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$CD4^+CD8\alpha^{low}$ T Cell Clonal Expansion Dependent on Costimulation in Patients With Rheumatoid Arthritis

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Objective. $CD4^+CD8^+$ T cells are increased in patients with rheumatoid arthritis (RA). They are not only associated with joint erosions in established disease but are also present in the preclinical stages of RA. This study aims to further investigate their expansion in the context of T cell clonality in patients with RA, as well as their responsiveness to T cell–targeted treatment.

Methods. Single-cell RNA (scRNA) and single-cell T cell receptor (TCR) sequencing data were used to determine coreceptor expression and TCR sequences to assess the clonality of $CD4^+CD8^+$ T cells in patients with RA (n = 3) and healthy controls (n = 2). Peripheral $CD4^+CD8^+$ T cells and their subpopulations were measured in patients with RA (n = 53), patients with psoriatic arthritis (PsA; n = 52), and healthy donors (n = 50) using flow cytometry. In addition, changes in $CD4^+CD8^+$ T cell frequency were prospectively observed in patients with RA receiving therapy with abatacept for 12 weeks.

Results. We observed an increase of $CD4^+$ T cells expressing CD8α in patients with RA, both in comparison to patients with PsA and healthy controls. Clonality analysis revealed that these $CD4^+CD8\alpha^{low}$ T cells are part of large T cell clones, which cluster separately from $CD4^+CD8^-$ T cell clones in the scRNA sequencing (scRNA-seq) gene expression analysis. Treatment with abatacept significantly reduced the frequency of peripheral $CD4^+CD8\alpha^{low}$ T cells, and this was linked to reduction in disease activity.

Conclusion. In patients with RA, clonal expansion of $CD4^+$ T cell culminates in the emergence of peripheral $CD4^+CD8\alpha^{low}$ T cells, which are associated with disease activity and diminished upon abatacept treatment and could contribute to disease pathogenesis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by perturbations in the adaptive immune system that occur already in the preclinical phase of the disease before first symptoms occur.[1] After onset of disease, the leading clinical symptom is a chronic arthritis of small- and medium-sized joints. When left untreated, this leads to erosive destruction of bone and joint structures.[2]

The pivotal role of the adaptive immune system, and in particular of T lymphocytes, is emphasized by several experimental and clinical observations. The T cell pool of patients with RA is characterized by clonal expansions that are simultaneously detectable in peripheral blood and RA synovium. As a consequence, T cell receptor (TCR) diversity is decreased compared to age-matched controls, indicating a lack of naive, not clonally expanded T cells with unique TCR specificity.[3,4] Functionally, pathogenically relevant T cells in patients with RA have been

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shown to secrete proinflammatory cytokines but also to express natural killer cell receptors[5,6] and exhibit cytotoxic potential.[2] Several subpopulations of pathologically relevant T cells have been described, including $CD4^+CD28^-$ clones,[7] PD-1^+ follicular helper-like T cells,[8,9] or $CCR7^-CD27^-$ peripheral T helper cells.[10,11] The pathogenic role of T cells in patients with RA is emphasized by the clinical efficacy of a blockade of costimulatory CD80/CD86 interaction with abatacept, which ameliorates disease activity in patients with RA and, most importantly, prevents bone destruction and disability of patients.[12]

We were first to describe another T cell subpopulation, $CD4^+CD8\alpha^+$ T cells, as a pathologic subset in patients with RA, which is also detectable in peripheral blood as well as in rheumatoid synovium.[13,14] Importantly, $CD4^+CD8\alpha^{low}$ T cells were recently reported to emerge in the synovial membrane of healthy individuals who are anti–cyclic citrullinated peptide (CCP) positive despite a lack of clinical inflammation and who are considered to be at risk to develop RA. This could indicate a profound pathogenetic role of $CD4^+CD8\alpha^+$ T cells in the development of RA, even before arthritis occurs.[15]

The CD8 coreceptor is expressed on T cells as a dimer with two different isoforms, CD8αβ or CD8αα. CD8αβ is mainly expressed on cytotoxic $CD8^+$ T cells. When $CD4^+$ T cells upregulate CD8αα, it is expressed as CD8αα. Based on their expression level of CD4 and CD8α, $CD4^+CD8\alpha^+$ T cells can be divided into $CD4^+CD8\alpha^{low}$ and $CD8^+CD4^{low}$ T cells.[16] Until now, functional studies for $CD4^+CD8\alpha^+$ T cells were rare, but virus- and disease-specific functions were suspected.[16,17]

Phenotypically, these cells were described as polyfunctional $CD4^+CD8^{dim}$ T cells.[15] We report here that expanded $CD4^+CD8\alpha^+$ T cells in patients with RA preferentially have a $CD4^+CD8\alpha^{low}$ phenotype and are always part of large T cell clones, the bulk of which is $CD4^+CD8^-$. $CD4^+CD8\alpha^{low}$ T cells are increased in patients with more active disease, which implies their participation in disease pathogenesis. Clinically efficient therapy with abatacept is able to significantly reduce the frequency of those T cells, which corroborates the hypothesis that they are driving disease pathogenesis.

PATIENTS AND METHODS

Patients. Patients with RA fulfilling the American College of Rheumatology/EULAR 2010 classification criteria[18] were recruited from the Rheumatology Clinic of Leipzig University. For detailed clinical characterization of patients, see Supplementary Table 1. Age-matched healthy donors served as controls (58.45 vs 57.22 years of age, no significant difference, $P = 0.6$). Patients with psoriatic arthritis (PsA; with arthritis as the primary clinical manifestation) served as a disease control. Data from an independent cohort of patients with RA were used for LIR-1/PD-1 analysis. Written consent was obtained from all donors. This study was conducted with the approval of the University of Leipzig Ethics Committee (253/21-EK).

Longitudinal RA study. Two subgroups of patients with early RA were recruited into a longitudinal study. The first cohort consisted of patients with RA who were naive to disease-modifying drugs and who began treatment with receiving methotrexate (MTX). The second cohort comprised patients with RA in whom treatment with receiving MTX alone was not effective and who began additional treatment with receiving abatacept (CTLA4-Ig). $CD4^+CD8\alpha^+$ T cells were determined at the three time points: before initiation (visit 1), after 6 ± 1 weeks (visit 2) and after 12 ± 2 weeks (visit 3) of treatment initiation.

Cell preparation and flow cytometry. Peripheral mononuclear cells were isolated using Ficoll density gradient. Peripheral blood mononuclear cells (PBMCs) were stained for surface expression of the following markers: LIVE/DEAD fixable viability dye (405 nm; ThermoFisher Scientific); CD3, CD4, and CD8α (all rabbit polyclonal antibodies; Miltenyi Biotec); and CD8β (BD Biosciences). Cells were acquired on FACS LSR Fortessa (BD Biosciences) and analyzed using FlowJo version 10 software.

Single-cell RNA sequencing. For single-cell RNA sequencing (scRNA-seq), T cells were isolated from PBMCs from three patients with RA and two healthy controls (Supplementary Table 2) using Pan T cell isolation Kit (Miltenyi Biotec). T cells were then labeled for CD4 and CD8α using TotalSeq-C-antibodies (BioLegend): CD4 clone RPTA-T4, barcode sequence TGTTC CCGCTCAACT; CD8α clone RPTA-T8; barcode sequence GCTGCGCTTTCCATT. The cells were loaded with a maximum concentration of 1,000 cells/μL and a maximum cell number of 17,000 cells on a Chromium Chip G (10x Genomics). Gene expression, hashtag, and TCR libraries were generated according to the manufacturer's instruction using the Chromium version 1.1 chemistry (10x Genomics). Sequencing was conducted with a NovaSeq 6000 cartridge (Illumina) with 20,000 reads per cell for gene expression libraries and 5,000 reads per cell for TCR libraries.

Preprocessing and cell type annotation. Count matrixes have been created with 10x Genomics Cell Ranger (version 6.0.1) with the count function from raw data.[19] Data preprocession of gene expression count matrixes as well as antibody-derived tag (ADT) counts and TCR sequencing data have been conducted in R (version 4.2.3) with Seurat (version 4.3.0).[20] Each sample has been preprocessed individually. Ambient RNA has been removed with SoupX (version 1.5.2) to remove unwanted variance and improve clustering results.[21] TCR sequence data were imported with the djvdj package (version 0.1.0).[22]

Filtering has been applied on cell level and cells with a number of unique molecular identifiers (UMIs) <500, number of genes <300 and >4,500, genes per UMI >0.8, and fraction of mitochondrial genes >0.1 have been removed from further analysis. Filtering on gene level was applied to remove unwanted variance due to strong interfering biologic effects, which would otherwise affect the cluster analysis. Hence, genes were removed that relate to the apoptosis pathway in the Kyoto Encyclopedia of Genes and Genomes[23] as well as genes coding for TCRα, TCRβ, and TCRγ variables because they tend to create individual clusters. Among the remaining genes, only those were kept for further analysis, which have been expressed in at least 10 cells.

Subsequently, cell matrixes have been processed following the Seurat best practices. Count matrixes have been normalized, and cell cycle scoring has been applied to exclude the possibility of strong variation due to cell cycle effects. Count matrix transformation was applied with SCTransform() (version 0.3.5),[24] and the percentage of mitochondrial genes has been adjusted for. Principal component analysis was conducted with 50 components, and Seurat functions runUMAP(), FindNeighbors(), and FindClusters() were applied with the first 50 principal components and clustering resolution of 0.5 to compute preliminary clusters. Doublet detection has been performed with the scDblFinder() function from the scDblFinder package (version 1.12).[25]

Cell type prediction was conducted with the Seurat functions FindTransferAnchors() and MapQuery(), for which we annotated cells in the query data sets based on the previously published atlas of human PBMCs.[26] Feature barcodes were normalized and denoised with the dsb package (version 1.0.3).[27] After individual preprocessing, all samples were integrated with scvi_tools (version 0.20.3)[28] in R with reticulate (version 1.26)[29] with default settings while removing the effect of the laboratory covariate. A final clustering analysis was conducted on the integrated data set with Seurat functions FindNeighbors(), FindClusters(), and runUMAP() using the first 10 dimensions from the scvi assay. Subsequently, only cells were included if they were annotated as $CD4^+$ or $CD8^+$ and not as proliferating T cells and not classified as doublets and carried exactly one TCRα and one TCRβ sequences.

T cell analysis. By visual inspection, CD4 and CD8α thresholds were defined for ADT signal levels. Next, T cell clonotypes are cells with the same paired TCRα and TCRβ sequences. TCR sequences were assigned to the aforementioned ADT classes. TCR sequences, the copies of which occurred in $CD4^+CD8^+$ as well as $CD4^+CD8^-$ T cells, have been marked as $CD4^+CD8\alpha^+$ with $CD4^+$ origin and $CD4^+CD8\alpha^+$ with $CD8^+$ origin, respectively. Figures were created with ggplot2 (version 3.3.5).[30]

To determine the size of expanded T cell clones, the number of individual cells expressing identical receptors was used. Because the number of $CD4^+CD8^+$ T cells within expanded clones is likely to increase in proportion to the number of cells sequenced, an in silico experiment was performed by iteratively decreasing the data set by randomly selecting a subset of cells and computing the number of clonally expanded cells. The results show that the number of detected expanded clones increases with increasing numbers of analyzed cells, but this increase is stagnating and converges to an upper limit, with the result that a maximum appears to be reached at 100% of the original sample. This would suggest that the larger the data set, the better the true fraction of expanded clonotypes is captured. Nevertheless, it cannot be excluded that the frequency of rare cells detected would increase if significantly larger data sets were available (Supplementary Figure 1).

Statistical analysis. Statistical analysis of flow cytometry data was performed using GraphPad Prism 9.0 (GraphPad Software). First, a normality test was performed. Differences among groups in medians or means were analyzed with unpaired *t*-test or Mann–Whitney rank sum test. The Wilcoxon test and the paired *t*-test were used to evaluate differences in the course of therapy. Correlations were assessed using Pearson's product-moment correlation or Spearman's rank correlation coefficient. *P* values less than 0.05 were considered statistically significant.

RESULTS

$CD4^+CD8\alpha^+$ T cells are associated with patients with RA but not those with PsA. To investigate the relevance of $CD4^+CD8\alpha^+$ T cells in patients with RA independently of treatment effects of long-standing immunosuppression, we recruited a study cohort of patients with recent-onset RA (n = 53, median disease duration two years; Supplementary Table 1). A total of 46 patients with RA had not previously been treated with biologics or JAK inhibitors, and 21 were disease-modifying antirheumatic drug (DMARD) naive entirely. As a control, patients with PsA (n = 52) were enrolled in the study. In addition, healthy donors (n = 50) were recruited, and both control groups were meticulously matched for age and sex.

Flow cytometric analysis of CD4 and CD8α coreceptor expression confirmed the previously observed increase of the frequency of $CD4^+CD8\alpha^+$ T cells in patients with RA in comparison to healthy controls (median 1.5% vs 1.15%, $P = 0.008$; Figure 1A and B). No significant difference between patients with PsA and controls was discernible. We could also confirm the previously reported correlation between the frequency of $CD4^+CD8\alpha^+$ T cells and an individuals' age both in patients with RA ($r = 0.53$, $P < 0.001$; Figure 1C) and with PsA ($r = 0.3$, $P = 0.03$, data not shown). This trend did not reach statistical significance in healthy controls (data not shown).

In the cohort with RA, median disease activity was comparatively high, as indicated by a Disease Activity Score in 28 joints (DAS28) of 3.8 (interquartile range 2.6–4.6), whereas erosive disease was rare. Notably, disease activity was higher in patients with an expanded $CD4^+CD8\alpha^+$ T cell population (>2% of $CD3^+$ T cells), as evident in a higher DAS28 score (median 4.2 vs 3.6,

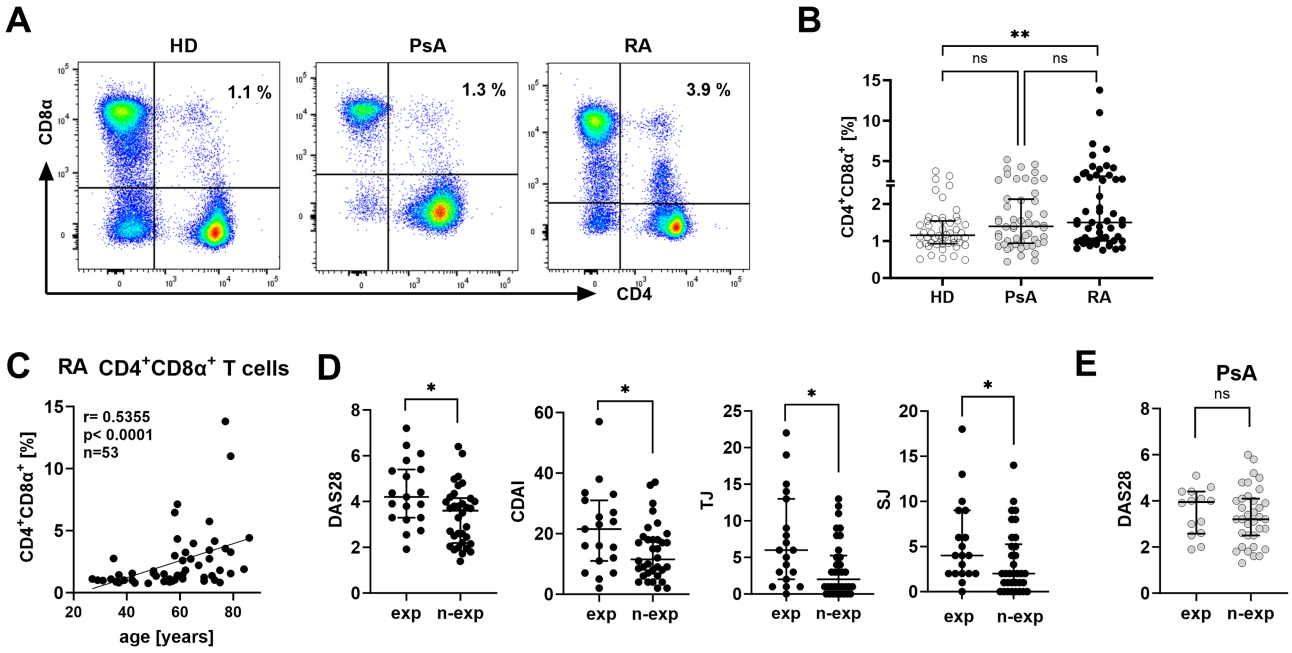

Figure 1. $CD4^+CD8\alpha^+$ T cells are expanded (exp) in patients with rheumatoid arthritis (RA) but not in patients with psoriatic arthritis (PsA) or healthy donors (HDs). Representative (A) dot plots and (B) graph show frequencies of peripheral blood $CD4^+CD8\alpha^+$ T cells from patients with RA (n = 53) in comparison to patients with PsA (n = 52) and HDs (n = 50). $^{**}P \leq 0.01$. (C) Frequencies of $CD4^+CD8\alpha^+$ T cell in association with age of patients with RA. (D) Graphs show Disease Activity Score in 28 joints (DAS28), Clinical Disease Activity Index (CDAI), number of tender joints (TJs), and swollen joints (SJs) of patients with RA (from left to right) or (E) patients with PsA with exp (frequency > 2%) and nonexpanded (n-exp; <2%) $CD4^+CD8\alpha^+$ T cells. $^{*}P \leq 0.05$. Data are shown as median (± interquartile range). Dots represent individual donors. Statistical analysis was performed using Mann–Whitney U test or Spearman correlation. ns, not significant.

$P = 0.016$), higher Clinical Disease Activity Index (CDAI) score (median 21.5 vs 11.5, $P = 0.049$), and its components (Figure 1D). This association between $CD4^+CD8\alpha^+$ T cell expansion and joint-related disease activity did not reach significance in patients with PsA (Figure 1E).

ADTs can be used to distinguish between $CD4^+$ and $CD8^+$ T cells. $CD4^+CD8\alpha^+$ T cells are a heterogeneous T cell population reaching from $CD4^+CD8^{low}$ to $CD8^+CD4^{low}$ T cells. Mechanisms underlying the emergence of these cells as well as their origin are currently unclear. In early studies, the CD4/CD8 expression pattern has been suggested as a determinator of lineage origin of the cells. $CD4^+CD8\alpha^+$ T cells have been suspected to be of CD4 origin if high expression of CD4 was combined with low CD8α expression,[31] whereas $CD8^+CD4^{low}$ T cells were suggested to be of CD8 origin.[32,33] However, this principle of assignment has not been confirmed experimentally.

To address this question for $CD4^+CD8\alpha^+$ T cells in patients with RA, we separated peripheral blood $CD3^+$ T cells from three patients with RA and two healthy controls and performed 10x 5′ scRNA sequencing in combination with TCRαβ sequencing (Figure 2A; Supplementary Table 2). To facilitate identification of $CD4^+CD8^+$ T cells, we preincubated the separated $CD3^+$ T cells with precoded anti-CD4 and anti-CD8α antibodies (ADTs). After quality control, all transcriptomic data were integrated into one data set, and T cell clusters were identified based on genomic RNA expression profiles (Patients and Methods section; Figure 2B; Supplementary Figure 2A and B). Variability among samples had no impact on cluster analysis.

The intensity of ADT signals from anti-CD4 and anti-CD8α antibodies was used to quantify coreceptor surface expression on the single-cell level. To identify cells expressing CD4, CD8α, or both, data were visualized in two-dimensional dot plots (Figure 2C; Supplementary Figure 2C). $CD4^+$ and $CD8^+$ T cells could be differentiated in the cluster analysis by ADTs (Figure 2D). Spatial distribution of the intensity of CD4 and CD8 ADT signals in the uniform manifold approximation and projection (UMAP) plot was comparable to that of CD4, CD8A, and CD8B gene expression (Figure 2D). Furthermore, we compared gene expression level of CD4, CD8A, and CD8B among the ADT-based cell populations on the individual donor level. The results confirmed a significantly higher expression level of CD8A in $CD4^+CD8^+$ T cells based on ADTs (Supplementary Figure 3). Labeling cells in the UMAP based on CD4 and CD8 ADT signals from all five donors allowed distinct separation of $CD4^+CD8^-$ and $CD8^+CD4^-$ as well as $CD4^+CD8^+$ T cells in the cluster analysis (Figure 2E; Supplementary Figure 4A and B).

Analysis of T cell clonality by TCRαβ sequencing can be used to distinguish between $CD4^+CD8^+$ T cells of either CD4 or CD8 origin. TCRαβ sequencing was used to

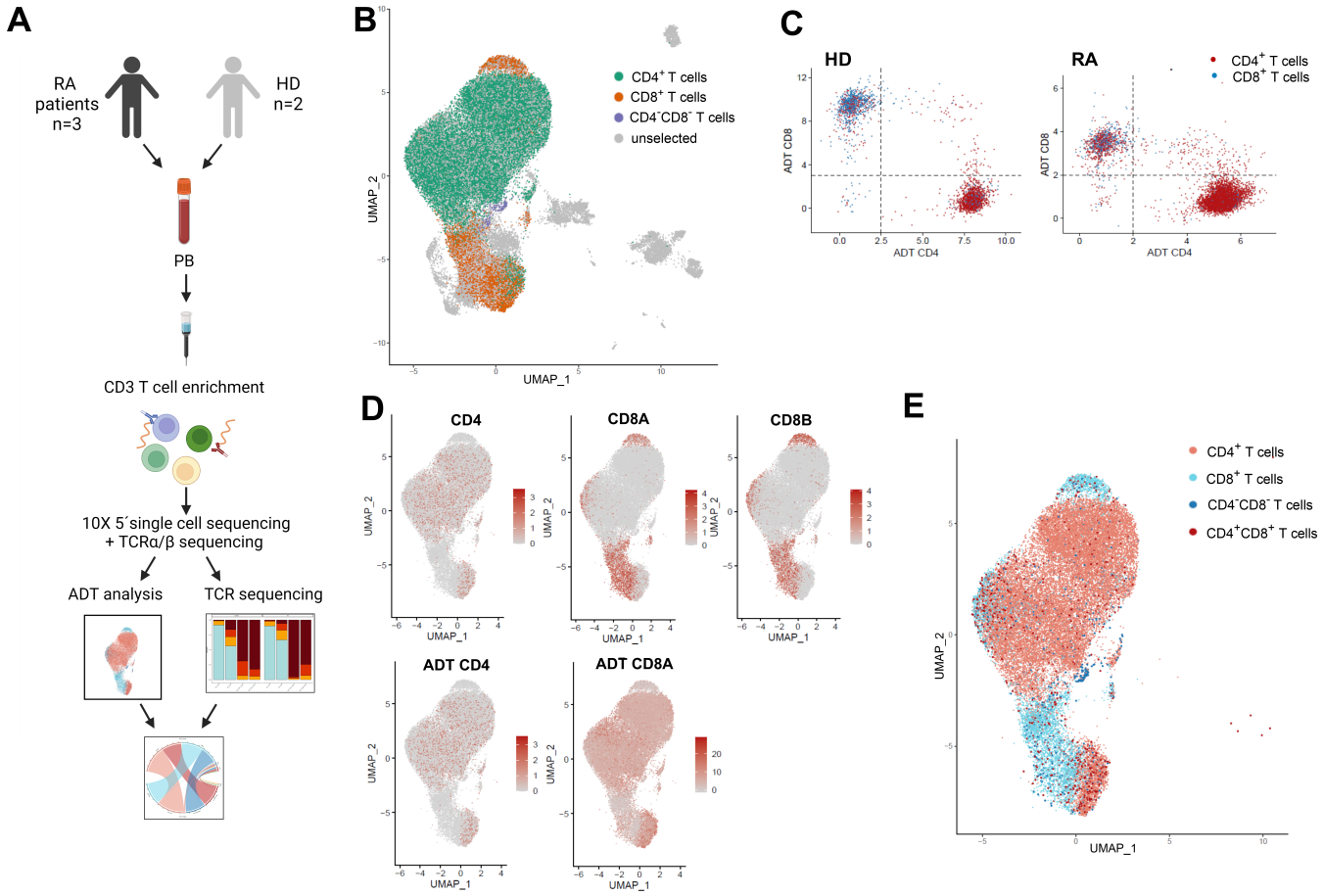

Figure 2. Characterization of peripheral blood (PB) T cells in patients with RA and healthy donors (HDs) by ADTs and scRNA sequencing. (A) Experimental workflow including T cell enrichment and 10x 5′ single cell coupled to ADT analysis and TCR and scRNA sequencing of three patients with RA and two HDs (created with Biorender.com). (B) UMAP displaying all TCRα/β$^+$ T cells in the analysis, color coded as indicated in the legend. Assignment of CD4$^+$ and CD8$^+$ T cells based on genomic RNA expression profiles. (C) Antibody-derived tags (ADTs) for CD4 and CD8 represent CD4 and CD8 surface expression on T cells in representative dot plots of one HD and one patient with RA. (D) UMAPs showing distribution of CD4 and CD8 gene expression (upper row) and of ADT signal for CD4 and CD8 on T cells (lower row) of one representative donor. (E) Integrated data of ADT signals of five donors. Assignment of CD4 and CD8 positivity is according to labels derived from ADTs. Cells are color coded as indicated in the legend based on ADTs. Dots represent individual cells. RA, rheumatoid arthritis; scRNA, single-cell RNA; TCR, T cell receptor; UMAP, uniform manifold approximation and projection.

identify clonally expanded T cells (Figure 3A–D). Two or more cells sharing identical TCRα and -β chains were considered to belong to an expanded T cell clone. Accordingly, we were able to discriminate T cells with unique TCRs, termed singletons according to Moon et al,[34] and expanded T cell clones (two or more cells with identical TCR sequences; absolute numbers and percentage of clone size are presented in Supplementary Tables 3 and 4).

T cell clones containing CD4$^+$CD8$^+$ T cells were mostly found to also contain either CD4$^+$CD8$^-$ or CD8$^+$CD4$^-$ cells, which made it possible to unequivocally assign the whole clone to either CD4 or CD8 origin. Importantly, only very few of the CD4$^+$CD8$^+$ T cell clones (<0.1%) were found to simultaneously contain both CD4$^+$CD8$^-$ and CD8$^+$CD4$^-$ cells. These results further confirmed the assignment based on the CD4 and CD8 ADT signal.

In the next step, for representation in the UMAP plot, cells were labeled as CD4$^+$, CD8$^+$, or CD4$^+$CD8$^+$ according to ADTs in combination with TCR sequencing results. First, singletons with unique TCRs were assigned to CD4$^+$CD8$^-$ or CD8$^+$CD4$^-$ T cells based on ADT (Figure 3A). Next, T cells from clones without CD4$^+$CD8$^+$ cells were labeled CD4$^+$CD8$^-$ or CD8$^+$CD4$^-$ (Figure 3B). Finally, T cell clones containing one or more CD4$^+$CD8$^+$ cells were assigned as CD4$^+$CD8$^+$ of CD4 or CD8 origin based on clonal ADT assignment (Figure 3C and D). The UMAP plots show that CD4$^+$ and CD8$^+$ singletons tended to cluster separately from all clonally expanded cells (Figure 3A). In comparison to clonally expanded CD4$^+$CD8$^-$ T cells (Figure 3B), CD4$^+$CD8$^+$ T cell clones of CD4 origin are concentrated in only one isolated cluster (Figure 3C). This differential clustering most likely represents differences in genomic RNA expression between

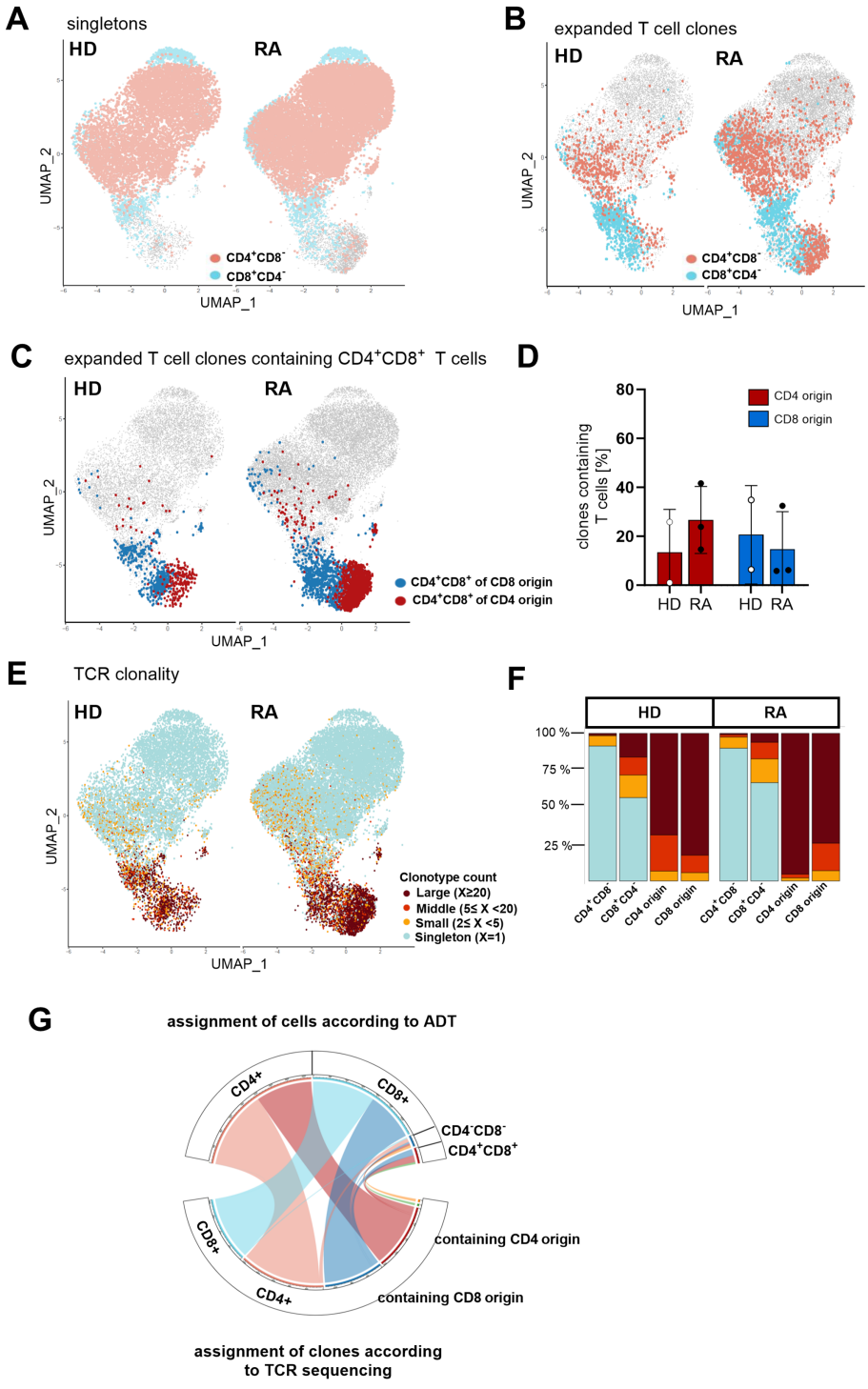

Figure 3. $CD4^+CD8^+$ T cells of CD4 origin have clonally expanded TCR sequences. (A–C) UMAP of integrated data of healthy donors (HDs; n = 2) and patients with RA (n = 3) showing the (A) distribution of singletons, (B) expanded $CD4^+CD8^-$ and $CD8^+CD4^-$ T cell clones without $CD4^+CD8^+$ cells, and (C) T cell clones containing $CD4^+CD8^+$ cells of either CD4 or CD8 origin. (D) Graphs show frequencies of $CD4^+CD8^+$ T cells of CD4 or CD8 origin among all clonal T cells for patients with RA and controls. Cells are colored according to labels derived from *TCRα/β* sequencing and ADT analysis, as indicated in the legends. (E) UMAP shows the distribution of T cell clones of the indicated sizes. X represents the frequency of each clonotype defined by its unique paired *TCRαβ* sequence. Large expanded clones ($X \geq 20$), medium expanded clones ($5 \leq X < 20$), small expanded clones ($2 \leq X < 5$), and singletons ($X = 1$) are color coded as indicated. (F) Bar graph depicts fraction of total T cells occupied by T cell clones in the size ranges indicated in HDs and patients with RA. (G) Circos plot displaying integrated analysis of shared clones according to *TCRα/β* sequencing in comparison to ADT data. ADT, antibody-derived tag; RA, rheumatoid arthritis; TCR, T cell receptor; UMAP, uniform manifold approximation and projection.

naive and antigen-experienced T cells and requires further phenotypic and functional investigation.

Clones containing $CD4^+CD8^+$ T cells appeared more prominent in patients with RA compared to controls because high frequencies of clonal T cells of CD4 origin occurred in all three patients with RA (Figure 3C and D). We also found a higher frequency of massively expanded very large T cell clones in patients with RA (Figure 3E and F). Importantly, the population of $CD4^+CD8^+$ T cells of CD4 and CD8 origin comprised almost exclusively of very large clones, which differentiates it from all other T cells (Figure 3F; Supplementary Figure 4C). Visualization of the relation between ADT-based categories and TCR sequencing–based clone assignment shows that only a small fraction of cells in CD4 clones are $CD4^+CD8^+$ (Figure 3G).

$CD4^+CD8^+$ T cells of CD4 origin match the flow cytometrically determined $CD4^+CD8\alpha^{low}$ T cell population. Previously, lineage and origin discrimination of $CD4^+CD8\alpha^+$ T cells have been described based on expression of the CD8β chain. Although $CD4^+CD8^+$ T cells of CD4 origin were reported to express CD8αα homodimers,[31,35] $CD8^+CD4^+$ T cells of CD8 origin are characterized by expression of both the CD8α and CD8β chain.[33,36] Analysis of CD8B gene expression in our data set confirmed that $CD4^+CD8^+$ T cells of CD8 origin expressed CD8B messenger RNA, whereas those of CD4 origin did not (Figure 4A).

Next, in order to enable determination of lineage origin of $CD4^+CD8\alpha^+$ T cells in a larger cohort of patients, multicolor flow cytometry was used to quantify surface expression of CD8α and CD8β on $CD4^+CD8\alpha^+$ T cells in 15 patients with RA and 15 healthy controls (gating strategy in Figure 4B; Supplementary Figure 5B and C). The results show that only $CD8^+CD4^{low}$ T cells express relevant levels of CD8β, which strongly suggests their CD8 origin, whereas $CD4^+CD8\alpha^{low}$ cells do not, which indicates their CD4 origin (Figure 4C; Supplementary Figure 5). In alternative analyses, gating for CD8β-negative $CD4^+$ T cells produced only $CD4^+CD8\alpha^{low}$ T cells, whereas CD8β-positive cells consisted exclusively of $CD8^+CD4^{low}$ T cells (Supplementary Figure 5). Analysis of multidimensional flow cytometric data from this cohort in t-distributed stochastic neighbor embedding plots confirmed that CD8β expression was present only in two T cell clusters, one of which contained $CD8^+CD4^-$ T cells, whereas the other one contained $CD8^+CD4^{low}$ T cells of CD8 origin (Figure 4D and E).

Taken together, the results of scRNA and single-cell TCR sequencing and the flow cytometric data for CD8β confirmed that $CD4^+CD8\alpha^{low}$ T cells are of CD4 origin. Therefore, we used this expression pattern to determine frequencies of $CD4^+CD8\alpha^+$ T cells of CD4 origin in all subsequent analyses. Accordingly, for the remaining part of the manuscript, the designation $CD4^+CD8\alpha^{low}$ T cells will refer to $CD4^+CD8^+$ cells that express CD8α at low levels and that are of CD4 origin.

RA-associated $CD4^+CD8\alpha^+$ T cells are primarily of CD4 origin. When $CD4^+CD8\alpha^+$ T cells were assigned to either CD4 or CD8 origin, we found that only $CD4^+CD8\alpha^{low}$ cells of CD4 origin were increased in patients with RA compared to healthy controls (Figure 4F), whereas no difference in $CD8^+CD4^{low}$ was detectable (data not shown). Patients with PsA did not differ from healthy donors and had significantly less $CD4^+CD8\alpha^{low}$ T cells than patients with RA (Figure 4F).

$CD4^+CD8\alpha^{low}$ T cells are associated with latent cytomegalovirus infection and characterized by increased expression of LIR-1 and PD-1. Several groups including our own have previously reported a close association between latent cytomegalovirus (CMV) infection and altered T cell subsets in patients with RA, including emergence of $CD4^+CD8\alpha^+$ T cells, loss of CD28, or gain of the inhibitory receptor LIR-1.[37] Therefore, we investigated the frequency of latent CMV infection and its influence on the frequency of $CD4^+CD8\alpha^{low}$ T cells in patients with RA and controls. The results showed that the frequency of CMV seropositivity was significantly higher in patients with RA than in healthy controls (58.5% vs 36%, $P = 0.0159$; Supplementary Table 1), which confirmed previous reports.[13,37–43] In addition, we found that individuals with IgG-positive CMV have significantly higher frequencies of $CD4^+CD8\alpha^{low}$ T cells compared to individuals who were CMV negative in all three cohorts analyzed. Importantly, $CD4^+CD8\alpha^{low}$ T cells were also more frequent in patients with RA who were CMV positive in comparison to healthy controls who were CMV positive. Furthermore, patients with RA who were CMV negative also had significantly higher frequencies of $CD4^+CD8\alpha^{low}$ T cells compared to patients with PsA who were CMV negative, which confirms RA specificity of this expansion (Figure 4G).

In view of the observed impact of latent CMV status on the expression of the inhibitory receptor LIR-1 in patients with RA, we took advantage of flow cytometry data available for a previously published unrelated cohort of patients with RA and analyzed LIR-1 expression on $CD4^+CD8\alpha^{low}$ versus $CD8^+CD4^{low}$ T cells. $CD4^+CD8\alpha^{low}$ T cells were found to express LIR-1 significantly more frequently compared to $CD4^+CD8^-$ T cells (Figure 4H). As reported previously, the highest frequency of LIR-1 expression was found in $CD8^+CD4^-$ T cells in both individuals with RA and healthy individuals (Figure 4H). Simultaneously, PD-1 was more frequently expressed on $CD4^+CD8\alpha^{low}$ T cells than on $CD4^+CD8^-$ T cells. Both the LIR-1 and the PD-1 expression on $CD4^+CD8\alpha^{low}$ T cells did not differ between patients with RA and controls (data not shown).

$CD4^+CD8\alpha^{low}$ T cells are associated with increased disease activity. Due to the stringent inclusion criteria, a comparatively high disease activity was present at study entry in the cohort with RA, as indicated by the median DAS28 of 3.8. The frequency of $CD4^+CD8\alpha^{low}$ T cells was significantly linked to disease

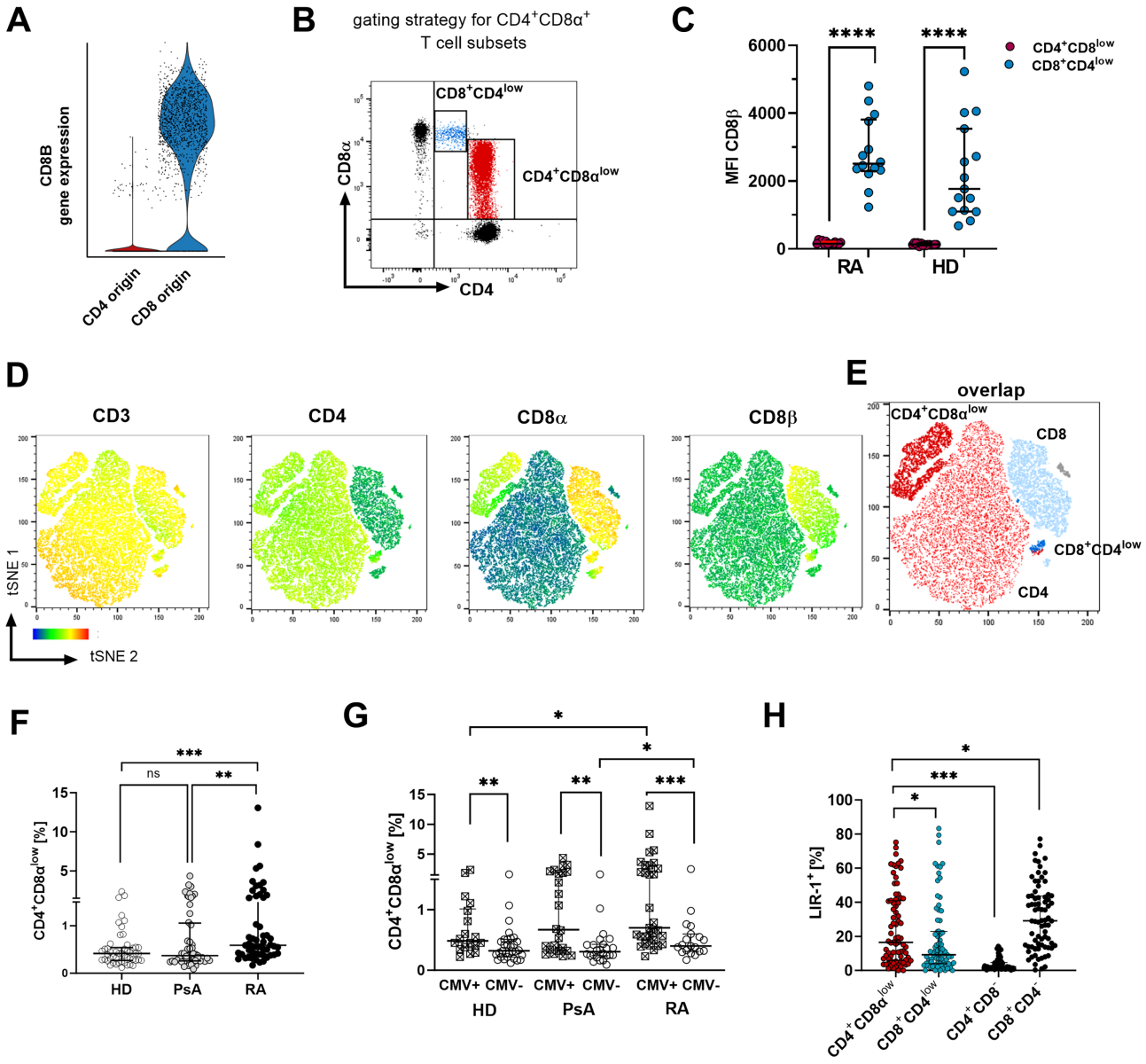

Figure 4. CD4$^+$CD8$^+$ T cells of CD4 origin express CD8α, but not CD8β, and show a differentiated phenotype. (A) Violin plot displaying CD8B messenger RNA expression based on single-cell RNA sequencing in CD4$^+$CD8$^+$ T cells of CD4 and CD8 origin. (B) Surface expression of CD4, CD8α, and CD8β of peripheral T cells was determined by flow cytometry. Dot plot shows gating of CD4$^+$CD8α^{low} and CD8$^+$CD4^{low} subpopulations. (C) Graph displaying mean fluorescence intensity (MFI) of CD8β expression on CD4$^+$CD8α^{low} and CD8$^+$CD4^{low} T cells among patients with RA (n = 14) and healthy donors (HDs, n = 15). ****$P \leq 0.0001$. (D) tSNE analysis of flow cytometry data of one representative patient with RA shows the expression of the indicated marker. Coloring correlates with the intensity of the expression. Red indicates high expression; blue indicates the absence. (E) Merged tSNE plot for CD4, CD8α, and CD8β expression showing distribution of indicated T cell subsets. (F and G) Graphs show frequencies of CD4$^+$CD8α^{low} T cells from patients with RA (n = 53) in comparison to (F) patients with PsA (n = 52) and HDs (n = 50) and comparison between (G) individuals who were CMV$^+$ and CMV$^-$. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$. (H) Surface expression of LIR-1 on CD4$^+$CD8α^{low} and CD8$^+$CD4^{low} T cells in patients with RA. Each dot represents individual donors, lines depict median ± interquartile range. *$P \leq 0.05$, ***$P \leq 0.001$. Statistical analysis was performed using Mann–Whitney U test. CMV, cytomegalovirus; ns, not significant; PsA, psoriatic arthritis; RA, rheumatoid arthritis; tSNE, t-distributed stochastic neighbor embedding.

activity because it was found to correlate with DAS28 (r = 0.35, P = 0.01), C-reactive protein (CRP; r = 0.35, P = 0.01), tender joint count (TJC; r = 0.29; P = 0.03), and swollen joint count (SJC; r = 0.30, P = 0.03). Accordingly, when the median frequency of 0.59% was used as a cutoff for an increased percentage of CD4$^+$CD8α^{low} T cells, we found increased CRP, TJC, SJC, DAS28, and CDAI in patients with an expansion of those cells (Figure 5A). No effect of individual treatment regimen including steroid dosage or MTX treatment was discernible in the cross-sectional analysis.

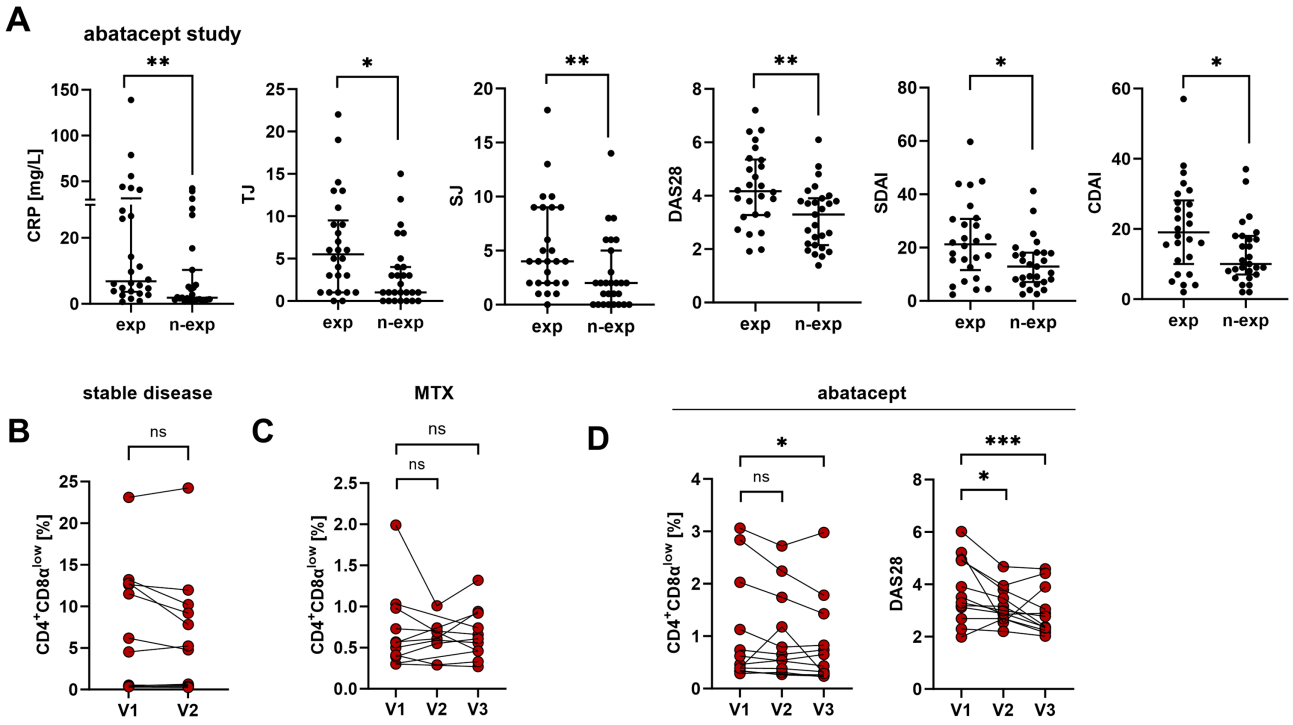

Figure 5. Frequencies of $CD4^+CD8\alpha^{low}$ T cells are associated with increased disease activity in patients with RA and declined activity under abatacept treatment. (A) Graphs show C-reactive protein (CRP), number of tender joints (TJs) and swollen joints (SJs), Disease Activity Score in 28 joints (DAS28), Short Disease Activity Index (SDAI), and Clinical Disease Activity Index (CDAI) of patients with RA with expanded (exp; >0.59%, n = 26) and nonexpanded (n-exp; ≤0.59%, n = 27) $CD4^+CD8\alpha^{low}$ T cells. $^*P \le 0.05$, $^{**}P \le 0.01$. (B) Frequencies of $CD4^+CD8\alpha^{low}$ T cells in patients with RA with stable disease (n = 11, visit [V] 1 and V2 were at least three months apart). (C and D) Frequencies of $CD4^+CD8\alpha^{low}$ T cells in patients with RA before and after treatment with receiving (C) MTX (n = 11) or (D) abatacept (n = 12) at the following time points: before treatment (V1) and 6 and 12 weeks (V2 and V3, respectively) after initiation of treatment. Dots represent individual donors. $^*P \le 0.05$, $^{***}P \le 0.001$. Statistical analysis was performed using (A) Mann–Whitney U test or (B–D) Wilcoxon test. MTX, methotrexate; ns, not significant; RA, rheumatoid arthritis.

Next, we performed longitudinal studies to determine the time course of $CD4^+CD8\alpha^{low}$ T cells in relation to disease activity. In patients with RA with stable disease and unchanged treatment for at least six months (n = 11), the median frequency of $CD4^+CD8\alpha^{low}$ T cells did not change significantly (median change −0.06%, $P = 0.24$; Figure 5B). Next, we investigated a cohort of patients with recent-onset RA and no history of immunosuppressive treatment (n = 12) in whom MTX therapy was initiated. The results showed that treatment with MTX had no significant influence on the frequency of $CD4^+CD8\alpha^{low}$ T cells (median change −0.03%, $P = 0.83$; Figure 5C). Finally, a cohort of patients with RA with incomplete response to MTX was recruited (n = 12) in whom treatment with the T cell–directed anti-CTLA4-Ig construct abatacept was initiated (Figure 5D). In this anti-CCP positive, biologic DMARD–naive cohort of patients with highly active, recent-onset RA, a clinical response to abatacept was evident in the vast majority of patients. The results in this cohort show a significant decrease of the frequency of $CD4^+CD8\alpha^{low}$ T cells after three months of treatment (median change −0.09%, $P = 0.02$), which was accompanied by a significant reduction in DAS28 (median change −0.075, $P = 0.001$; Figure 5D).

DISCUSSION

Taken together, our results show that $CD4^+CD8\alpha^{low}$ T cells are the only subpopulation of $CD4^+CD8\alpha^+$ T cells that is associated specifically with RA. A recent study described the important observation that $CD4^+CD8\alpha^{low}$ T cells occur very early in the disease process and are already present in healthy individuals positive for anti-CCP antibodies, which are at risk to develop RA later on in life.[15] $CD4^+CD8\alpha^+$ T cells are present in the rheumatoid synovium[13] and appear to be more frequent in the joints than in the peripheral circulation,[15] which puts them into the center of disease pathogenesis. Previously, $CD4^+CD8\alpha^+$ T cells were thought to either be the result of inadequate thymic escape of immature, double-positive T cells or to develop during the long-standing disease process due to extensive homeostatic proliferation or clonal expansion of T cells. More recently, however, $CD4^+CD8\alpha^+$ T cells have also been observed in the vicinity of solid tumors like melanoma.[44] This study showed that TCR stimulation by dominant tumor antigens leads to up-regulation of the second coreceptor gene, both in CD4 and in CD8 T cells. Subsequently, it was also shown that major histocompatibility complex (MHC) restriction is influenced by this coreceptor expression because

TCR signals can be triggered in $CD4^+CD8\alpha^+$ T cells by MHC I–presented antigens and in $CD8^+CD4^+$ T cells by antigens bound to MHC II molecules.[44] If this mechanism was also relevant in patients with RA, it would offer an entirely new hypothesis for the generation of autoimmunity because MHC II–restricted CD4 T cells, which become $CD4^+CD8\alpha^{low}$, could subsequently respond to MHC I–presented autoantigens for which they have not been negatively selected in the thymus.

$CD4^+CD8\alpha$ T cells of CD4 origin in patients with RA have been shown to have a polyfunctional phenotype because they produce excessive amounts of interferon (IFN) γ upon activation,[13,15] increase their use of oxidative phosphorylation, and are highly polyfunctional producing IFNγ, interleukin (IL)-2, and IL-4 if present in the rheumatoid synovial tissue.[15] Such a pathologic phenotype appears to be characteristic for autoimmune arthritis because polyfunctionality of CD4 T cells has also been observed in patients with juvenile idiopathic arthritis and Down syndrome–associated arthritis[45] and in those with PsA.[46,47] Besides autoimmunity, polyfunctional cells are also known to occur in certain infectious situations, in particular in patients with viral infections (eg, CMV)[48,49] and in patient with tuberculosis,[50] in whom $CD4^+CD8\alpha^+$ T cells also occur.

Our findings should be interpreted within the context of certain limitations. One limitation is the comparatively small size and the monocentric recruitment of our study of patients with RA treated with receiving abatacept. A larger, multicenter cohort study will be necessary to corroborate our findings. Another limitation is that the cellular analyses are limited to peripheral blood T cells. Regarding synovial T cell populations, we are referring to our previous work, however, which clearly showed that $CD4^+CD8\alpha^+$ T cells can also be found in the rheumatoid joint and are functionally characterized by increased production of IFNγ, IL-21, and IL-4.[13] Accordingly, they likely play a proinflammatory role while also providing autoreactive T cell help.

The combined genomic RNA and TCR sequencing performed in this current study allows, for the first time, a quantitative and qualitative assessment of TCR oligoclonality in patients with RA on a single-cell level. The results show that increases in clonal size and deviations of genomic RNA expression from the naive state of singleton cells can be traced during clonal expansion and in particular in $CD4^+$ clone cells that up-regulate the CD8α chain to become $CD4^+CD8\alpha^{low}$. This process is already detectable in elderly individuals age matched to a population with RA and in particular in individuals with latent CMV infection. Both the numeric increase and the functional deviation of $CD4^+CD8\alpha^{low}$ T cells is more aggravated in patients with RA, however, because both patients with RA who were CMV positive and who were CMV negative had higher frequencies of $CD4^+CD8\alpha^{low}$ T cells than healthy controls who were CMV positive and who were CMV negative, respectively.

We have shown previously that latent CMV infection associates with a more severe disease in patients with RA.[14,43] The increased rate of CMV positivity in patients with RA in the present study and the fact that the highest percentages of $CD4^+CD8\alpha^{low}$ T cells are detectable in patients with RA who were CMV positive corroborates that chronic immune deviation due to latent CMV infection aggravates the pathogenic T cell response in patients with RA. In conclusion, our study shows that $CD4^+CD8\alpha^{low}$ T cells are present very early in the course of RA, which is in line with the observations reported even for patients with preclinical RA.[15] The result of the clinical study with abatacept is of particular interest because the decrease of $CD4^+CD8\alpha^{low}$ T cell clones upon a clinically efficacious T cell–directed therapy implies that it might be beneficial to target those cells in patient with RA or even in individuals at risk to develop RA.

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AUTHOR CONTRIBUTIONS

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

Study conception and design. Beck, Nguyen, Hagemann, Rothe, Wagner.

Acquisition of data. Beck, Nguyen, Loyal, Melzer, Apel, Pierer, Krasselt, Seifert, Glimm, Hagemann, Rothe, Wagner.

Analysis and interpretation of data. Beck, Nguyen, Hoffman, Loyal, Thiel, Hagemann, Rothe, Wagner.

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