Nguyen Phuong (Orcid ID: 0000-0003-2403-0418)
Krasselt Marco Lothar (Orcid ID: 0000-0002-1842-0754)
Rothe Kathrin (Orcid ID: 0000-0002-7376-180X)

$CD4^+CD8\alpha^{low}$ T cells in rheumatoid arthritis are clonally expanded and dependent on co-stimulation

Felix Beck[1#], Phuong Nguyen[1#] MD, Anne Hoffmann[2] PhD, Lucie Loyal[3,4] PhD, Andreas Thiel[3,4] MD, Marc Melzer[1] MD, Hannah Apel[1], Matthias Pierer[1] MD, Marco Krasselt[1] MD, Olga Seifert[1] MD, Anne-Marie Glimm[1] MD, Tobias Hagemann[2§], Kathrin Rothe[1§] PhD and Ulf Wagner[1§] MD.

Affiliation:

[1] Department of Rheumatology, Medizinische Klinik III, Universität Leipzig, Medizinische Fakultät, Leipzig, Germany

[2] Helmholtz Institute for Metabolic, Obesity and Vascular Research (HI-MAG) of the Helmholtz Zentrum München at the University of Leipzig and University Hospital Leipzig, Leipzig, Germany

[3] Berlin Institute of Health (BIH) at Charité – Universitätsmedizin Berlin, Immunomics - Regenerative Immunology and Aging; Berlin, Germany.

[4] Si-M / "Der Simulierte Mensch" a science framework of Technische Universität Berlin and Charité - Universitätsmedizin Berlin; Berlin, Germany.

#,§ contributed equally

Correspondence to Prof. Ulf Wagner, Department of Rheumatology, Medizinische Klinik III, Liebigstraße 19, Medical Faculty Universität Leipzig, 04103 Leipzig; Ulf.Wagner@medizin.uni-leipzig.de; Tel: +49 3419724707; Fax: +49 3419725959

Acknowledgements and affiliations:

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$CD4^+CD8\alpha^{low}$ T cells in rheumatoid arthritis are clonally expanded

Abstract

Objectives:

$CD4^+CD8^+$ 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 pre-clinical 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-(sc)RNA- and scTCR-sequencing data were used to determine co-receptor expression and T cell receptor sequences to assess clonality of $CD4^+CD8^+$ T cells in RA (n=3) patients and healthy controls (n=2). Peripheral $CD4^+CD8^+$ T cells and their subpopulations were measured in patients with RA (n=53), PsA (n=52) and healthy donors (n=50) using flow cytometry. In addition, changes in $CD4^+CD8^+$ T cell frequency were prospectively followed in RA patients receiving therapy with abatacept for 12 weeks.

Results:

We observed an increase of $CD4^+$ T cells expressing $CD8\alpha$ in RA patients, both in comparison to PsA patients and to 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-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 RA, clonal expansion of $CD4^+$ T cell clones culminates in the emergence of peripheral $CD4^+CD8\alpha^{low}$ T cells, which are associated with disease activity and diminished upon abatacept treatment, and which could contribute to disease pathogenesis.

Introduction

Rheumatoid arthritis is a chronic autoimmune disease characterized by perturbations in the adaptive immune system that occur already in the pre-clinical 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, TCR diversity is decreased compared to age matched controls, indicating a lack of naïve, not clonally expanded T cells with unique TCR specificity[3,4]. Functionally, pathogenically relevant T cells in RA have been 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 RA is emphasized by the clinical efficacy of a blockade of costimulatory CD80/CD86 interaction with abatacept, which ameliorates disease activity in 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 pathological subset in 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-CCP positive despite a lack of clinical

inflammation and who are considered to be at risk to develop rheumatoid arthritis. 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 CD8α, it is expressed as CD8αα. Based on their expression level of CD4 and CD8α, $CD4^+CD8\alpha^+$ T cells can be devided into $CD4^+CD8\alpha^{low}$ and $CD8^+CD4^{low}$ T cells [16]. Until now, functional studies for $CD4^+CD8\alpha^+$ T cells are rare, but virus- and disease-specific functions are 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 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.

Material and Methods

Patients

RA patients fulfilling the ACR/EULAR 2010 classification criteria were recruited from the Rheumatology Clinic of Leipzig University. For detailed clinical characterization of patients, see Supplemental 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 (with arthritis as the primary clinical manifestation) served as a disease control. Data from an independent cohort of RA patients was used for LIR-1/PD-1 analysis. Written consent was obtained from all donors. This study was carried out with the approval of the University of Leipzig ethics committee (253/21-EK).

Longitudinal RA study

Two subgroups of early RA patients were recruited into a longitudinal study. The first cohort consisted of RA patients naïve to disease modifying drugs who began treatment with methotrexate (MTX). The second cohort comprised RA patients in whom treatment with MTX alone was not effective, and who began additional treatment with abatacept (CTLA4-Ig). $CD4^+CD8\alpha^+$ T cells were determined at the three time points: before initiation (V1), after 6±1 weeks (V2) and after 12±2 weeks (visit 3, V3) of treatment initiation.

Cell preparation and flow cytometry

Peripheral mononuclear cells were isolated using Ficoll density gradient. PBMCs were stained for surface expression of the following markers: LIVE/DEAD™ fixable viability dye (405nm, ThermoFisher Scientific), CD3, CD4, CD8α (all Miltenyi Biotec, all REA

antibodies) and CD8β (BD Biosciences). Cells were acquired on FACS LSR Fortessa (BD Biosciences) and analyzed using FlowJo V10 software.

scRNA-sequencing

For scRNA-seq, T cells were isolated from PBMCs from 3 RA patients and 2 healthy controls (Supplemental 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: TGTTCCCGCTCAACT; CD8α-Clone: RPTA-T8; Barcode Sequence: GCTGCGCTTTCCATT.

The cells were loaded with a maximum concentration of 1000 cells/µl and a maximum cell number of 17,000 cells on a Chromium Chip G (10xGenomics). Gene expression, hashtag and TCR libraries were generated according to the manufacturer's instruction using the Chromium v1.1 chemistry (10xGenomics). Sequencing was conducted with a NovaSeq 6000 cartridge (Illumina) with 20,000 reads per cell for GEX libraries and 5,000 reads per cell for TCR libraries.

Pre-processing and cell type annotation

Count matrices have been created with 10X Genomics Cell Ranger (v. 6.0.1) with the count function from raw data [18]. Data pre-procession of gene expression count matrices as well as antibody-derived tag (ADT) counts and T cell receptor (TCR) sequencing data has been conducted in R (v.4.2.3) with Seurat (v. 4.3.0) [19]. Each sample has been preprocessed individually. Ambient RNA has been removed with SoupX (v.1.5.2) to remove unwanted variance and improve clustering results [20]. TCR sequence data was imported with the djvdj package (v.0.1.0) [21].

Filtering has been applied on cell level and cells with a nUMI (number of unique molecular identifiers) < 500, number of genes < 300 and > 4500, 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 biological effects, which would otherwise affect the cluster analysis. Hence, genes were removed which relate to the apoptosis pathway in the Kyoto Encyclopedia of Genes and Genomes (KEGG) [22] as well as genes coding for T cell receptor alpha, beta and gamma variables as they tend to create individual clusters. Among the remaining genes, only those were kept for further analysis, which have been expressed in at least ten cells.

Subsequently, cell matrices have been processed following the Seurat best practices. Count matrices 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() (v.0.3.5) (21) and 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 (v.1.12) [24].

Cell type prediction was conducted with the Seurat functions FindTransferAnchors() and MapQuery() where we annotated cells in the query datasets based on the previously published atlas of human peripheral blood mononuclear cells [25]. Feature barcodes were normalized and denoised with the dsb package (v.1.0.3) [26].

After individual preprocessing, all samples were integrated with scvi_tools (v.0.20.3) [27] in R with reticulate (v.1.26) [28] with default settings while removing the effect of the laboratory covariate. A final clustering analysis was conducted on the integrated dataset with Seurat

functions FindNeighbors(), FindClusters() and RunUMAP() using the first ten dimensions from the scvi assay.

Subsequently, only cells were included if they were annotated as $CD4^+$ or $CD8^+$ and not as proliferating T cells, not classified as doublets and carried exactly one alpha and one beta TCR 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 alpha and beta TCR sequences. TCR sequences were assigned to the aforementioned ADT classes. TCR sequences, whose copies 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 (v.3.3.5) [29].

To determine the size of expanded T cell clones, the number of individual cells expressing identical receptors was used. Since 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 dataset 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 analysed 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 dataset 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 (Supplemental Figure 1).

Statistical analysis

Statistical analysis of flow cytometry data was performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). First, a normality test was performed. Differences between 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 of <0.05 were considered statistically significant.

Results

CD4+CD8α+ T cells are associated with RA, but not PsA

To investigate the relevance of CD4+CD8α+ T cells in RA independently of treatment effects of longstanding immunosuppression, we recruited a study cohort of patients (n=53) with recent onset RA (median disease duration 2 years, Supplemental Table 1). 46 RA patients had not previously been treated with biologicals or JAK inhibitors, and 21 were DMARD naïve entirely.

As a control, patients with psoriatic arthritis (PsA, n=52) were enrolled in the study. In addition, healthy donors (HD, n=50) were recruited, and both control groups were meticulously matched for age and sex.

Flow cytometric analysis of CD4 and CD8α co-receptor expression confirmed the previously observed increase of the frequency of CD4+CD8α+ T cells in rheumatoid arthritis in comparison to healthy controls (median 1.5% vs. 1.15%, p=0.008; Figure 1, A-B). No significant difference between psoriatic arthritis patients and controls was discernible. We

could also confirm the previously reported correlation between the frequency of CD4+CD8α+ T cells and an individuals' age both in patients with RA (r=0.53, p<0.001; Figure 1C) and with psoriatic arthritis (r=0.3, p=0.03, data not shown). This trend did not reach statistical significance in healthy controls (data not shown).

In the RA cohort, median disease activity was comparatively high as indicated by a DAS28 of 3.8 (IQR 2.6 to 4.6), while erosive disease was rare. Notably, disease activity was higher in patients with an expanded (>2 % of CD3+ T cells) CD4+CD8α+ T cell population, as evident in a higher DAS28 score (median 4.2 vs. 3.6, p=0.016), CDAI score (median 21.5 vs. 11.5, p=0.049), and its components (Figure 1D). This association between CD4+CD8α+ T cell expansion and joint-related disease activity did not reach significance in psoriatic arthritis (Figure 1E).

Antibody-derived tagging (ADT) can be used to distinguish between CD4+ and CD8+ T cells

CD4+CD8α+ T cells are a heterogeneous T cell population reaching from CD4+CD8low to CD8+CD4low 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+CD8α+ T cells have been suspected to be of CD4 origin, if high expression of CD4 was combined with low CD8α expression [30], while CD8+CD4low T cells were suggested to be of CD8 origin [31,32]. However, this principle of assignment has not been confirmed experimentally.

To address this question for CD4+CD8α+ T cells in RA, we separated peripheral blood CD3+ T cells from three RA patients and two healthy controls and performed 10X 5´single-cell RNA sequencing in combination with TCRαβ sequencing (Figure 2A, Supplemental Table 2).

To facilitate identification of $CD4^+CD8^+$ T cells, we pre-incubated the separated $CD3^+$ T cells with pre-coded anti-CD4 and anti-CD8α antibodies (ADT). After quality control, all transcriptomic data were integrated into one data set, and T cell clusters were identified based on genomic RNA expression profiles (see material and methods and Figure 2B, Supplemental Figure 2A-B). Variability between samples had no impact on cluster analysis.

The intensity of ADT signals from anti-CD4 and anti-CD8α antibodies was used to quantify co-receptor 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, Supplemental Figure 2C). $CD4^+$ and $CD8^+$ T cells could be differentiated in the cluster analysis by ADT (Figure 2D). Spatial distribution of the intensity of CD4 and CD8 ADT signals in the 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 between the ADT based cell populations on individual donor level. The results confirmed a significantly higher expression level of CD8A in $CD4^+CD8^+$ T cells based on ADT (Supplemental Figure 3). Labelling cells in the UMAP based on CD4 and CD8 ADT signals from all 5 donors allowed distinct separation of $CD4^+CD8^-$ and $CD8^+CD4^-$ as well as $CD4^+CD8^+$ T cells in the cluster analysis (Figure 2E; Supplemental Figure 4A-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 identify clonally expanded T cells (Figure 3, A-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 [33], and expanded T cell clones (≥ 2 cells with identical TCR

sequences, absolute numbers and percentage of clone size are presented in Supplemental Table 3-4).

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 labelled as $CD4^+$, $CD8^+$ or $CD4^+CD8^+$ according to ADT 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 labelled $CD4^+CD8^-$ or $CD8^+CD4^-$ (Figure 3B). Finally, T cell clones containing one or more $CD4^+CD8^+$ cell were assigned as $CD4^+CD8^+$ of CD4 or CD8 origin based on clonal ADT assignment (Figure 3C-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 naïve and antigen-experienced T cells and requires further phenotypic and functional investigation.

Clones containing $CD4^+CD8^+$ T cells appeared more prominent in RA patients compared to controls, since high frequencies of clonal T cells of CD4 origin occurred in all three RA patients (Figure 3C-D). We also found a higher frequency of massively expanded very large T cell clones in RA (Figure 3E-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 flowcytometrically determined $CD4^+CD8\alpha^{low}$ T cell population

Previously, lineage and origin discrimination of $CD4^+CD8\alpha^+$ T cells has been described based on expression of the CD8β chain. While $CD4^+CD8^+$ T cells of CD4 origin were reported to express CD8αα homodimers [30,34], $CD8^+CD4^+$ T cells of CD8 origin are characterized by expression of both the CD8α and CD8β chain [32,35].

Analysis of CD8B gene expression in our dataset confirmed, that $CD4^+CD8^+$ T cells of CD8 origin expressed CD8B mRNA, while 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 RA patients and 15 healthy controls (see gating strategy in Figure 4B and Supplemental Figure 5B-C). The results show that only $CD8^+CD4^{low}$ T cells express relevant levels of CD8β, which strongly suggests their CD8 origin, while $CD4^+CD8\alpha^{low}$ cells do not, which indicates their CD4 origin (Figure 4C, Supplemental Figure 5). In alternative analyses, gating for CD8β negative $CD4^+$ T cells produced only $CD4^+CD8\alpha^{low}$ T cells, while CD8β positive cells consisted exclusively of $CD8^+CD4^{low}$ T cells (Supplemental Figure 5). Analysis of multidimensional flow cytometric data from this cohort in tSNE plots confirmed, that CD8β expression was present only in two T cell clusters, one of which contained $CD8^+CD4^-$ T cells, while the other one contained $CD8^+CD4^{low}$ T cells of CD8 origin (Figure 4 D-E).

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

RA associated CD4+CD8α+ T cells are primarily of CD4 origin

When CD4+CD8α+ T cells were assigned to either CD4 or CD8 origin we found that only CD4+CD8α low cells of CD4 origin were increased in RA compared to healthy controls (Figure 4F), while no difference in CD8+CD4low was detectable (data not shown). Psoriatic arthritis patients (PsA) did not differ from healthy donors and had significantly less CD4+CD8α low T cells than RA patients (Figure 4F).

CD4+CD8α low T cells are associated with latent CMV 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 CMV infection and altered T cell subsets in RA, including emergence of CD4+CD8α+ T cells, loss of CD28, or gain of the inhibitory receptor LIR-1 [36]. Therefore, we investigated the frequency of latent CMV infection and its influence on the frequency of CD4+CD8α low T cells in RA patients and controls. The results showed, that the frequency of CMV seropositivity was significantly higher in RA patients than in healthy controls (58.5% vs. 36 %, p= 0.0159, Supplementary Table 1), which confirmed previous reports [13,36–42]. In addition, we found, that CMV IgG positive individuals have significantly higher frequencies of CD4+CD8α low T cells

compared to CMV negative individuals in all three cohorts analyzed. Importantly, $CD4^+CD8\alpha^{low}$ T cells were also more frequent in CMV positive RA patients in comparison to CMV positive healthy controls. Furthermore, CMV negative RA patients had also significantly higher frequencies of $CD4^+CD8\alpha^{low}$ T cells compared to CMV negative patients with psoriatic arthritis, 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 RA, we took advantage of flow cytometry data available for a previously published unrelated cohort of RA patients and analyzed LIR-1 expression on $CD4^+CD8\alpha^{low}$ vs. $CD8^+CD4^{low}$ 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 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 RA patients 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 RA cohort, as indicated by the median DAS28 of 3.8. The frequency of $CD4^+CD8\alpha^{low}$ T cells was significantly linked to disease activity, since it was found to correlate with DAS28 (r=0.35, p=0.01), CRP (r=0.35, p=0.01), tender joint count (r=0.29; p=0.03) and swollen joint count (r=0.30, p=0.03). Accordingly, when the median frequency of 0.59 % was used as a cut-off for increased percentage of $CD4^+CD8\alpha^{low}$ 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 methotrexat (MTX) treatment was discernible in the cross-sectional analysis.

$CD4^+CD8\alpha^{low}$ T cells in rheumatoid arthritis are clonally expanded

Next, we performed longitudinal studies to determine the time course of $CD4^+CD8\alpha^{low}$ T cells in relation to disease activity. In RA patients 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 prior history of immunosuppressive treatment (n=12), in whom methotrexate therapy was initiated. The results showed, that treatment with methotrexate 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 RA patient with incomplete response to methotrexate 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, bDMARD-naïve cohort of patients with highly active recent-onset rheumatoid arthritis, 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 3 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+CD8α low T cells are the only subpopulation of CD4+CD8α+ T cells that is associated specifically with RA. A recent study described the important observation that CD4+CD8α 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 rheumatoid arthritis later on in life [15].

CD4+CD8α+ 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+CD8α+ T cells were thought to either be the result of inadequate thymic escape of immature, double positive T cells, or develop during the long-standing disease process due to extensive homeostatic proliferation or clonal expansion of T cells. More recently, however, CD4+CD8α+ T cells have also been observed in the vicinity of solid tumors like melanoma [43]. This study showed that T cell receptor stimulation by dominant tumor antigens leads to upregulation of the second co-receptor gene, both in CD4 and in CD8 T cells. Subsequently, it was also shown that MHC restriction is influenced by this co-receptor expression, since TCR signals can be triggered in CD4+CD8α+ T cells by MHC I presented antigens, and in CD8+CD4+ T cells by antigens bound to MHC II molecules [43]. If this mechanism was also relevant in rheumatoid arthritis, it would offer an entirely new hypothesis for the generation of autoimmunity since MHC II restricted CD4 T cells, which become CD4+CD8α low, could subsequently respond to MHC I presented autoantigens, for which they have not been negatively selected in the thymus.

CD4+CD8α T cells of CD4 origin in RA have been shown to have a polyfunctional phenotype since they produce excessive amounts of IFN-γ upon activation [13,15], increase their utilisation of oxidative phosphorylation, and are highly polyfunctional producing IFN-γ, IL-2 and IL-4 if present in the rheumatoid synovial tissue [15]. Such a pathological phenotype appears to be

characteristic for autoimmune arthritis, since polyfunctionality of CD4 T cells has also been observed in juvenile idiopathic arthritis and Down syndrome-associated arthritis [44] and in psoriatic arthritis [45,46]. Besides autoimmunity, polyfunctional cells are also known to occur in certain infectious situations, in particular in viral infections e.g. CMV [47,48] and in tuberculosis [49], where $CD4^+CD8\alpha^+$ 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 RA patients treated with 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 pro-inflammatory 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 T cell receptor oligoclonality in patients with rheumatoid arthritis on a single cell level. The results show that increases in clonal size and deviations of genomic RNA expression from the naïve state of singleton cells can be traced during clonal expansion, and in particular in $CD4^+$ clone cells that upregulate the CD8α chain to become $CD4^+CD8\alpha^{low}$. This process is already detectable in elderly individuals age-matched to a RA population, 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 RA patients, however, since both CMV positive and CMV negative RA patients had higher frequencies of $CD4^+CD8\alpha^{low}$ T cells than CMV positive and CMV negative healthy controls, respectively.

$CD4^+CD8\alpha^{low}$ T cells in rheumatoid arthritis are clonally expanded

We have shown previously, that latent CMV infection associates with a more severe case of rheumatoid arthritis [14,42]. The increased rate of CMV positivity in RA patients in the present study and the fact that the highest percentages of $CD4^+CD8\alpha^{low}$ T cells are detectable in CMV positive RA patients corroborates, that chronic immune deviation due to latent CMV infection aggravates the pathogenic T cell response in 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 preclinical RA [15]. The result of the clinical study with abatacept is of particular interest, since 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 RA or even in individuals at risk to develop RA.

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Figure 1: $CD4^+CD8\alpha^+$ T cells are expanded in RA but not in PsA or healthy controls

Representative dot plots **(A)** and graph **(B)** show frequencies of peripheral blood $CD4^+CD8\alpha^+$ T cells from patients with rheumatoid arthritis (RA, n=53) in comparison to patients with psoriatic arthritis (PsA; n=52) and healthy donors (HD, n=50). **(C)** Frequencies of $CD4^+CD8\alpha^+$ T cell in association with age of RA patients. **(D)** Graphs show Disease Activity Score 28 (DAS28), Clinical Disease Activity Index (CDAI), number of tender joints (TJ) and swollen joints (SJ) of RA patients (from left to right) or **(E)** PsA patients with expanded (exp, frequency >2%) and non-expanded (n-exp, <2%) $CD4^+CD8\alpha^+$ T cells. Data are shown as median (± interquartile range). Dots represent individual donors. Statistical analysis using Mann-Whitney-U test or Spearman correlation. For all panels: "ns" denotes not significant; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

Figure 2: Characterization of peripheral blood T cells in RA and HD by ADT and scRNA-Seq

(A) Experimental workflow including T cell enrichment and 10X 5'single-cell coupled to ADT analysis and TCR and scRNA-sequencing of 3 RA patients and 2 healthy controls (HD, created using Biorender). (B) UMAP displaying all $TCR\alpha/\beta^+$ 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 (ADT) for CD4 and CD8 represent CD4 and CD8 surface expression on T cells in representative dot plots of one healthy donor and RA patient**. (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 5 donors. Assignment of CD4 and CD8 positivity is according to labels derived from ADT. Cells are color-coded as indicated in the legend based on ADT. Dots represent individual cells.

Figure 3: $CD4^+CD8^+$ T cells of CD4 origin have clonally expanded TCR sequences

(A-C) UMAP of integrated data of healthy donors (n=2) and RA patients (n=3) showing the distribution of singletons **(A)**, expanded $CD4^+CD8^-$ and $CD8^+CD4^-$ T cell clones without $CD4^+CD8^+$ cells **(B)**, and T cell clones containing $CD4^+CD8^+$ cells of either CD4 or CD8 origin **(C)**. (**D**) Graphs show frequencies of $CD4^+CD8^+$ T cells of CD4 or CD8 origin amongst all clonal T cells for RA patients and controls. Cells are colored according to labels derived from *TCRα/β* sequencing and ADT analysis as indicated in the legends.

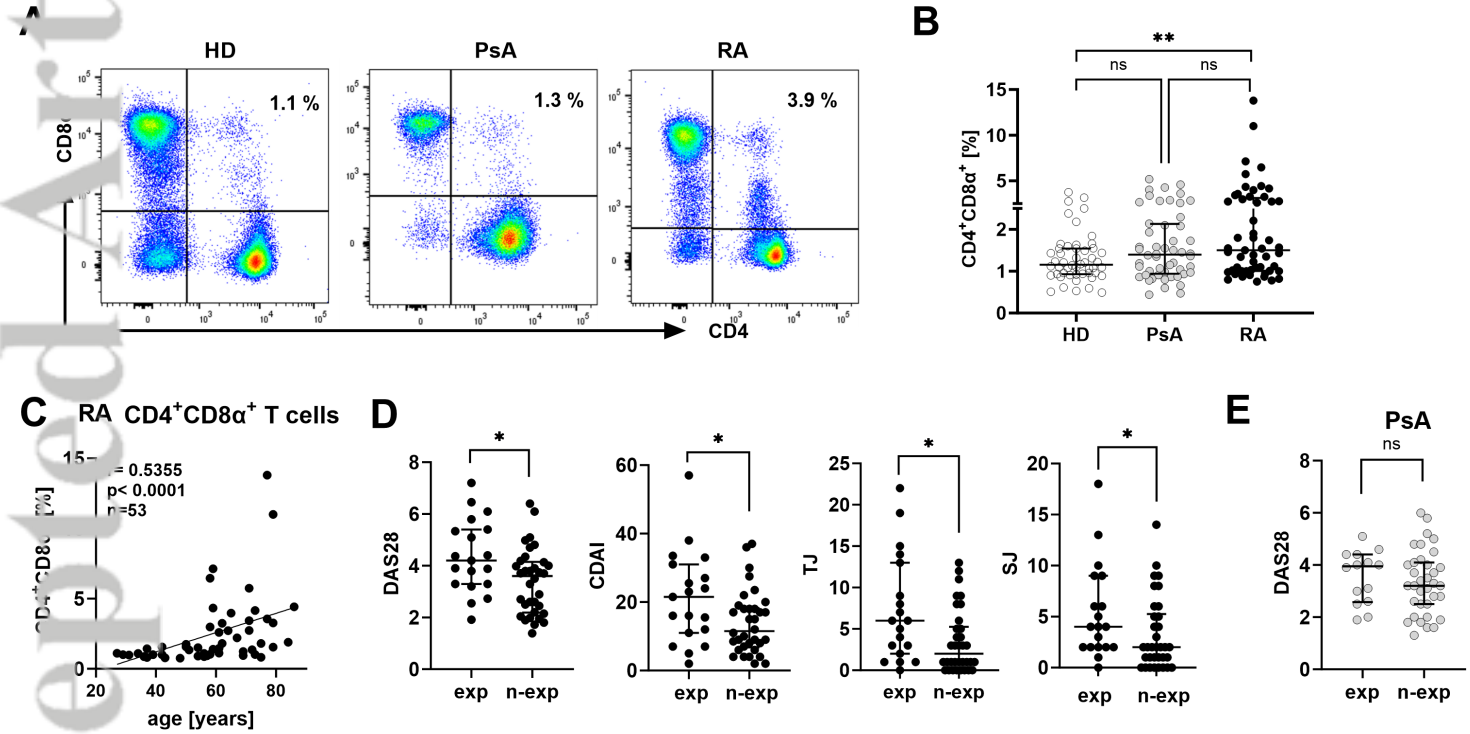
(E) UMAP shows the distribution of T cell clones of the indicated sizes. X represents the frequency of each clonotype defined by its unique paired *TCRαβ* sequence. Large expanded clones ($X \geq 20$), medium-expanded clones ($5 \leq X < 20$), small expanded clones ($2 \leq X < 5$) and singletons ($X=1$) 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 HD and RA. **(G)** Circos plot displaying integrated analysis of shared clones according to *TCRα/β* sequencing in comparison to ADT data.

Figure 4: $CD4^+CD8^+$ T cells of CD4 origin express CD8α, but not CD8β, and show a differentiated phenotype

(A) Violin plot displaying CD8B mRNA expression based on scRNA sequencing in $CD4^+CD8^+$ T cells of CD4 and CD8 origin. **(B)** Surface expression of CD4, CD8α and CD8β of peripheral T cells determined by flow cytometry. Dot plot shows gating of $CD4^+CD8\alpha^{low}$ and $CD8^+CD4^{low}$ subpopulations. **(C)** Graph displaying mean fluorescence intensity (MFI) of CD8β expression on $CD4^+CD8\alpha^{low}$ and $CD8^+CD4^{low}$ T cells among RA patients (RA, n=14) and healthy donors (HD, n=15). **(D)** tSNE analysis of flow cytometry data of one representative RA patient 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,G)** Graphs show frequencies of $CD4^+CD8\alpha^{low}$ T cells from RA patients (n=53) in comparison to PsA patients (n=52) and healthy donors (HD, n=50) **(F)** and comparison between CMV+ and CMV- individuals **(G)**. **(H)** Surface expression of LIR-1 on $CD4^+CD8\alpha^{low}$ and $CD8^+CD4^{low}$ T cells in RA patients. Each dot represents individual donors, lines depict median ± interquartile range. Statistical analysis using Mann-Whitney-U test. For all panels: "ns" denotes not significant; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

Figure 5: Frequencies of $CD4^+CD8\alpha^{low}$ T cells are associated with increased disease activity in RA, and decline under abatacept treatment

(A) Graphs show C-reactive protein (CRP), number of tender joints (TJ) and swollen joints (SJ), Disease Activity Score 28 (DAS28), Short Disease Activity Index (SDAI) and Clinical Disease Activity Index (CDAI) of RA patients with expanded (exp; >0.59%, n=26) and non-expanded (n-exp; <=0.59%, n=27) $CD4^+CD8\alpha^{low}$ T cells. **(B)** Frequencies of $CD4^+CD8\alpha^{low}$ T cells in RA patients with stable disease (n=11, visits V1 and V2 were at least 3 months apart). **(C,D)** Frequencies of $CD4^+CD8\alpha^{low}$ T cells in RA patients before and after treatment with MTX (n=11) **(C)** or abatacept (n=12) **(D)** at the following time points: before (V1), 6 and 12 weeks (V2 and V3) after initiation of treatment. Dots represent individual donors. Statistical analysis using Mann Whitney test (5A) or Wilcoxon test (B-D). For all panels: "ns" denotes not significant; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.



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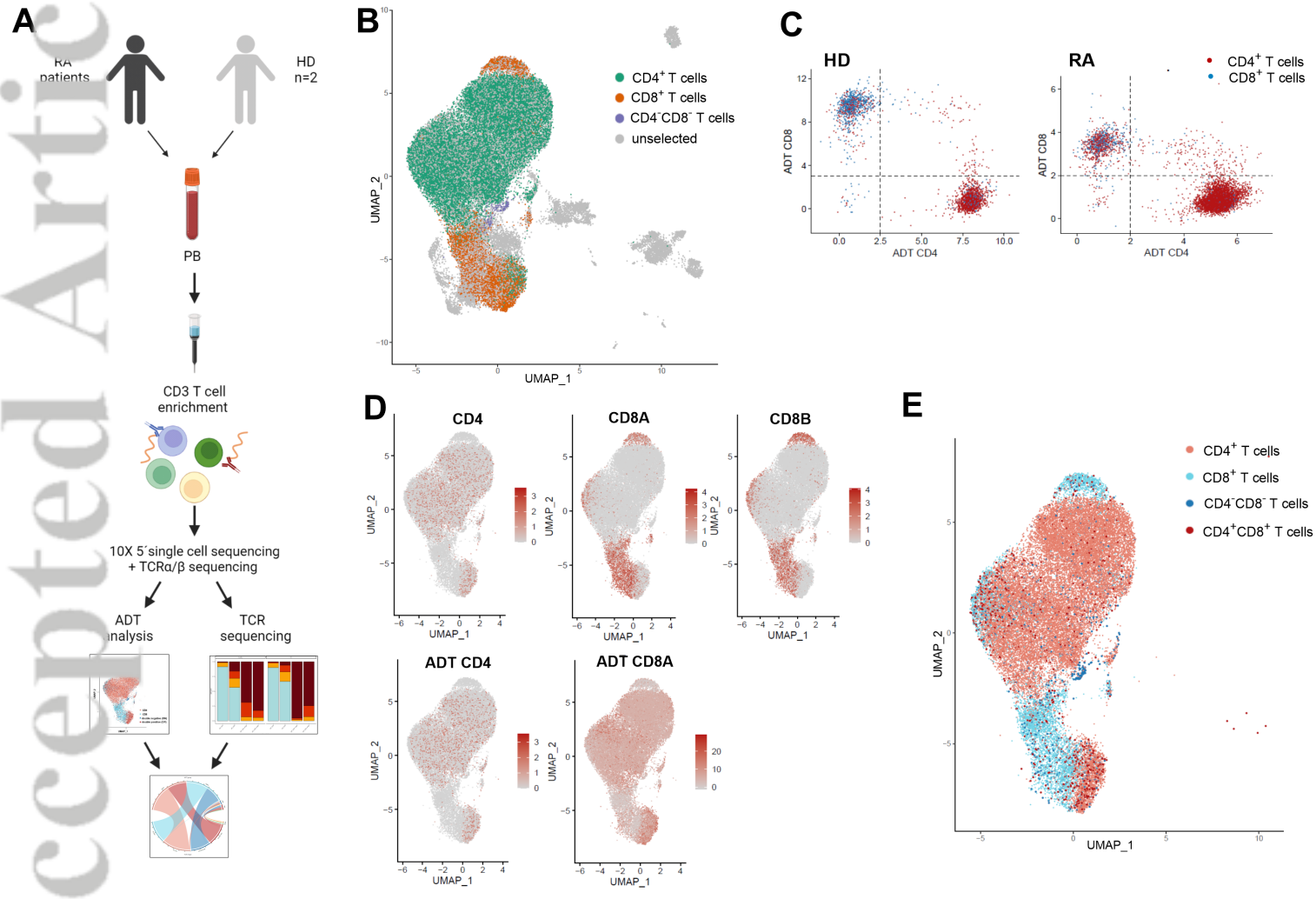

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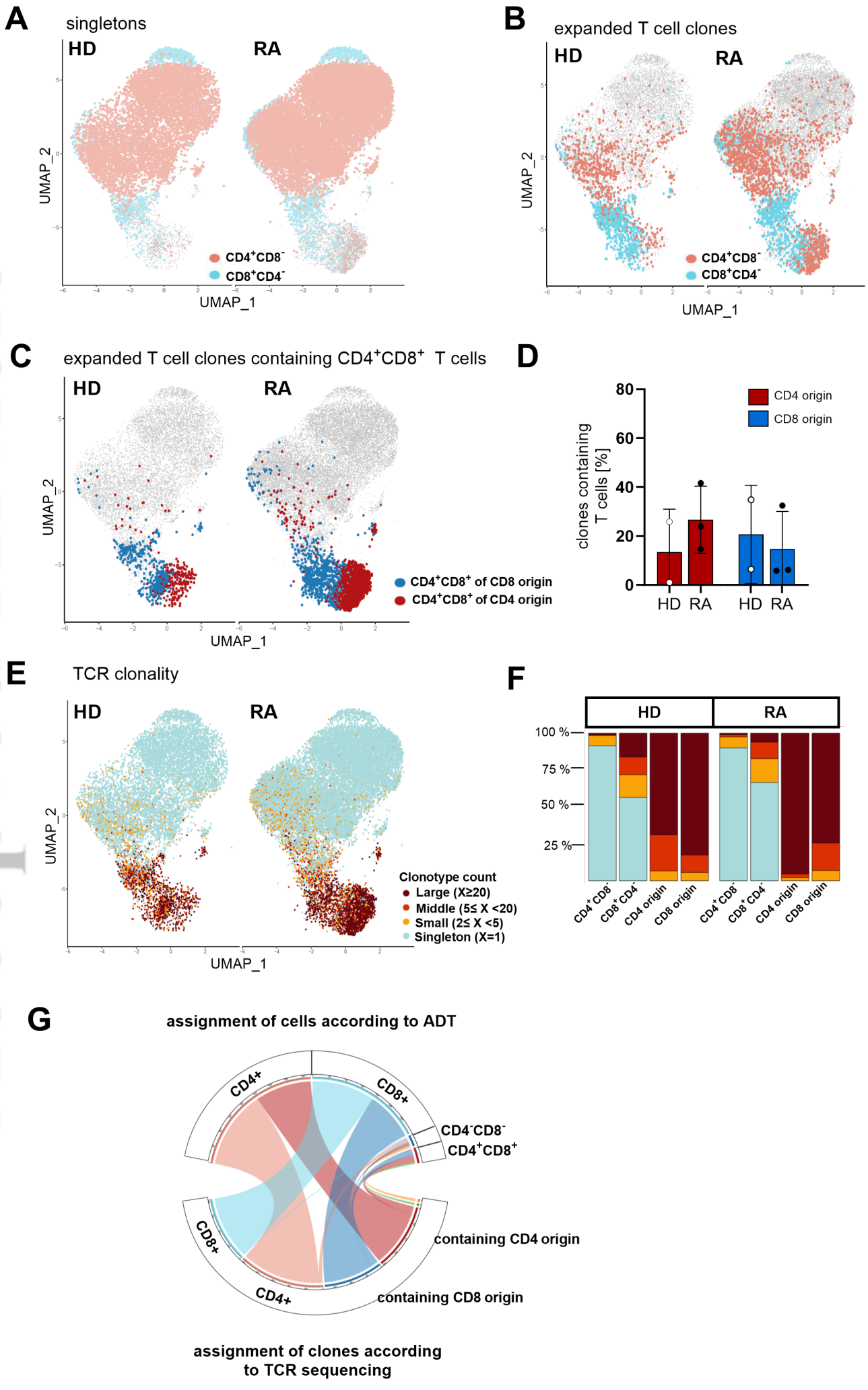

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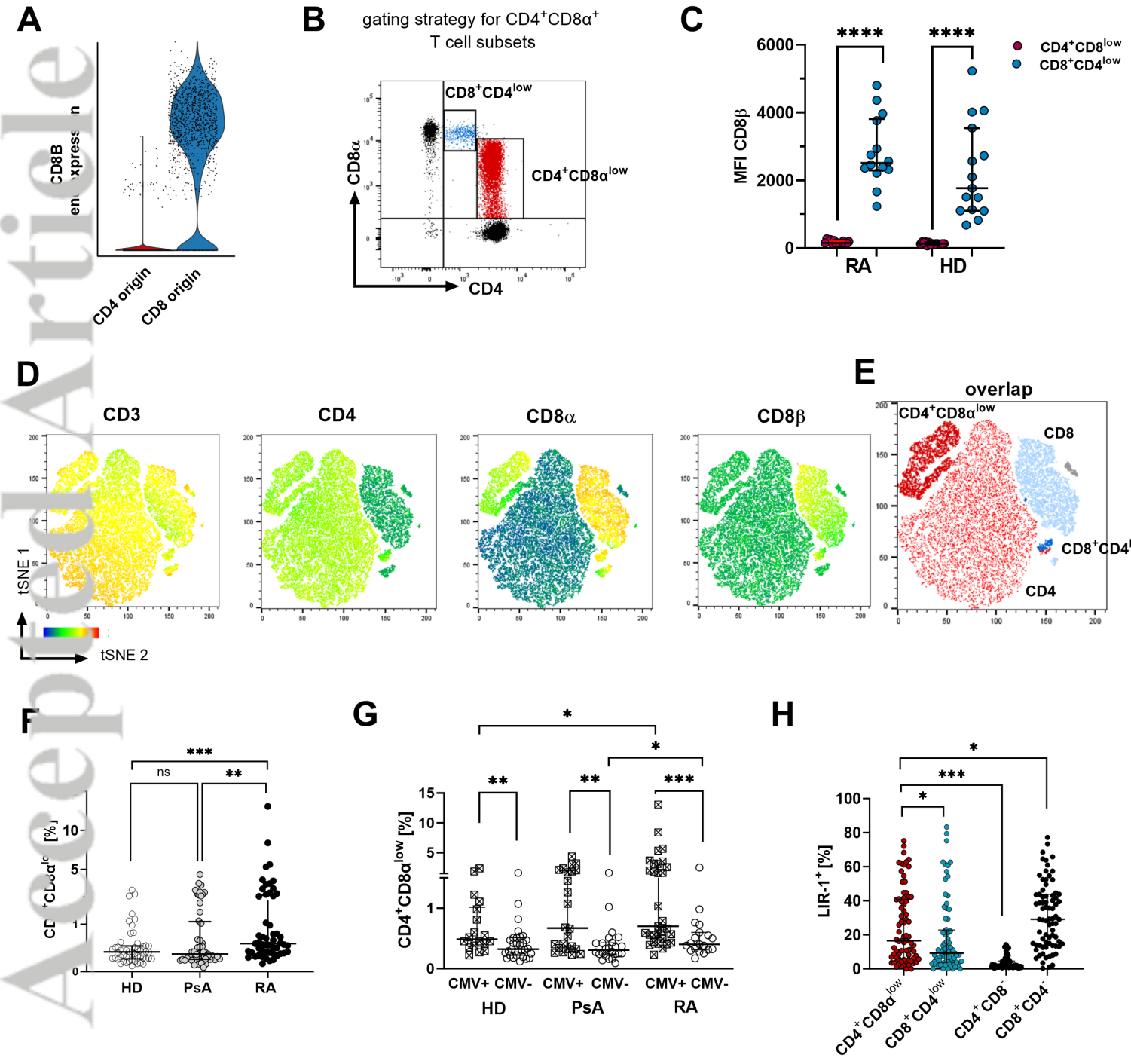

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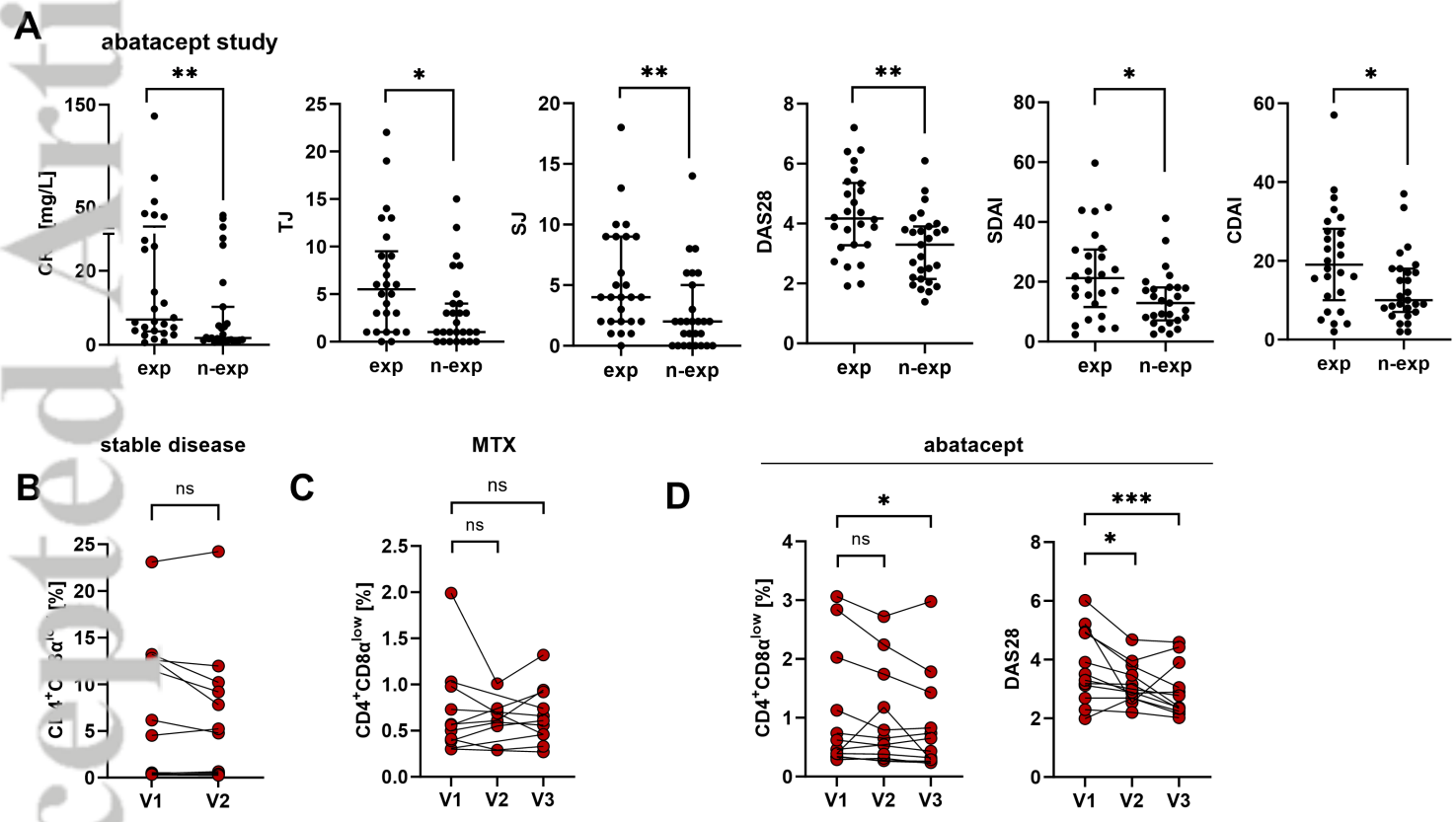

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