELOVL4*, a gene involved in FA elongation. In the effector compartment (EMRA T cells), both RA and control CD8+ T cells displayed an increased expression of genes involved in FA and cholesterol synthesis and FA transport (*SREBF2*, *DGAT1*, and *FABP3* in RA CD8+ T cells; *ACSS1*, *ACSS2*, and *CNTAR2* in control CD8+ T cells). In the Tem cell population, RA CD8+ T cells down-regulated the expression of a variety of genes for FA transport and sensing (*PKAA2*, *FAPB2*, *FABP9*, *CD36*, and *SLC27A2*) as well as for acetyl-CoA synthesis (*ACSS1*, *ACSM2A*, *ACSM2B*, and *ACSM5*), acetyl-CoA breakdown (*ACADSB*), and FA oxidation (*HADHB*). Similarly, in RA patients, Tem cell genes involved in FA synthesis (*ELOVL6* and *ACLY*) and in FA oxidation (*ACADVL*) were increased compared to controls. The gene expression in the Tcm cell compartment resembled that of the Tem cell compartment. Gene expression differences between RA patients and controls in the Tem and Tcm cell subsets were mostly related to FA synthesis–related, transport-related, and sensing-related genes. Control CD8+ Tem and Tcm cell subsets increased the expression of *FFAR1, FFAR3*, *ACSM2A*, and *DGAT2*, while in RA equivalents there was increased expression of *FABP2*, *CD36*, *CPT1B*, *CNTAR2, ACSL6*, *ELOVL2*, and *ELOVL4*. Paired partial least-squares discriminant analysis with 3 principal components of the subgroup that did not receive any type of therapy at baseline and afterwards received DMARD therapy for the first 6 months showed a significant separation of the 2 time points (accuracy 87.5%; R^2 = 0.935; Q^2 = 0.535) (Figure 1D and Supplementary Figure 1B, https://onlinelibrary.wiley.com/doi/10.1002/art.42456).

Next, we determined whether RA patients who responded to therapy after 6 months changed their FA metabolism–related gene expression profile. We observed an extensive alteration in gene expression in patients who responded to MTX therapy (combined with other DMARDs or as monotherapy) but not in patients who responded to other therapeutic regimens, and only few changes were observed in those patients who did not respond to MTX therapy (Figure 1E). To validate the influence of clinical parameters on the gene expression at baseline and to evaluate if baseline gene expression might be a possible predictor of DAS28 improvement, we performed a Spearman's correlation analysis. However, we found only weak to medium correlations (Supplementary Figure 1C, https://onlinelibrary.wiley.com/doi/10.1002/art.42456).

FA transporter expression on CD8+ T cells from RA patients receiving different therapies. In the analysis of the GEO data sets, we observed a greater difference between baseline and 6-month time points in the subgroup of RA patients who were therapy naive upon study enrollment in the genes encoding for the FA transporters CD36 and GPR84 (Supplementary Figure 2A, https://onlinelibrary.wiley.com/doi/10.1002/art.42456), which transport long-chain and medium-chain FA (22,23), respectively. Together with FABP4, they are the main FA transporters in T cells (reviewed in ref. 24). Furthermore, we had previously observed that upon TCR-mediated stimulation, CD8+ T cells, particularly from RA patients, imported considerable amounts of FA from the cell culture medium (4). We therefore analyzed the expression of these FA transporters at the gene (using the retrieved GEO data) and protein levels on the surface of total CD8+ T cells and their functional subsets that we isolated from controls and RA patients receiving different therapeutic strategies (Table 1).

At the gene expression level, *CD36* expression on the total CD8+ T cell pool did not differ between controls and RA patients and was not influenced by the type of therapy, while *FABP4* and *GPR84* displayed a significant up-regulation and down-regulation, respectively, in CD8+ T cells from RA patients treated with DMARDs versus those from controls (Figure 2A). When subdividing the CD8+ T cell pool into its functional subsets, we did not observe any therapy-related changes in the gene expression of *CD36*, *FABP4*, and *GPR84* within each subset (Supplementary Figure 2B, https://onlinelibrary.wiley.com/doi/10.1002/art.42456).

To confirm that the observations from the gene expression analyses could be reproduced at the protein level, we recruited RA patients and controls and isolated peripheral blood CD8+ T cells (Table 1). By flow cytometry analysis, we determined the influence of different therapies on the distribution of CD8+ T cell functional subsets (Figure 2B) within the total isolated CD8+ T cell pool. RA patients overall displayed a change in the distribution of the functional CD8+ T cell subsets when compared to controls, while RA patients receiving JAK inhibitor treatment and biologics therapy had significantly different subset distributions (X^2 = 8.2, P = 0.043 for all RA patients versus controls; X^2 = 8.4, P = 0.0391 for RA patients receiving JAK inhibitors versus RA patients receiving biologics therapy).

FABP4 and GPR84 median fluorescence intensity (MFI) values were elevated in CD8+ T cells isolated from RA patients in all memory subsets when comparing the pooled RA cohort and the RA subgroup receiving DMARD therapy with the control group, (Figure 2C). Even though the number of samples from RA patients receiving JAK inhibitor therapy was small, there was still a significant decrease in GPR84 expression in the memory subsets from this group compared to cells from patients receiving DMARD therapy (Figure 2C). The frequency of FA transporter populations within each functional subset was increased in RA patients compared to controls for all 3 transporters; however, this increase was less pronounced in the naive T cell compartment (Figures 2D and 2E). Differences from the control group were also observed for patients receiving DMARD or biologics therapy (Figure 2E). Therapy did not change the frequency or surface expression of CD36 and FABP4 within the total CD8+ T cell pool; however, GPR84 frequency (but not expression) was lower in RA patients receiving JAK inhibitor therapy compared to those receiving DMARD therapy (Supplementary Figure 2B, https://onlinelibrary.wiley.com/doi/10.1002/art.42456).

These findings could also be partially reproduced in CD8+ T cells from PsA and SpA patients compared to healthy controls

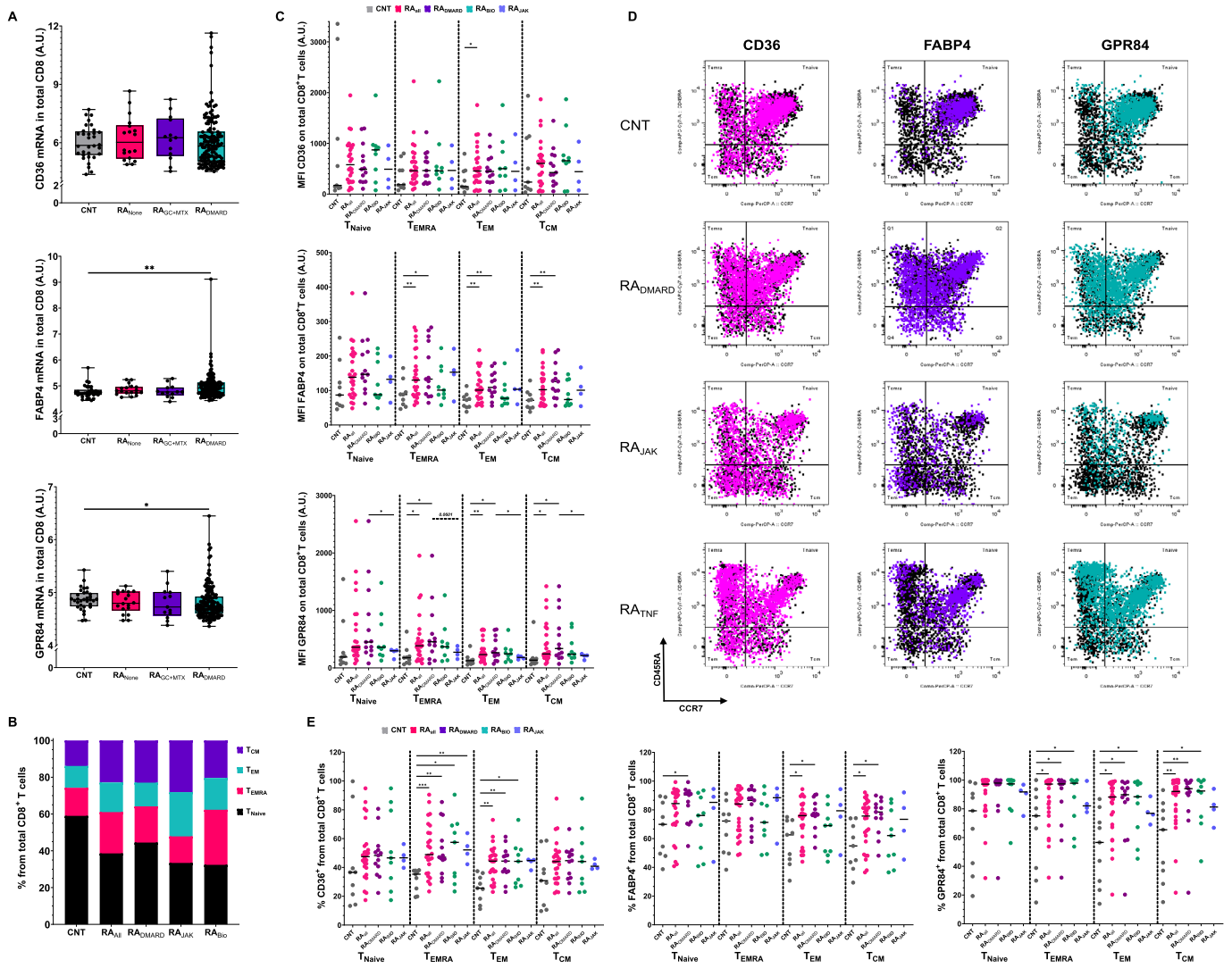

Figure 2. Fatty acid (FA) transporter expression on CD8+ T cell functional subsets from healthy controls (CNT) and RA patients. **A**, Expression of CD36, FABP4, and GPR84 were analysed on total CD8+ T cells and their functional subsets using data from 32 controls and RA patients stratified according to therapy (18 patients not receiving therapy, 13 patients receiving a glucocorticoid [GC] plus MTX, 149 patients receiving DMARDs). Gene expression data were retrieved from the GEO database (accession nos. GSE97475, GSE97948, and GSE97810). **B**, Distribution of the CD8+ T cell functional subsets in controls and RA patients stratified by therapy received. **C**–**E**, Fluorescence-activated cell sorting analysis of the FA transporters on CD8+ T cell functional subsets subdivided by therapy received, showing median fluorescence intensity (MFI) values (**C**), representative overlays of the FA transporter expression population on the CD45RA CCR7 distribution (**D**), and percentage CD36+, FABP4+, and GPR84+ in total CD8+ T cells (**E**). In **B**, **C**, and **E**, data are for 9 controls and 26 total RA patients, with 13 patients receiving DMARDs, 4 patients receiving JAK inhibitors, and 9 patients receiving biologic DMARDs (RA_{BIO}). In box plots, symbols represent individual patient samples. Boxes represent the 25th to 75th percentiles. Lines inside the boxes represent the median; whiskers show the range. In dot plots, symbols represent individual patient samples. Lines show the median. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. RA_{TNF} = RA patients receiving anti–tumor necrosis factor therapy. See Figure 1 for other definitions.

(Supplementary Figure 3, https://onlinelibrary.wiley.com/doi/10.1002/art.42456). No correlations were found between the expression levels or frequency of FA transporter–expressing CD8+ T cells and clinical or demographic parameters (Supplementary Tables 2–4).

Metabolic profiling of patients with autoimmune arthritis. To evaluate metabolic activity, we incubated the isolated CD8+ T cells in RPMI medium containing ^{13}C-enriched glucose either with or without TCR-mediated stimulation. Confirming our previous findings (4), CD8+ T cells from RA patients took up more glucose uptake and produced more lactate at baseline and upon stimulation. CD8+ T cells from RA patients receiving biologics therapy had higher fold changes in glucose uptake and lactate production upon stimulation compared to those from controls (P = 0.0454 for fold change in glucose

uptake; $P = 0.0363$ for fold change in lactate production) and compared to those from RA patients receiving DMARD therapy ($P = 0.0114$ for fold change in glucose uptake; $P = 0.0172$ for fold change in lactate production) (Figures 3A and 3B). Also, FA uptake (quantified by BODIPY C_{16} fluorescence intensity) was up-regulated in RA CD8+ T cells. However, FA uptake was not influenced by therapies or by other clinical or demographic parameters (Figure 3C and Supplementary Table 5, https://onlinelibrary.wiley.com/doi/10.1002/art.42456). The intracellular concentrations of palmitate (16:0), stearate (18:0), and arachidonate (20:4) did not significantly differ between the groups and upon in vitro stimulation (Figure 3D). However, the concentration of unsaturated oleate (18:1) was elevated in CD8+ T cells from SpA patients compared to those from controls and PsA patients (Figure 3D). Since FA free serum was not used in the culture media, we were unable to quantify the percentage of FA derived from ^{13}C-enriched glucose.

Altered CD8+ T cell immune functions after in vitro inhibition of FA metabolism. To further investigate the FA metabolism in RA patients and the impact of the 3 transporters on CD8+ T cell functions, we used in vitro the following inhibitors: etomoxir (FA oxidation inhibition), TOFA (FA synthesis inhibition), sulfosuccinimidyl oleate sodium (CD36 inhibitor), CAS 300657-03-8 (FABP4 inhibitor), and GPR84 antagonist 8 (GPR84 inhibitor). None of the inhibitors were observed to affect cell viability

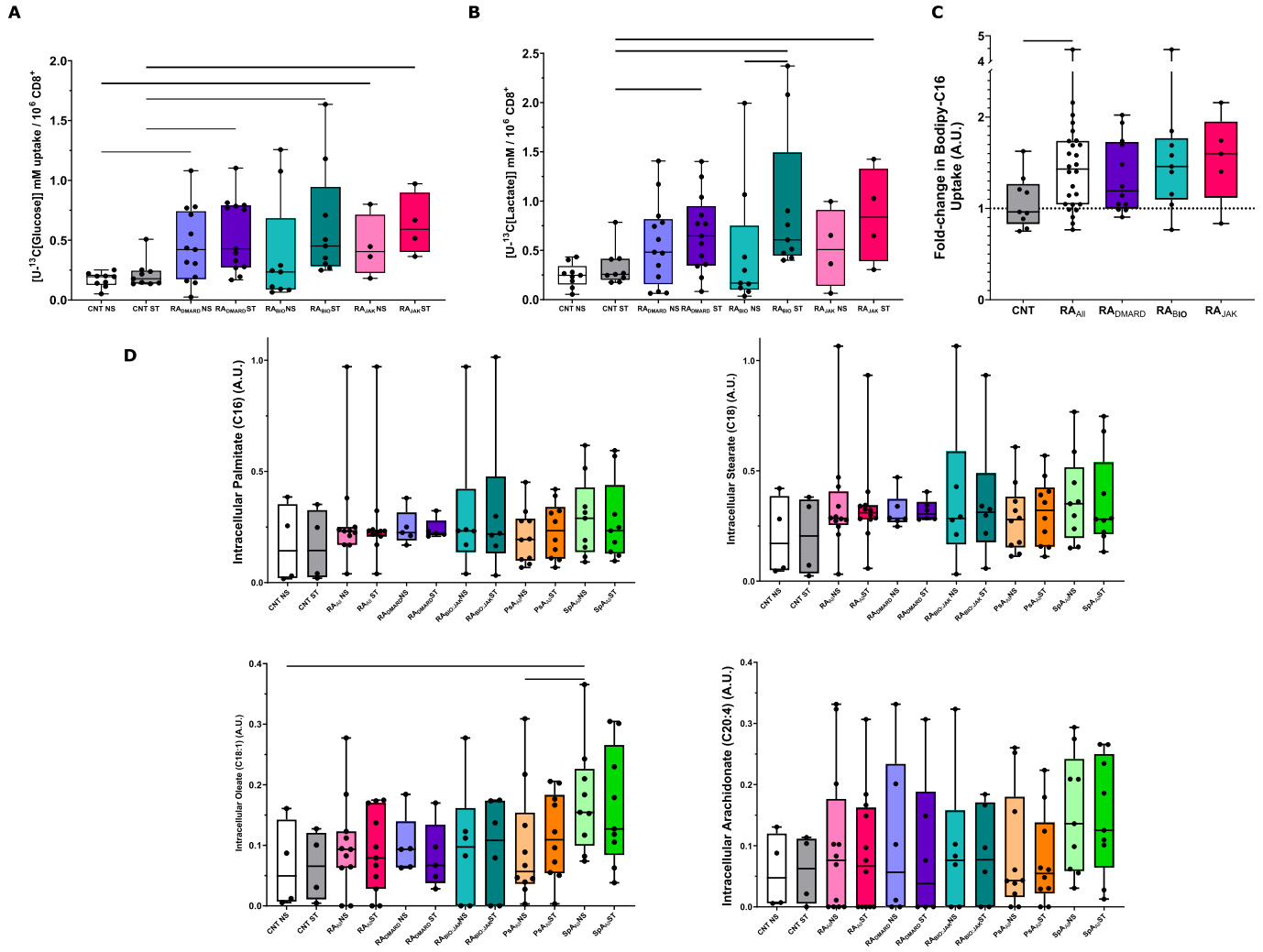

Figure 3. Metabolic analysis of CD8+ T cells from healthy controls (CNT) and RA patients receiving different therapies. Total CD8+ T cells were cultured in vitro unstimulated (NS) or under T cell receptor–mediated stimulation (ST). **A**–**C**, Cellular ^{13}C-enriched (U-^{13}C) glucose uptake (**A**), concentration of ^{13}C-enriched lactate in the cell culture supernatant (**B**), and fluorescence-activated cell sorting analysis of intracellular BODIPY C_{16} uptake (**C**) in samples from 9 controls, 13 RA patients receiving DMARDs, 9 RA patients receiving biologic DMARDs (RA_{BIO}), and 5 RA patients receiving JAK inhibitors. **D**, Mass spectrometry analysis of intracellular FA concentrations of palmitate, stearate, oleate, and arachidonate in samples from 4 controls, 11–12 total RA patients (5–6 patients receiving DMARDs, 6 patients receiving biologic DMARD or JAK inhibitor), 10 psoriatic arthritis (PsA) patients, and 9 spondyloarthritis (SpA) patients. Symbols represent individual patient samples. Boxes represent the 25th to 75th percentiles. Lines inside the boxes represent the median; whiskers show the range. * = $P < 0.05$. See Figure 1 for other definitions.

(Supplementary Figure 4A, https://onlinelibrary.wiley.com/doi/10.1002/art.42456). RA CD8+ T cells accumulated more neutral lipids upon TCR-mediated stimulation than those from healthy controls. While the presence of the different inhibitors during stimulation did not significantly reduce lipid accumulation in control CD8+ T cells, in RA CD8+ T cells, CD36 inhibitor, FABP4 inhibitor, and GPR84 inhibitor were able to reduce the neutral lipid content (Figures 4A and 4B). In vitro stimulation induced proliferation (quantified by Ki-67 expression) and activation (quantified by CD69 expression) in RA CD8+ T cells to a significantly greater extent than in control CD8+ T cells. However, their proliferative capacity and activation potential were slightly reduced in RA CD8+ T cells in the presence of etomoxir, FABP4 inhibitor, and GPR84 inhibitor and in control CD8+ T cells in the presence of CD36 inhibitor, GPR84 inhibitor, and TOFA (Figures 4C–F). The cytotoxic capacity of the cells was assessed by granzyme B expression levels. Etomoxir reduced the granzyme B–expressing CD8+ T cell population as well as the MFI in RA CD8+ T cells. TOFA treatment also induced a decrease of granzyme B MFI in RA CD8+ T cells but not in control CD8+ T cells. In contrast to RA CD8+ T cells, the granzyme B–positive population in control CD8+ T cells was decreased by CD36 inhibitor, etomoxir, and GPR84 inhibitor (Figures 4G–I).

For functional readouts, cytokine release by the cells was analyzed as well as the influence of secreted mediators present in the cell culture supernatants on the proliferation of the human lung adenocarcinoma cell line H838. As expected, upon TCR-mediated stimulation, RA CD8+ T cells released more cytokines and cytotoxic mediators than control CD8+ T cells (Supplementary Figures 4B and 5, https://onlinelibrary.wiley.com/doi/10.1002/art.42456). In accordance with the above findings, CD36 inhibitor, etomoxir, and GPR84 inhibitor affected control CD8+ T cells and decreased the secretion of cytotoxic and cytolytic molecules. Etomoxir, FABP4 inhibitor, and GPR84 inhibitor treatment resulted in a decrease in IL-10 secretion in RA CD8+ T cells and an increase in IL-4 and IL-2 secretion. H838 cell proliferation was significantly reduced upon addition of the conditioned media from stimulated CD8+ T cells from both RA patients and controls (Figure 4J). In the presence of inhibitors, H838 cell proliferation in conditioned media from stimulated control CD8+ T cells did not significantly change compared to conditioned

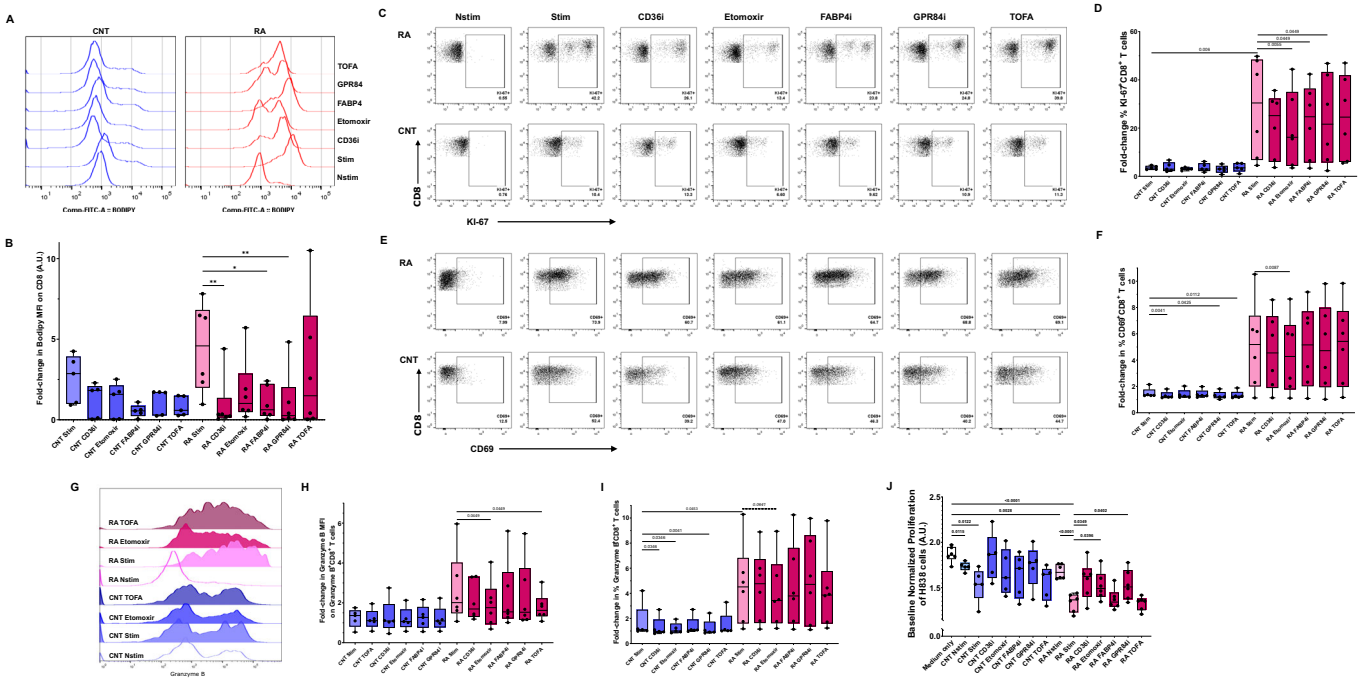

Figure 4. Effect of FA metabolism inhibitors on immunofunctional properties of CD8+ T cells from 5 healthy controls (CNT) and 6 RA patients. CD8+ T cells were cultured in vitro unstimulated (Nstim) or under T cell receptor–mediated stimulation (Stim) and with the following inhibitors: etomoxir (CPT1 inhibitor), CD36 inhibitor (CD36i), FABP4 inhibitor (FABP4i), GPR84 inhibitor (GPR84i), and TOFA (FAS inhibitor). Viability and effector functions were analyzed by fluorescence-activated cell sorting (FACS). **A** and **B**, Cell viability was assessed using fixable viability staining. FACS analysis of BODIPY (neutral lipids) uptake by total CD8+ T cells in the presence of the FA transporter inhibitors, with representative histograms (**A**) and cumulative fold change in median fluorescence intensity (MFI) (**B**) shown. **C**–**F**, FACS analysis of the proliferation capacity (Ki-67) (**C** and **D**) and activation (CD69) (**E** and **F**) of CD8+ T cells from controls and RA patients. Fold changes in **D** and **F** are compared to unstimulated samples. **G**–**I**, FACS analysis of granzyme B expression shown as a histogram (**G**) and box plots of fold changes in granzyme B MFI (**H**) and percentage granzyme B (**I**) in total CD8+ T cells. **J**, Assessment of the proliferation inhibition capacity of the secreted mediators in the supernatants on H838 cells (adenocarcinoma; non–small-cell lung cancer) using an Incucyte system. In box plots, symbols represent individual patient samples. Boxes represent the 25th to 75th percentiles. Lines inside the boxes represent the median; whiskers show the range. * = $P < 0.05$; ** = $P < 0.01$. See Figure 1 for other definitions.

medium lacking inhibitors. However, H838 cells cultured in conditioned media from RA CD8+ T cells treated with CD36 inhibitor, etomoxir, or GPR84 inhibitor proliferated significantly more than those cultured with conditioned media from TCR-stimulated CD8+ T cells without inhibitors. We could not find any statistically significant correlations between reduction in the concentrations of cytolytic mediators and H838 proliferation (data not shown).

DISCUSSION

Research on the role of lipid metabolism in the maturation process of CD8+ T cells has shown that FA oxidation is crucial for memory formation, while FA uptake from the extracellular milieu and FA synthesis are essential steps in the metabolic rewiring of effector CD8+ T cells (11,25). Studies on tumor-infiltrating CD8+ T cells have shown that hijacking CD8+ T cell lipid metabolism induced by the tumor microenvironment leads to a cytoplasmatic overaccumulation of oxidized lipids, which undergo peroxidation and compromise the anti-tumoral effector functions (13). However, while defects in lipid metabolism and how they contribute to inflammation have been thoroughly studied for RA CD4+ T cells (5), data on the role of lipid metabolism for the proinflammatory phenotype of RA CD8+ T cells are still largely missing. Additionally, it has been shown that RA patients treated with either MTX, the TNF blocker infliximab, or the JAK inhibitor tofacitinib, have a significant increase in serologic cholesterol levels and FA (26–29), which suggests that such therapies might equally lead to alterations in lipid metabolism at the cellular level for CD8+ T cells.

A recent large-scale, multiomics study on isolated total CD8+ T cells from British RA patients showed a significantly different gene expression profile compared to those from healthy vaccinated controls (17). Another gene expression analysis of sorted CD4+ and CD8+ T cell functional subsets from small groups of Japanese RA patients under different therapies revealed specific disease-related changes compared to controls (19). Since none of these studies focused on lipid metabolism, we reanalyzed the 2 gene data sets focusing on the expression of genes directly related to lipid metabolism. Additionally, the simultaneous analysis of these 2 independent cohorts allowed us to validate the findings and overcome the challenge of multiple hypothesis testing. The analysis of the total CD8+ T cell data set revealed that at the gene expression level, lipid metabolism appears to be differentially regulated in untreated, early-stage RA patients compared to healthy controls. After these previously untreated early-stage RA patients underwent 6 months of conventional synthetic DMARD therapy, predominantly with MTX, their CD8+ T cell lipid metabolism–related gene expression profile dramatically changed toward increased FA elongation, synthesis, transport, and oxidation. In general, good clinical response to therapy with MTX (either alone or in combination with other DMARDs) was accompanied by a major change in the expression of FA metabolism–related genes, which did not occur in patients who had a good response to other types of DMARD therapies, while only a few genes were differentially expressed in the patients with no DAS28-CRP improvement after 6 months of MTX therapy. Thus, an increase in FA metabolism in CD8+ T cells appears to be a characteristic specific to good clinical response and to MTX therapy.

Data from a serologic study found that RA patients 6 months after MTX therapy initiation had higher concentrations of total cholesterol, with increases in both low- and high-density lipoprotein cholesterol (30). Another study in which MTX was administered to healthy rats reported an increase in hepatic lipid concentration but a decrease in serum lipid concentrations with a simultaneous increase in palmitoyl-CoA synthetase and carnitine palmitoyltransferase (CPT), supposedly due to reduced choline availability (31). Together with our gene expression data, these studies suggest connectivity between the main MTX mechanisms of action in T cell inhibition of adenosine release and impairment of JAK/STAT and NF-κB signaling (reviewed in ref. 32), and increased lipid metabolism in RA at the systemic, tissue, and CD8+ T cell levels. In our study, even though the number of samples for the CD8+ T cell functional subset analysis was small, which limits data interpretation, we could observe that in patients who did not receive treatment that the subsets with effector functions registered the largest number of total changes in lipid metabolism–related genes. It has been reported that altered FA metabolism can impair the antitumoral function of CD8+ T cells (32), and in CD4+ T cells, impaired FA oxidation inhibits the formation of the effector memory pool (32). This suggests that the observed alterations in the expression of lipid metabolism–related genes in the effector subsets might fundamentally alter the effector functions of RA CD8+ T cells toward a chronic inflammatory response.

In contrast to memory CD8+ T cells, effector counterparts express higher levels of the FA transporter CD36 and have increased intracellular palmitate levels (11). All functional subsets of RA CD8+ T cells expressed CD36 more often, as well as FABP4 and GPR84, than those from healthy controls, and the cell surface expression of FABP4 and GPR84 was especially higher in RA effector and memory subsets. In mice, macrophages polarize toward a proinflammatory phenotype upon activation of the JAK/STAT pathway by FABP4, while an agonist of GPR84 triggers TNF production in human peripheral blood mononuclear cells (23,33). These studies may explain the decrease in the expression of these 2 transporters in RA patients receiving JAK inhibitors or TNF blockers.

Incorporating FA into the metabolic portfolio might help cells to survive in the nutrient-deprived microenvironment of the synovium, akin to what has been seen in tumor-infiltrating cells. In those tumor cells, acetyl-CoA derived from imported palmitate was used as a carbon source for the tricarboxylic acid (TCA) cycle to compensate for reduced glucose levels and to enable cells to maintain their anti-tumoral function (34). Due to the limited total CD8+ T cells isolated from some patients and controls, we could only manage

intracellular FA quantification for 30–50% of all donor groups. Additionally, the limitation on sample numbers in our study resulted in considerable variation in intracellular FA content. Nevertheless, we could observe that arthritis patients seem to have more intracellular palmitate (16:0), oleate (18:1), and stearate (18:0). Palmitate uptake induces T cell activation and increases their proinflammatory profile via phosphatidylinositol 3-kinase/Akt signaling (34) and, in contrast to memory CD8+ T cells, effector CD8+ T cells take up higher concentrations of palmitate (11). Oleate and arachidonate (24:4) were shown to decrease proliferation and proinflammatory functions of Jurkat cells, though arachidonate was, however, already at a 10-fold lower concentration (35). And whereas high intracellular concentrations of stearate triggered an inflammatory profile and apoptosis in mouse peritoneal macrophages, in mouse chondrocytes it promoted proinflammatory cytokine and lactate production and increased lactate dehydrogenase A expression (36,37). Therefore, it is plausible that in RA CD8+ T cells these FAs promote similar proinflammatory and metabolic profiles. Interestingly, the intracellular content of arachidonate, which is a precursor for the synthesis of systemically proinflammatory prostaglandins, did not appear to be affected in arthritis patients.

As FA free serum culture medium was not used, we were unable to reconstruct whether these changes in intracellular FA concentrations were derived from increased FA uptake or FA synthesis. Therefore, to analyze the impact of FA import from the extracellular milieu, we cultured CD8+ T cells in the presence of several FA transporter inhibitors. Upon TCR-mediated activation, CD8+ T cells switch to glycolysis in a mechanistic target of rapamycin complex 1–dependent manner (38) and decrease oxidative phosphorylation, and in RA, even naive CD8+ T cells present such a metabolic profile (4). Following activation of CD8+ T cells from arthritis patients, ~60% of ATP results from glycolysis (15), leaving the TCA cycle mainly for anabolic processes. Since aerobic glycolysis forces most of the pyruvate to be converted to lactate rather than to acetyl-CoA, the TCA cycle relies on acetyl-CoA resulting from FA oxidation. The limiting step in FA oxidation is the transport of acetyl-CoA into the mitochondria by CPT1. Thus, without FA transport into the mitochondria, the already diminished mitochondrial acetyl-CoA pool in RA CD8+ T cells cannot be replenished. This may explain why RA CD8+ T cells exposed to the CPT1 inhibitor etomoxir had a considerable loss of their immune functional capacity.

In contrast, when FA synthesis was inhibited, RA CD8+ T cell functionality was maintained. This suggests that FA uptake from the extracellular milieu might compensate for the reduced FA synthesis, as we did not see a major change in the total intracellular lipid content of TOFA-treated cells, nor in most immune functions. Even though we observed a dampening of RA CD8+ T cell immune activity upon inhibition of the FA transporters, single inhibition of each of them individually did not promote the same kind of functional alterations, which suggests that they can be compensated for by other FA transporters.

Overall, our data show an increase in parameters related with FA synthesis and breakdown, which suggests an increased FA turnover and a more dynamic FA metabolism. Based on these data, and those on FA metabolism in RA CD4+ T cells (5), which are different from activated healthy T cells, the use of FA oxidation and FA transport inhibition could be considered as new directions for future therapeutic strategies in RA, especially since the combined use of atorvastatin together with DMARD therapy in a placebo-controlled study in RA patients has shown a significant improvement in DAS28 score (39). Nonetheless, the potential hepatic toxicity of systemic FA metabolism inhibition requires that such interventions must first be tested in chronic polyarthritis mouse models, or otherwise, methods for cell-specific drug delivery must be developed. Since some of these inhibitors are currently entering the clinical trial phase in cancer patients, this might expedite their use in RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content. All authors approved the final version to be published. Dr. Kraus, Mr. Keck, and Dr. Souto-Carneiro had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Klika, Carvalho, Lorenz, Souto-Carneiro.

Acquisition of data. Kraus, Keck, Klika, Graf, Souto-Carneiro.

Analysis and interpretation of data. Kraus, Keck, Klika, Carvalho, Lorenz, Souto-Carneiro.

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