

Single-cell transcriptomic profiling reveals a pathogenic role of cytotoxic CD4⁺ T cells in giant cell arteritis

Elio G. Carmona^{a,b}, José Luis Callejas-Rubio^b, Enrique Raya^c, Raquel Ríos-Fernández^b, Gonzalo Villanueva-Martín^a, María C. Cid^d, José Hernández-Rodríguez^d, Esteban Ballestar^e, Bernd Timmermann^f, Norberto Ortego-Centeno^g, Javier Martín^a, Ana Márquez^{a,*}

^a Institute of Parasitology and Biomedicine López-Neyra (IPBLN), Spanish National Research Council (CSIC), Granada, Spain

^b Systemic Autoimmune Diseases Unit, Hospital Universitario Clínico San Cecilio, Instituto de Investigación Biosanitaria de Granada ibs.GRANADA, Granada, Spain

^c Rheumatology Department, Hospital Universitario Clínico San Cecilio, Instituto de Investigación Biosanitaria de Granada ibs.GRANADA, Granada, Spain

^d Vasculitis Research Unit, Department of Autoimmune Diseases, Hospital Clínic, University of Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

^e Epigenetics and Immune Disease Group, Josep Carreras Research Institute (IJC), Badalona, Barcelona, Spain

^f Max Planck Institute for Molecular Genetics, Berlin, Germany

^g Department of Medicine, University of Granada, Instituto de Investigación Biosanitaria de Granada ibs.GRANADA, Granada, Spain

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ABSTRACT

Giant cell arteritis (GCA) is a systemic vasculitis mediated by an aberrant immunological response against the blood vessel wall. Although the pathogenic mechanisms that drive GCA have not yet been elucidated, there is strong evidence that CD4⁺ T cells are key drivers of the inflammatory process occurring in this vasculitis. The aim of this study was to further delineate the role of CD4⁺ T cells in GCA by applying single-cell RNA sequencing and T cell receptor (TCR) repertoire profiling to 114,799 circulating CD4⁺ T cells from eight GCA patients in two different clinical states, active and in remission, and eight healthy controls. Our results revealed an expansion of cytotoxic CD4⁺ T lymphocytes (CTLs) in active GCA patients, which expressed higher levels of cytotoxic and chemotactic genes when compared to patients in remission and controls. Accordingly, differentially expressed genes in CTLs of active patients were enriched in pathways related to granzyme-mediated apoptosis, inflammation, and the recruitment of different immune cells, suggesting a role of this cell type in the inflammatory and vascular remodelling processes occurring in GCA. CTLs also exhibited a higher clonal expansion in active patients with respect to those in remission. Drug repurposing analysis prioritized maraviroc, which targeted CTLs, as potentially repositionable for this vasculitis. In addition, effector regulatory T cells (Tregs) were decreased in GCA and showed lower expression of genes involved in their suppressive activity. These findings provide further insights into the pathogenic role of CD4⁺ T cells in GCA and suggest targeting CTLs as a potential therapeutic option.

1. Introduction

Giant cell arteritis (GCA) is a chronic systemic vasculitis characterized by the inflammation of medium- and large-size blood vessels, primarily the aorta and its major branches, including vessels supplying the optic nerve and retina and the temporal artery, which leads to severe complications such as aortic aneurysm or blindness [1]. GCA represents the most frequent vasculitis in elderly individuals from Western countries, with the highest incidence between the ages of 70–80 years [2].

This vasculitis shows a complex etiology in which the interaction between genetic and environmental factors results in an aberrant immune response against the vascular wall, leading to inflammation and vascular remodelling [3]. It is well known that CD4⁺ T cells play a crucial role in the pathogenesis of GCA, representing the dominant cell type in the vasculitic lesions of these patients. In particular, two CD4⁺ T cell subtypes, Th1 and Th17, have been found to drive this process and are, indeed, expanded in the peripheral circulation of patients with this vasculitis [4,5]. Regulatory T cells (Tregs) are also involved in GCA

* Corresponding author. Institute of Parasitology and Biomedicine López-Neyra, IPBLN, Consejo Superior de Investigaciones Científicas, CSIC, 18016, Granada, Spain.

E-mail address: anamaort@ipb.csic.es (A. Márquez).

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pathogenesis. In this regard, Tregs were shown to be decreased in peripheral blood of GCA patients [6,7]. Interestingly, it has been reported that glucocorticoid (GC) treatment, the first-line therapy for GCA, results in a reduction of the production of cytokines involved in promoting CD4⁺ T cell differentiation into Th17, whereas Th1 response and Treg dysregulation seem to persist after GC treatment [6–8].

In recent years, genetic and immunological studies have provided insight into the pathogenesis of GCA [9,10]. However, we are still far from fully understanding the mechanisms involved in the vascular inflammation that occurs in this disorder. Recently, evaluation of the transcriptomic profile at the single-cell level has emerged as a revolutionary tool to dissect the cellular heterogeneity of a specific tissue or cell type [11,12], thus revealing important insights into the pathogenesis of complex diseases and allowing the identification of novel cell subpopulations with a pathogenic role [13,14]. In this line, an expansion of cytotoxic CD4⁺ T cells (CTLs) in several immune-mediated diseases has been recently revealed using this approach [15–18]. Furthermore, single-cell TCR profiling coupled with RNA sequencing makes it possible to characterize T cell clonal expansion, hence providing a measure of T cell diversity and antigen specificity.

Considering the significance of CD4⁺ T cells in the pathogenesis of GCA, the objective of this study was to comprehensively delineate the role of this cell type in GCA by conducting single-cell RNA sequencing and TCR repertoire profiling of patients in two different clinical states of the disease, namely active and in remission.

2. Material and methods

2.1. Study samples

Eight patients with GCA and eight sex- and age-matched healthy controls were included in the study. GCA patients were collected at the Hospital Clínico San Cecilio of Granada and were diagnosed based on clinical symptoms, positive temporal artery biopsy and/or positive proof of GCA on imaging. All patients fulfilled the 1990 American College of Rheumatology classification criteria for this disease. In addition, GCA patients were classified according to their clinical status at the time of sample collection into active patients (n = 5; newly diagnosed patients or patients with a disease relapse during the follow-up) and patients in remission (n = 3; patients presenting disease in remission on low doses of prednisone (<10 mg/day)). Active disease was defined as new, persistent, or worsening clinical signs and/or symptoms of GCA while remission was defined as absence of clinical signs or symptoms attributed to active GCA. All patients with GCA were receiving GC treatment at the time of sample collection. The main characteristics of the individuals included in the study are shown in [Supplemental Table 1](#). The local ethics committee approved the study and all participants signed an informed consent form in accordance with the ethical guidelines of the 1975 declaration of Helsinki.

After the collection of fresh whole blood samples from patients and controls, peripheral blood mononuclear cells (PBMCs) were immediately isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). Then, CD4⁺ T cells were isolated from PBMCs using the EasySep™ Human CD4⁺ T Cell Isolation Kit (StemCell Technologies) according to the manufacturer's instructions. After assessing cell counting and viability, CD4⁺ T cells were resuspended in freezing medium (FBS, 10% DMSO) and cryopreserved in liquid nitrogen until processed.

2.2. Single-cell library preparation and sequencing

Single-cell libraries were prepared from isolated CD4⁺ T cells using the Chromium Next GEM Single Cell V(D)J Reagent Kits v1.1 (10x Genomics). Cell suspensions were loaded onto the 10x Genomics Chromium Chip together with gel beads containing barcoded oligonucleotides (unique molecular identifier, UMIs) and partitioning

oil to generate single-cell gel beads-in-emulsion (GEMs). Barcoded cDNAs generated within each individual GEM by reverse transcription were PCR-amplified. Specifically, single-cell RNA sequencing libraries were generated by using the Chromium Single Cell 5' Library & Gel Bead Kit and the single-cell TCR sequencing libraries were constructed by using the V(D)J Enrichment Kit, Human T Cell. Single-cell libraries were sequenced on an Illumina NovaSeq 6000 platform to generate 2 × 101-bp paired-end reads.

2.3. Single-cell data analysis

Data processing and quality control. Raw single-cell RNA sequencing data were mapped to the human transcriptome GRCh38 and UMI counting was performed using the CellRanger software (v.4.0.0). Output data files were then individually prepared for quality control with the Seurat V.3 R package [19].

Cells expressing less than 400 or more than 2500 unique genes, cells with a fraction of mitochondrial (MT) genes higher than 8 %, and cells with a percentage of ribosomal genes below 15 % were removed from the analysis. Non-CD3⁺ and CD8⁺ cells (*CD8A*, *CD8B*) were also removed ([Supplemental Table 2](#)).

Doublet detection. DoubletFinder V2.0 [20] was implemented in order to predict and eliminate doublets from our data. This step was performed after the filtering and normalization steps but before the integration, as recommended by the developer. Based on the estimated multiplet rate from 10X Genomics, 540 putative doublets were removed on average for each sample dataset ([Supplemental Table 2](#)).

Data normalization and integration: Each sample was individually normalized using SCTransform V2 [21], accounting for 3000 highly variable genes. Next, integration was performed with the Reciprocal Principal Component Analysis (RPCA) embedded in Seurat v4.0.

Cell cycle score and MT genes regression: We determined the cell cycle phase of each cell and assigned a score with the Seurat *CellCycleScoring()* function. Then, gene expression data were adjusted by linear regression using this score and the percentage of MT genes as covariates to remove their influence.

Cell clustering and projection: Cell clustering was performed based on the first 19 principal components, which explain ~90 % of the total variance in our data ([Supplemental Fig. 1](#)). As a result, 13 clusters were generated using the Louvain algorithm with a resolution parameter of 0.8. Graphical projections of this clustering were built with the Uniform Manifold Automated Projection (UMAP) technique to visualize different expression profiles of cells in a reduced dimensional space.

Cell type annotation: Genes overexpressed in each cluster with respect to the others were identified using the Wilcoxon rank-sum test under the *FindMarkers()* function (\log_2 fold change (FC) ≥ 0.20 ; $P < 0.05$ after Bonferroni correction). Cell type annotation was performed with the automated annotation package SingleR [22], which compared the transcriptome of each cluster with the reference dataset Database of Immune Cell Expression (DICE) [23]. In addition, we then refined cell cluster annotation considering the expression of canonical and previously reported CD4⁺ T cell markers [24]. [Supplemental Table 3](#) shows the list of markers used to annotate cell clusters. Three residual populations (n < 30) annotated as B cells or monocytes were removed.

Differential abundance analysis: To compare differences in cell composition between GCA patients and healthy controls as well as between subgroups of GCA patients, we employed edgeR package [25] and its function *glmQLFTest()*, which utilizes a negative binomial generalized linear model (NB-GLM) to model count data with limited replication while taking into account overdispersion of data.

Differential expression analysis: Differentially expressed genes (DEGs) between GCA patients and healthy controls as well as between subgroups of GCA patients were identified separately for each cell type using the Wilcoxon rank-sum test under the *FindMarkers()* function. Since the number of males and females among the different subgroups was slightly different, gender was added as a covariate to control its

influence on the results. Bonferroni was used to correct for multiple testing and those genes showing \log_2 fold changes ≥ 0.2 and adjusted p-values < 0.05 were considered as DEGs.

Enrichment analysis for Gene Ontology (GO) Biological Processes terms across the set of DEGs identified in each CD4⁺ T cell subtype and in each comparison (active vs. healthy controls; active versus in remission; in remission vs. healthy controls) was performed using STRING [26].

2.4. Flow cytometric analysis

PBMC samples from 25 GCA patients classified into three clinical subgroups according to disease activity and treatment, including nine treatment-naïve patients with active disease, seven patients in remission without treatment, and nine patients in remission treated with GCs, and ten healthy controls were collected at the Hospital Clinic of Barcelona. Detailed characteristics of these individuals are reported in a previous study [27] and summarized in Supplemental Table 4. We employed a FacSymphony™ unit (BD Biosciences) and the software FlowJo v10.7 to analyze the percentage of CD4⁺ T cells expressing any of these two cytotoxic markers, granzyme B (GZMB) and perforin 1 (PRF1). First, we designed a flow cytometry panel including the following antibodies: anti-human CD3-APC, anti-human CD4-APC-H7, anti-human GZMB-PE, and anti-human PRF1-FITC, purchased from BD Pharmingen.

Cells were first treated with the Fixable Viability Dye eFluor™ 450 (eBioscience™), followed by staining of surface protein antibodies (CD3 and CD4). They were then washed and treated with permeabilization buffer (IntraPrep™, Beckman Coulter) before staining with intracellular protein antibodies (GZMB and PRF1) and cell fixation (IOtest3™, Beckman Coulter).

All samples were first gated by FSC/SSC and then filtered by doublets and viability (Supplemental Fig. 2). Four samples were discarded due to irregular parameters or low viability percentages. For the remaining 31 samples (eight untreated active GCA patients, six untreated GCA patients in remission, eight GCA patients in remission treated with GC, and nine healthy controls) the proportion of CD4⁺ T lymphocytes expressing GZMB and PRF1 was calculated. Differences in the mean proportion of CD4⁺ T cells expressing GZMB or PRF1 between pairs of groups were determined by performing Welch two-sample T-test. P-values lower than 0.05 were considered statistically significant.

2.5. Single-cell TCR analysis

Single CD4⁺ T cells were simultaneously employed to obtain TCR repertoire sequence using 10X Immune profiling technology (10X genomics) (Supplemental Table 5). Enriched cDNA from TCR- α and TCR- β chains was used to construct the libraries. After sequencing, reads were assembled using CellRanger VDJ (10x Genomics, v4.0.0). Only productive TRA-TRB contigs were added as metadata and analyzed with scRepertoire v1.8.0 [28] R package. Expanded clonotypes, defined as a TCR sequence detected in at least two cells, were identified and quantified as small ($1 < X \leq 5$), medium ($5 < X \leq 20$), large ($20 < X \leq 100$) and hyperexpanded ($100 < X \leq 600$) based on their frequency. Inverse Shannon entropy index for diversity analysis was calculated with the *clonalDiversity()* function included in scRepertoire.

2.6. Pseudotime analysis

We applied Monocle 3 [29] methodology to discover potential cell differentiation trajectories. After transformation of the Seurat Object into a cell data set object, effect sizes were calculated using the *estimate_size_factors()* function and partitioning of the raw data was performed with the *cluster_cell()* function. Subsequently, the *learn_graph()* function was used to infer branches of cell differentiation and thus assign a pseudotime value to each cell. Based on the higher expression of naïve T cell genes (C-C motif chemokine receptor 7 (CCR7), lymphoid enhancer

binding factor 1 (LEF1), and selectin L (SELL)) observed in cluster 3, we selected it as the pseudotime starting root. Additionally, the Destiny package (v3.12.0) [30] was applied to conduct an alternative unsupervised dimensionality reduction analysis via diffusion mapping. This projection of the data measured the probability of transitioning from one point to another based on the construction of a diffusion kernel.

2.7. Transcription factor analysis

We employed the decoupleR package [31] to estimate transcription factor (TF) activity scores. This software allows to infer TF activity at the single-cell level based on the weighted expression of target genes.

First, we accessed CollecTRI gene regulatory network data through OmniPathR. CollecTRI compiles data from 12 different sources (ExTRI, HTRI, TRRUST, TFAcTS, IntAct, SIGNOR, CytReg, GEREDB, Pavlidis, DoRothEA A, NTNU curations), which result in extensive coverage of interactions between TFs and their target genes. For our analysis, we used the human set of CollecTRI, containing information on 1178 unique TFs. We then applied the Univariate Linear Model (ulm) method to assign TF activity scores to each cell in the study. This approach involved fitting a linear model for each TF in the CollecTRI network, using our single-cell RNAseq expression dataset as input. A positive score indicated TF activity, while a negative score indicated TF inactivity.

To identify TFs showing differential activity between both groups of GCA patients and healthy controls as well as between active and in remission patients, we conducted Wilcoxon rank-sum tests and Hedge's G statistical tests to assess mean differences and effect sizes, respectively. We selected relevant TFs that met FDR < 0.05 and Hedge's G > 0.5 , which indicates a medium-sized effect.

2.8. Single-cell resolution drug targeting

We used the ASGAR (A Single-cell Guided Pipeline to Aid Repurposing of Drugs) R software [32] to identify potential drug candidates targeting specific CD4⁺ T cell subtypes. Using differentially expressed genes as inputs, we used the *GetDrug()* function to identify drugs that can significantly (single-cluster FDR < 0.05) reverse their expression levels in the L1000 drug response dataset [33].

3. Results

3.1. Single-cell landscape of CD4⁺ T cells in GCA

We performed single-cell transcriptome profiling on CD4⁺ T cells from eight GCA patients, five in the active state of the disease and three in remission, and eight healthy controls (Fig. 1a). After quality control, a total of 114,799 cells were analyzed, including 49,960 cells from patients and 64,839 cells from controls (Supplemental Table 2).

Based on their expression profiles, cells were clustered and visualized in 2D using UMAP. We identified 13 clusters (Fig. 1b and Supplemental Table 6), which were assigned to six different CD4⁺ T cell subpopulations according to the expression of both well-established and previously reported cell type markers [24] (Fig. 1c and d, and Supplemental Table 7), including naïve T cells (T_N) (clusters 0, 3, 4, 5, 6, and 11), characterized by expression of naïve markers, such as CCR7 and SELL; central memory T cells (T_{CM}) (clusters 1, 2, and 9), expressing higher levels of interleukin 7 receptor (IL7R) together with lower levels of CCR7; effector memory T cells (T_{EM}) (cluster 12), expressing genes related to effector functions, such as GZMA, annexin A2 (ANXA2), galectin 1 (LGALS1), and S100 calcium binding protein A4 (S100A4); a T cell subpopulation (cluster 7) showing high expression of granzyme K (GZMK⁺); cytotoxic T cells (CTL) (cluster 10), characterized by high expression levels of cytotoxicity-related genes (granulysin (GNLY), natural killer cell granule protein 7 (NKG7), PRF1, GZMB); and regulatory T cells (Tregs) (cluster 8), which expressed forkhead box P3

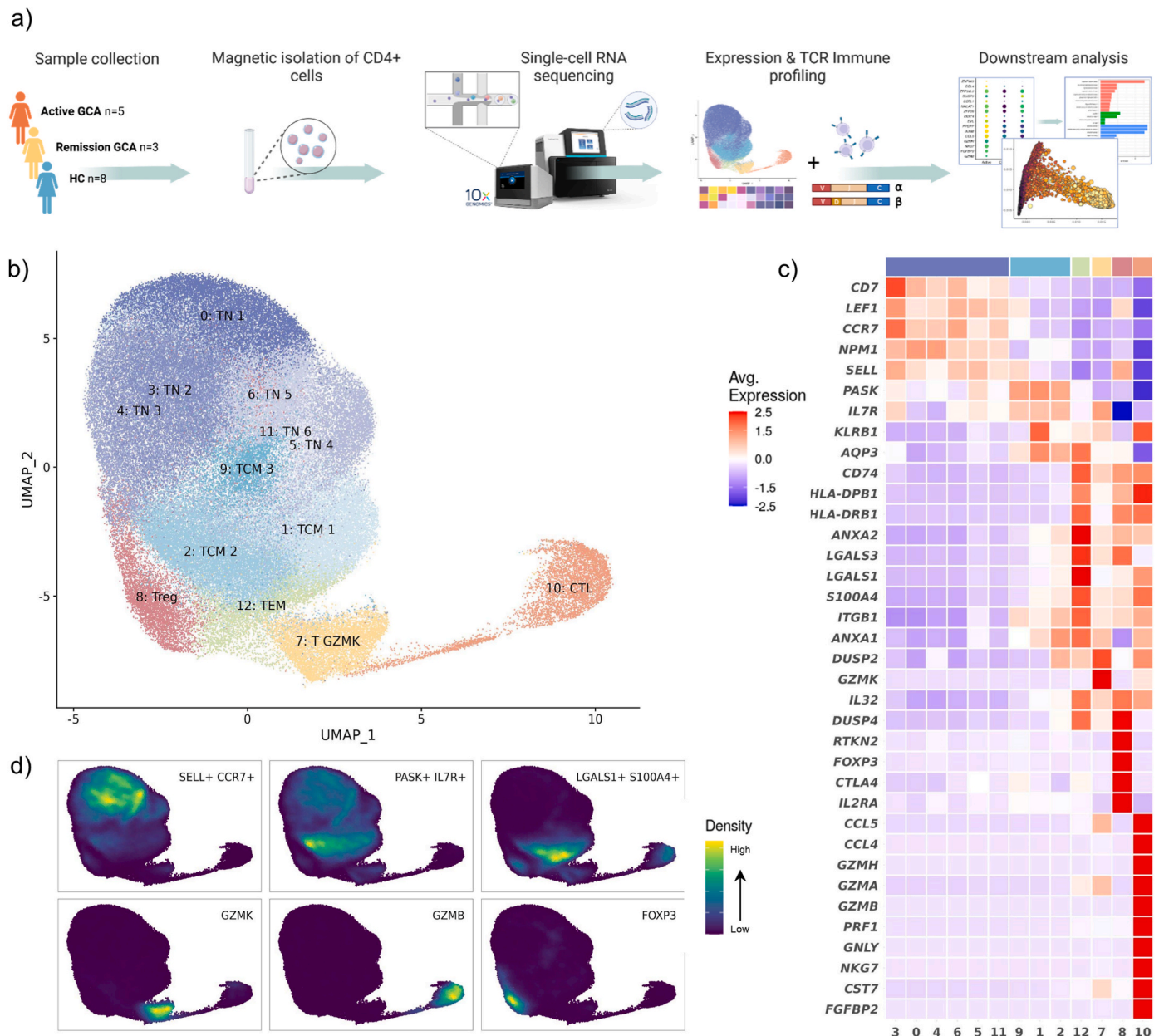


Fig. 1. Single-cell transcriptome profiling of $CD4^+$ T cells of patients with GCA in the active phase of the disease, patients in remission and healthy controls. (a) Schematic representation of the overall study design. Single-cell RNA sequencing and T cell receptor profiling were applied to circulating $CD4^+$ T cells. (b) UMAP visualization of single-cell RNA-seq data of 114,799 $CD4^+$ T cells. Colors represent cells in the 13 defined clusters, including 6 clusters of naïve T cells (TN), 3 clusters of central memory T cells (TCM), 1 cluster of effector memory T cells (TEM), 1 cluster of granzyme K positive (GZMK $^+$) T cells, 1 cluster of cytotoxic T cells (CTL), and 1 cluster of regulatory T cells (Treg). (c) Heatmap showing the average expression levels per cluster of canonical cell type markers and the top signature genes of each cluster. (d) Density plots showing the expression of canonical markers of the six $CD4^+$ T cell subpopulations. UMAP, uniform manifold approximation and projection.

(*FOXP3*) and *IL2RA*.

3.2. $CD4^+$ CTLs are expanded in active GCA patients compared to those in remission

After identification of $CD4^+$ T cell subtypes, changes in cellular composition were assessed by comparing the frequency of these cell subtypes between GCA patients and controls (Fig. 2a and Supplemental Fig. 3).

No differences were identified considering the global disease; however, when patients were stratified according to disease activity, we found a marked expansion of $CD4^+$ CTLs in active patients compared to patients in remission (5.11 % vs. 0.69 %; $p = 0.0011$) and a reduction in

patients in remission compared to controls (0.69 % vs. 4.84 %; $p = 0.033$) (Fig. 2a). An expansion of $CD4^+$ CTLs has been described in several autoimmune diseases with respect to healthy individuals; however, although the percentage of CTLs was slightly higher in active patients compared with controls (5.11 % vs. 4.84 %), it did not differ significantly.

It has been previously reported that GC treatment results in a reduction of cytotoxic $CD4^+$ T cells [34]. Considering this, we hypothesized that the low number of CTLs observed in active patients could be a consequence of GC treatment. To test this hypothesis and validate the expansion of CTLs in active patients, we used flow cytometry to analyze the percentage of $CD3^+CD4^+$ T cells expressing GZMB or PRF1 in an independent cohort of GCA patients classified in three different

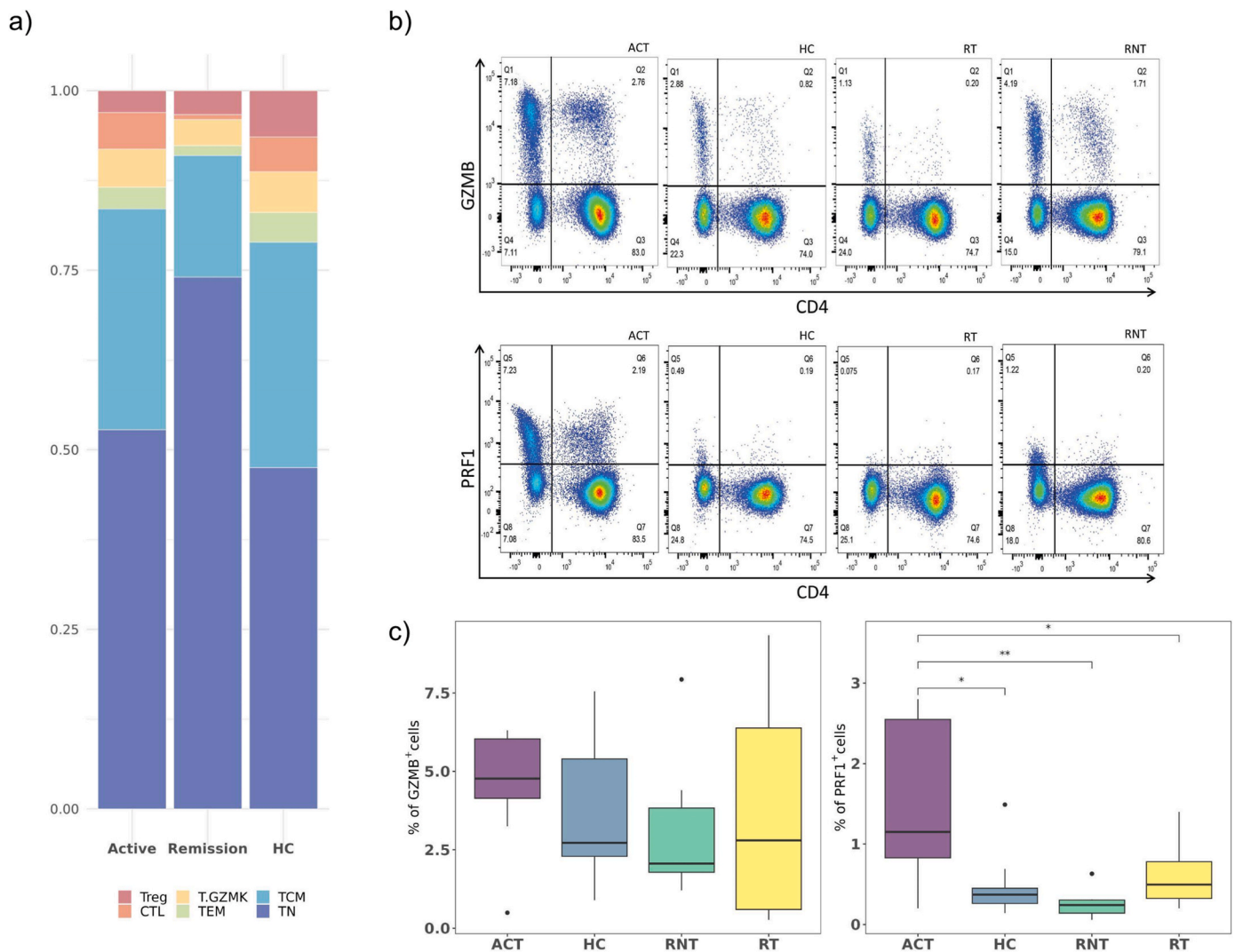


Fig. 2. Expansion of cytotoxic T cells in active GCA patients. (a) Relative percentage of each CD4⁺ T cell subtype among total cells in active GCA patients, patients in remission and healthy controls (HC). (b) Representative flow cytometry profiles of four of the 31 analyzed individuals, including 1 treatment-naïve active patient (ACT), 1 healthy control (HC), 1 patient in remission without treatment (RNT), and 1 patient in remission treated with glucocorticoids (RT). The plots for the remaining individuals are shown in [Supplementary Fig. 4](#). Cells gated on CD3⁺ were profiled using CD4 (x axis) and PRF1 or GZMB (y axis). (c) Boxplot showing the percentage of CD4⁺GZMB⁺ T cells (left panel) and CD4⁺PRF1⁺ T cells (right panel) among the total of CD4⁺ T cells of the 31 analyzed individuals (8 ACT, 9 HC, 6 RNT, and 8 RT). $P < 0.05$ (Welch two-sample T-test).

subgroups according to disease activity and treatment, including eight treatment-naïve active patients, eight patients in remission treated with GC, six patients in remission without treatment, and nine healthy controls (Fig. 2b and [Supplemental Fig. 4](#)).

Notably, the percentage of PRF1⁺ cells in the total of CD3⁺CD4⁺ T cells was statistically higher in active patients compared with any of the other subgroups, healthy controls ($p = 0.025$), patients in remission without treatment ($p = 0.0097$) and patients in remission treated with GCs ($p = 0.043$) (Fig. 2c). A higher percentage of CD4⁺GZMB⁺ cells was also observed in active patients with respect to healthy controls and patients in remission; however, these differences were not statistically significant (Fig. 2c).

On the other hand, a reduced proportion of other CD4⁺ subtypes, namely T_{CM} ($p = 0.017$) and T_{EM} ($p = 0.017$), was also found in patients in remission compared with healthy controls, whereas the percentage of T_N tended to be higher in the subgroup of patients with no sign of disease ($p = 0.084$) (Fig. 2a).

3.3. Effector Tregs are decreased in GCA patients

Comparison of the relative cell proportions also evidenced differences in the percentage of Tregs between GCA patients and controls. Specifically, the number of Tregs was reduced in active patients compared with healthy individuals (3.02 % vs. 6.44 %; $p = 0.023$) (Fig. 2a). A similar trend was observed in the subgroup of patients in remission, although this difference did not reach statistical significance (3.31 % vs. 6.44 %; $p = 0.154$).

Considering the functional heterogeneity of the Treg population, we decided to perform a subclustering to further characterize this cell subtype. The Treg cluster was split into eight new subclusters based on DEGs signatures (Fig. 3a). According to previously defined markers of naïve and effector Tregs and the top signature genes of each cluster, four subclusters (clusters 1, 2, 3 and 5), expressing *CCR7* and *IL7R*, matched a naïve or central memory phenotype, while the remaining four subclusters (clusters 0, 4, 6, and 7) showed an effector phenotype with increased expression of genes involved in the suppressive function of Tregs, including cytotoxic T-lymphocyte associated protein 4 (*CTLA4*), *IL10RA* and *LGALS1* (Fig. 3b and [Supplemental Table 8](#)). It is worth

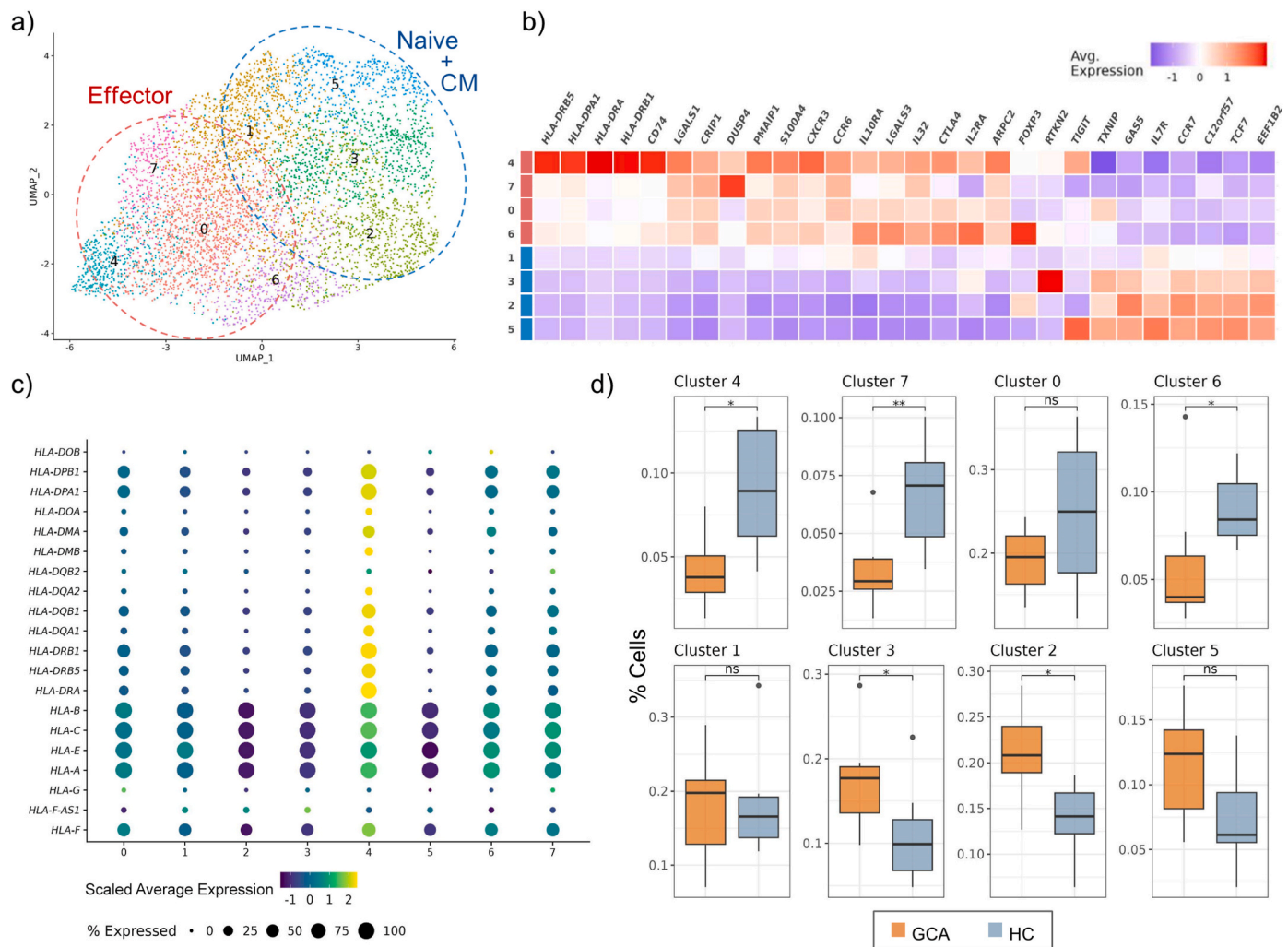


Fig. 3. Subclustering of regulatory T cells (Tregs). (A) UMAP visualization of single-cell RNA-seq data of Tregs. Colors represent cells in the eight defined subclusters, including four subclusters of naive/central memory Tregs and four subclusters of effector Tregs. (B) Heatmap showing the average expression levels per subcluster of previously defined markers of naive and effector Tregs and the top signature genes of each subcluster. (C) Dot plot displaying the expression of HLA class II genes for eight Treg subclusters. The size of each circle represents the percentage of cells in the subcluster expressing the gene and the color indicates the average expression. (D) Relative percentage of the different Treg subclusters among total Tregs in GCA patients and healthy controls (HC). UMAP, uniform manifold approximation and projection.

mentioning that cluster 4, one of the effector subclusters, was characterized by high expression of HLA class II genes (Fig. 3c), which have a key role in the regulatory functions of Tregs [35], and the chemokines C-X-C motif chemokine receptor 3 (*CXCR3*) and C-C motif chemokine receptor 6 (*CCR6*), involved in cell migration to inflammation zones.

Interestingly, most of the effector clusters, specifically clusters 4, 6, and 7, were decreased in patients with GCA compared with healthy controls ($p = 0.01$, $p = 0.021$, and $p = 0.0047$, respectively). On the contrary, clusters with a less activated phenotype, including clusters 2 and 3, were increased in GCA ($p = 0.01$ and $p = 0.038$, respectively) (Fig. 3d), likely at the expense of the decrease in the effector cell compartment.

3.4. $CD4^+$ T cells from active patients present higher expression levels of cytotoxicity-related genes and decreased regulatory activity

To identify transcriptomic changes between GCA patients and healthy individuals, we performed differential expression analysis separately for each cell type (Supplemental Tables 9–11). DEGs were found in the different subpopulations of $CD4^+$ T cells and in all three comparisons.

In the case of CTLs, compared with healthy individuals, active patients showed increased expression levels of several chemokines (C-C motif chemokine ligand 5 (*CCL5*), *CCL4* and C-C motif chemokine ligand 4 like 2 (*CCL4L2*)) and the transcription factor zinc finger protein 683 (*ZNF683*), also known as *HOBIT*, which is crucial for the cytotoxic function of $CD4^+$ T cells [36,37] (Fig. 4a and Supplemental Table 9). Interestingly, other genes involved in cytotoxicity, including *GZMH*, *GZMB*, fibroblast growth factor binding protein 2 (*FGFBP2*), and *NKG7*, were upregulated in active patients with respect to patients in remission (Fig. 4a and Supplemental Table 10). On the contrary, these same genes, together with other cytotoxic genes (*GNLY*, spondin 2 (*SPON2*), *PRF1*, and *GZMA*), showed decreased expression levels in the subgroup of patients in remission compared with controls (Fig. 4a and Supplemental Table 11). This suggests that CTL-mediated cytotoxicity is down-regulated in GCA patients with no signs of disease.

Regarding Tregs, genes involved in the suppressor function of this cell type, such as Major Histocompatibility Complex, Class II, DR Beta 1 (*DRB1*), *DQB1*, and *LGALS1* [35,38,39], showed lower expression levels in both active and in remission patients than in the control group (Fig. 4b and Supplemental Tables 9 and 11).

In addition, other $CD4^+$ T cell subtypes also showed aberrant

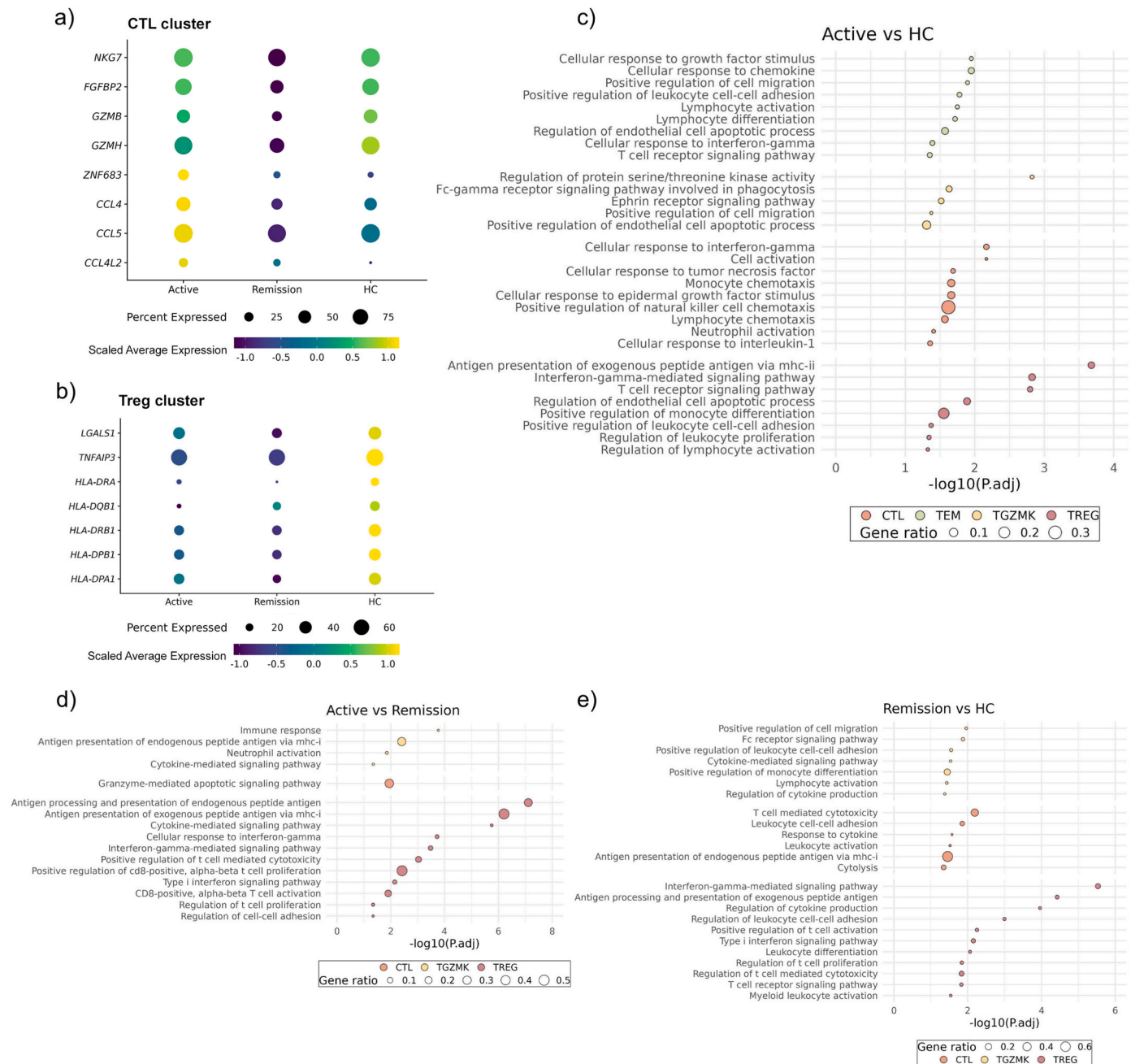


Fig. 4. Genes and pathways dysregulated in GCA patients. (a) Dot plot of differentially expressed cytotoxic and chemotactic genes in cytotoxic CD4⁺ T cells of active GCA patients compared with patients in remission and healthy controls (HC). The size of each circle represents the percentage of cells in the subcluster expressing the gene and the color indicates the average expression. P-value < 0.05 (Wilcoxon rank-sum test). (b) Dot plot of genes related to the regulatory function of Tregs differentially expressed in the Treg subpopulation of active and remission GCA patients compared with healthy controls. The size of each circle represents the percentage of cells in the subcluster expressing the gene and the color indicates the average expression. P-value < 0.05 (Wilcoxon rank-sum test). (c) Bubble plot representation of selected Gene Ontology (GO) biological processes enriched across the set of DEGs in active patients with respect to controls in different CD4⁺ T cell subtypes. (d) Bubble plot representation of selected GO biological processes enriched across the set of DEGs in active patients with respect to patients in remission in different CD4⁺ T cell subtypes. (e) Bubble plot representation of selected GO biological processes enriched across the set of DEGs in patients in remission with respect to controls in different CD4⁺ T cell subtypes.

expression levels of genes with a relevant role in the inflammatory process. In this regard, GZMK⁺ cells of patients in the active phase had increased levels of lymphotoxin beta (*LTB*) and *CCL5* compared with patients in remission (Supplemental Table 10), whereas this same cell type showed a downregulation of relevant genes, such as *ANXA1* and *CD74*, in patients in remission compared with controls (Supplemental Table 11).

Subsequently, we assessed the biological meaning of the identified

differential expression using a pathway enrichment analysis. This analysis evidenced several pathways that were altered in GCA (Fig. 4c, d, and 4e and Supplemental Tables 12–27).

Compared with healthy controls, DEGs in CTLs from active patients were enriched in pathways related to the recruitment and activation of different cell types, including neutrophils, monocytes, lymphocytes, and NK cells (Fig. 4c and Supplemental Table 12). Interestingly, other pathways with a known role in GCA pathogenesis, namely cellular

response to IL-1, cellular response to tumour necrosis factor (TNF), and cellular response to interferon-gamma (IFN- γ) were also enriched in CD4⁺ CTLs from active patients compared with healthy controls (Fig. 4c and Supplemental Table 12). In addition, several enriched pathways in active patients compared with controls were shared among different cell types, including some pathways with a potential role in the context of GCA pathogenesis, such as regulation of endothelial cell apoptotic process (Fig. 4c and Supplemental Tables 14–17).

An enrichment in relevant pathways was also observed for the set of

DEGs in active patients compared with patients in remission, including granzyme-mediated apoptotic signaling in CTLs (Fig. 4d and Supplemental Table 18), neutrophil activation in GZMK⁺ cells (Fig. 4d and Supplemental Table 21), and CD8-positive alpha-beta T cell activation and proliferation in different cell subtypes (Fig. 4d and Supplemental Tables 19 and 22).

In Tregs, enrichment in immune-relevant pathways, including T cell receptor signaling pathway, antigen processing and presentation of endogenous and exogenous peptide antigen via mhc class i and ii, and

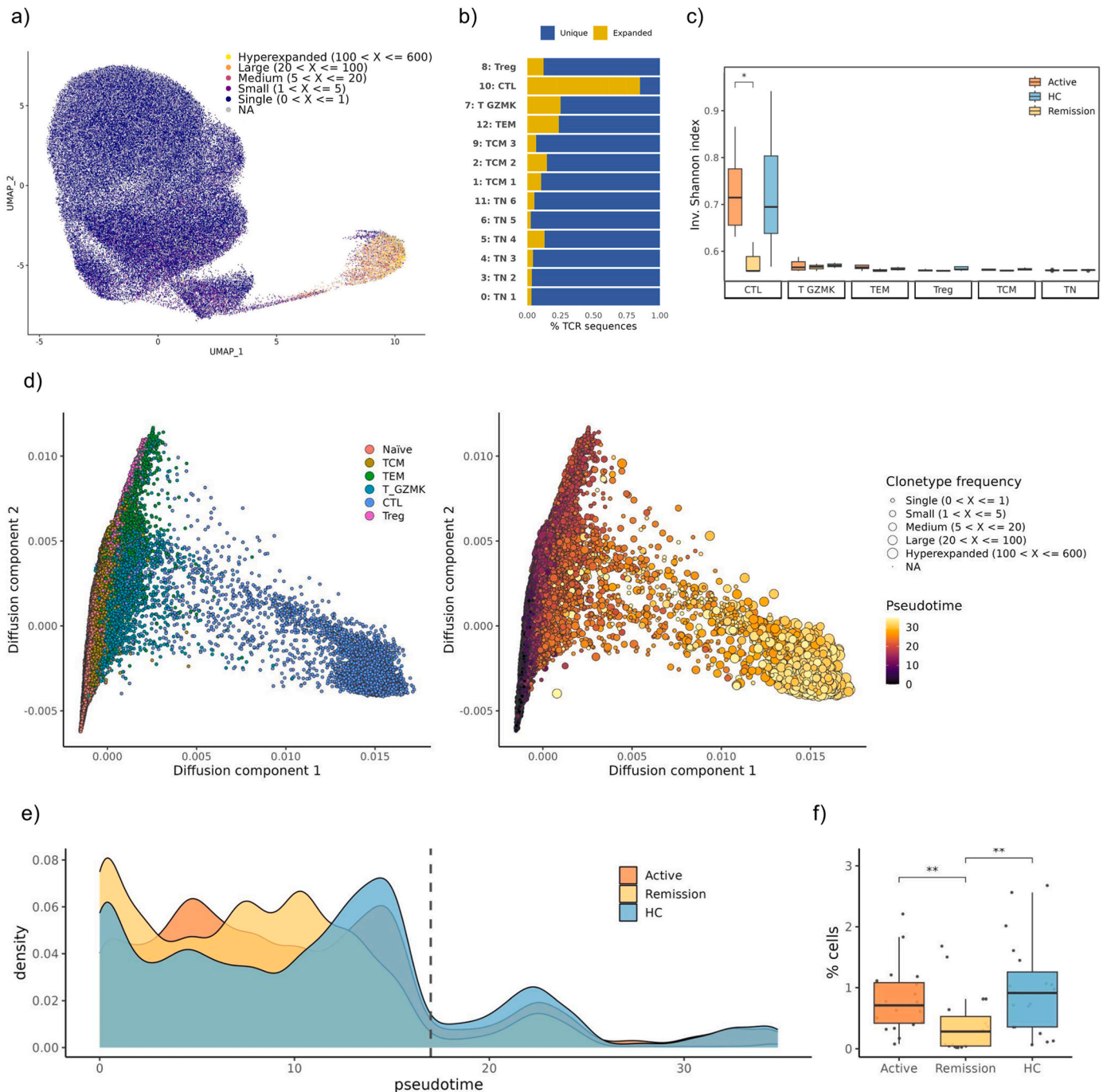


Fig. 5. Clonal expansion of T cell receptors (TCR) and trajectory analysis. (a) UMAP representation of the single-cell RNA-seq data of the analyzed CD4⁺ T cells colored by TCR clonality. (b) Bar plot showing percentage of expanded clonotypes in each cluster. (c) Diversity of the TCR repertoire measured with the inverse Shannon entropy index in each cluster across samples. (d) Diffusion map showing branched pseudotime trajectory of CD4⁺ T cells, each cell is colored by cell type (left panel) or pseudotime value (right panel). In the right panel, the size of the clones, from single to hyper-expanded clonotypes, is represented by the size of the dots. (e) Density plot showing the percentage of CD4⁺ T cells according to pseudotime value. (f) Barplot representing the percentage of CD4⁺ T cells with pseudotime values higher than 17 in active GCA patients, patients in remission, and healthy controls (HC). UMAP, uniform manifold approximation and projection.

interferon-gamma-mediated signaling pathway, among others, was observed in both types of GCA patients compared with healthy controls (Fig. 4c and e and Supplemental Tables 17 and 27).

With respect to healthy controls, patients in remission exhibited downregulation of genes involved in multiple relevant pathways, including T cell-mediated cytotoxicity and cytolysis in CTLs (Fig. 4e and Supplemental Table 23) and regulation of cytokine production, positive regulation of cell-cell adhesion and migration, Fc receptor signaling pathway, and positive regulation of monocyte differentiation, among others, in GZMK⁺ T cells (Fig. 4e and Supplemental Table 26).

3.5. CTLs show a marked clonal expansion

We used CellRanger to define clonotypes based on CDR3 sequences of both TCR alpha and beta chains. Of the 121,628 cells that were simultaneously interrogated for Immune profiling, we obtained paired TCR information for 76 % of them. After subsequent quality control, a total of 81,752 cells were included in this analysis (Supplemental Table 5).

Among the CD4⁺ populations, the CTL cluster showed the greatest clonal expansion and comprised almost all clones larger than 20 cells (Fig. 5a). Accordingly, the percentage of expanded clonotypes was markedly higher in CTLs (84.92 %) compared with the remaining clusters (<26 %) (Fig. 5b). We calculated the inverse Shannon's entropy index to evaluate the diversity of the TCR repertoire of each specific cluster in both subgroups of patients and in healthy controls. This index showed a lower clonal diversity of CTLs in active GCA patients compared with patients in remission (Wilcoxon rank-sum test $p = 0.036$) whereas a similar clonal diversity was evident between active patients and healthy controls (Fig. 5c). Nevertheless, the evaluation of overlap in CDR3 sequences among the analyzed individuals did not identify common clonotypes across GCA patients (Supplemental Fig. 5).

3.6. CD4⁺ T cells of GCA patients in remission shifted toward less differentiated states

To investigate the relationship among the CD4⁺ T cell subpopulations, we constructed developmental trajectories using Monocle v3 (Supplemental Fig. 6) and diffusion maps. Consistent with the T cell differentiation trajectory, a clear temporal separation between naïve and highly effector cells was evident (Fig. 5d). Cells at the beginning of the trajectory corresponded to T_N, which gradually progressed to T_{CM}, T_{EM}, GZMK⁺, and, finally, CTLs. A branching trajectory of Tregs along with some T_{EM} was also observed. In addition, most expanded clonotypes were preferentially located at the CTL branch, supporting the clonal expansion of this cell subtype (Fig. 5d).

To identify differences in the developmental trajectory of CD4⁺ T cells, we explored distributions of cells along pseudotime separately for the three subgroups of individuals, active patients, patients in remission and healthy controls (Fig. 5e). A lower percentage of CD4⁺ T cells showed later pseudotime values (>17) in patients in remission compared with active patients ($p = 0.0042$) and controls ($p = 0.0038$), thus showing a deviation towards less differentiated states (Fig. 5f).

3.7. Differential activity of relevant transcription factors in GCA patients

To identify TFs with a potentially relevant role in GCA, we inferred TF activity scores from single-cell RNAseq data. Several TFs involved in inflammation showed differential activity between subgroups (Supplemental Tables 28–30).

The comparison between active patients and healthy controls yielded only one TF, MYC, with differential activity and a moderate or large effect. Specifically, MYC, which is involved in controlling T cell proliferation [40], showed decreased activity in T_{CM} and GZMK⁺ cells from active GCA patients (Supplemental Table 28).

Comparison of patients in remission and controls (Supplemental

Table 29) showed lower activity of TFs related to the nuclear factor kappa B (NF- κ B) pathway in several CD4⁺ T cell subtypes, including NF- κ B1 in T_{CM} and Tregs, and NF- κ B, NF- κ B2, Rel, and RelA in Tregs. In addition, compared with healthy controls, Tregs of patients in remission exhibited decreased activity of SMAD Family Member 4 (SMAD4), a signaling molecule of the TGF- β pathway. Interestingly, all these TFs are crucial for the generation, maintenance and function of Tregs [40–42].

In contrast, when TF activity was compared between active patients and patients in remission (Supplemental Table 30), two TFs involved in the NF- κ B pathway, NF- κ B and RelA, showed higher activity in T_{EM} cells from active GCA patients. In this same cell subtype, we also observed higher activity of HLX, which has been shown to induce IFN- γ in CD4⁺ T cells [43]. In addition, CTLs from active patients exhibited increased activity scores of relevant TFs, including two members of the interferon regulatory factor (IRF) family, namely IRF5 and IRF6, and KLF13, which is crucial for regulating CCL5 expression in T cells [44].

3.8. Potentially repositionable drugs in GCA

We conducted a drug repurposing analysis to identify already approved therapies targeting DEGs in specific CD4⁺ T cell subtypes. Based on differential expression across the three comparisons (active vs. controls, active vs. in remission, and in remission vs. controls), this analysis prioritized 10 drugs as potentially useful for GCA treatment (Supplemental Fig. 7), some of them with a relevant effect in the context of this vasculitis.

Notably, maraviroc specifically targeted DEGs in CTLs in the comparison of active patients and patients in remission. This drug is an antagonist of CCR5, one of whose ligands is CCL5, a chemokine that showed increased expression in CTLs from active GCA patients, as mentioned above.

In addition, vorinostat, a histone deacetylase (HDAC) inhibitor, targeted naïve T cells. It has been reported that vorinostat has anti-inflammatory effects by decreasing expression levels of pro-inflammatory cytokines, such as IFN- γ and IL-17A, and TFs, including STAT1, STAT3, and RelA [45].

4. Discussion

The primary treatment for GCA is high-dose GCs; however, some patients may not sufficiently respond to this therapy or may experience relapses upon tapering [46]. Therefore, identifying novel pathogenic clues is crucial in order to develop new treatment approaches. Our results have highlighted molecular mechanisms that are altered in GCA patients, even after treatment with GCs. These mechanisms may be essential for identifying pathogenic pathways that are not adequately controlled by this therapy and, as a result, determining new therapeutic options.

Our single-cell atlas of CD4⁺ T cells showed an expansion of CD4⁺ CTLs in active patients in relation to those in remission. However, in contrast to what is described in other immune-mediated diseases, the frequency of CTLs was similar between active patients and healthy individuals, likely due to the effect of the GC treatment on this cell subtype. Indeed, in flow cytometry experiments, naïve-treatment active patients showed a higher proportion of CD4⁺ T cells expressing cytotoxic molecules compared with controls and patients in remission. In addition, differential expression analysis showed a higher expression of genes involved in cytotoxicity in CTLs from active patients with respect to healthy controls and patients with no sign of disease, thus demonstrating that both the proportion and function of CTLs are altered in the active phase of the disease. Although the cytotoxic activity of CD8⁺ T cells has been widely reported throughout the years, less is known about the mechanism of action of CD4⁺ T lymphocytes with similar capabilities. Nevertheless, due to the expansion of this subtype recently described in different immune-mediated diseases [15–18], the role of

CD4⁺ CTLs in autoimmunity is gaining more relevance. In this regard, it has been shown that this cell subpopulation is involved in the production of different proinflammatory cytokines, such as IFN- γ , IL-1 β and transforming growth factor beta (TGF- β) [18], all of which have been related to GCA. Interestingly, CD4⁺ CTLs were also found to play a role in the apoptosis of endothelial cells [47], a process involved in angiogenesis and vascular remodelling [48]. In this line, CTLs from active patients exhibited higher activity scores of TFs involved in the IFN pathway compared with patients in remission. Furthermore, DEGs in CTLs of active patients were enriched in pathways related to apoptosis (granzyme-mediated apoptotic signaling pathway) and inflammation (cellular response to IL-1, TNF, and IFN- γ), thus supporting a role of CTLs in both the vascular alterations and the inflammatory process occurring in GCA. In addition, regulation of the endothelial cell apoptotic process was enriched among the set of DEGs in different cell subpopulations of active patients, which points to a direct involvement of CD4⁺ T cells in vascular remodelling.

Moreover, CD4⁺ CTLs of active patients also expressed higher levels of chemokines compared with healthy controls and showed increased activity of KLF13, the TF that regulates the expression of CCL5, with respect to patients in remission. Indeed, as reflected in gene ontology analysis, CTLs seemed to be involved in the recruitment of different cell types, including monocytes and NK cells, as well as in activating neutrophils. Genes involved in the activation of neutrophils were also upregulated in the GZMK⁺ T cell subpopulation of patients in the active phase of the disease compared with those in remission. Although the mechanisms by which neutrophils affect this vasculitis are not fully understood, it has been recently reported that they could contribute by producing reactive oxygen species and neutrophil extracellular traps, which results in vascular damage [49,50]. Our findings underscore the involvement of CD4⁺ T cells in promoting the pathogenic role of neutrophils in GCA and emphasize the significance of this cell type in this disorder.

Taken together, these results suggest that CD4⁺ CTLs may play a significant role in the GCA pathogenesis, and targeting these cells may offer therapeutic efficacy. In this regard, a repurposing analysis of approved drugs prioritized maraviroc, which blocks the CCR5/CCL5 axis, as a potential treatment targeting CTLs. This finding is consistent with the recently reported role of this drug in decreasing systemic inflammation [51,52].

Previous studies have described a clonal expansion of CD4⁺ T cells in both arterial walls and peripheral blood of GCA patients [53,54]. Our results evidenced a marked clonal expansion of CTLs, which is consistent with their role in antiviral immunity [55,56] and with that reported in other autoimmune diseases [17,18]. This clonal expansion of CTLs was significantly higher in active patients, whereas patients in remission showed increased TCR repertoire diversity. This is in line with previous results in systemic lupus erythematosus and rheumatoid arthritis showing an increased number of expanded T clones in patients during active disease [57–59]. However, similar TCR diversity was observed between active patients and healthy individuals. This observation might indicate that GC treatment also affects clonal expansion and could be responsible for the lower clonal expansion observed in patients without disease symptoms. Nevertheless, analysing untreated samples would be necessary to validate this hypothesis. On the other hand, there was no clonal overlap across GCA patients, which does not support the existence of a disease-specific clonotype.

Comparison of the cell distribution between patients in remission and controls evidenced reduced percentages of other cell types with a more effector phenotype, T_{CM} and T_{EM}, and a trend toward a higher frequency of T_N. Accordingly, trajectory analysis showed that cells of patients in remission displayed a less differentiated phenotype, which could be reflecting the remission of the inflammatory process in patients with no sign of disease.

A reduction in the number of Tregs was also found in GCA patients, both during active disease and remission phases. This finding suggests

that GC treatment does not influence this specific cell subpopulation in GCA, as previously reported [7]. Notably, further subclustering of Tregs showed that the decrease specifically affected Treg clusters exhibiting an effector phenotype, including those with high expression levels of HLA class II molecules and chemokines involved in cell migration to inflammatory areas. Previous research has demonstrated that HLA class II expression identifies a distinct subtype of Tregs that displays a mature phenotype and a high regulatory activity [35]. In fact, in vitro antibody blockade of HLA-DR led to a loss of Treg regulatory capacity [39]. Additionally, recent evidence has shown that Tregs from GCA patients exhibit impaired suppressive capacity and are unable to control effector T cell proliferation [60]. Our results are consistent with these findings, as we observed that Tregs from GCA patients exhibited reduced expression of molecules involved in their regulatory activity. In addition, the activity scores of TFs involved in the regulatory function of Tregs, including SMAD4 and different subunits of the NF- κ B transcription factor complex [40–42], were also decreased in Tregs from patients in remission compared with controls. Thus, this dysregulation of the proportion and the transcriptome of Tregs may be impairing both the suppressive function and migratory ability of Tregs to affected arteries in GCA patients.

Our study has several limitations that should be considered. Firstly, the administration of GCs to patients with GCA may have influenced our results; however, it has been previously reported that certain dysregulated pathways in GCA may not respond completely to GC treatment [7]. In this regard, our approach provides an opportunity to investigate the molecular mechanisms that persistently undergo alteration even after treatment with GCs and that represent particularly relevant therapeutic targets to improve GCA treatment. Secondly, our study was focused on the analysis of circulating CD4⁺ T cells and, therefore, further studies into the role of CD4⁺ T cells in artery tissue from GCA patients will improve our understanding of this vasculitis.

5. Conclusions

In conclusion, through the first single-cell atlas of CD4⁺ T lymphocytes of GCA patients, we have redefined the role of this cell type in this vasculitis, highlighting the potential contribution of CD4⁺ CTLs to the inflammation and vascular remodelling that occurs in this vasculitis and opening new therapeutic possibilities. In addition, our findings support a quantitative defect of Tregs in GCA patients and show for the first time a dysregulation of genes involved in the regulatory functions of these cells, which could be involved in their lower suppressive capacity observed in GCA.

Contributors

E.G.C., B.T., and G.V.M.: performed experiments; E.G.C.: data analysis; E.G.C. and A.M.: interpretation of the results; E.G.C., J.M., and A.M.: manuscript drafting; J.L.C.R., N.O.C., E.R., R.R.F., M.C.C., J.H.R., E.B., and B.T.: sample and data collection; A.M.: study design. All authors read and approved the final version of the manuscript.

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Ethics approval

Written informed consent was obtained from all subjects with approval from the CSIC Ethic Committee and the Ethic Committee of Research of the Granada Province (CEIM/CEI).

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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