Journal of Autoimmunity 146 (2024) 103240

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Decoding CD4$^+$ T cell transcriptome in giant cell arteritis: Novel pathways and altered cross-talk with monocytes

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ARTICLE INFO

Keywords:
Giant cell arteritis
CD4$^+$ T cells
Transcriptomic
Cross-talk

ABSTRACT

Background: Giant cell arteritis (GCA) is an immune-mediated large-vessels vasculitis with complex etiology. Although the pathogenic mechanisms remain poorly understood, a central role for CD4$^+$ T cells has been demonstrated. In this context, understanding the transcriptome dysregulation in GCA CD4$^+$ T cells will yield new insights into its pathogenesis.

Methods: Transcriptome analysis was conducted on CD4$^+$ T cells from 70 patients with GCA with different disease activity and treatment status (active patients before treatment and patients in remission with and without glucocorticoid treatment), and 28 healthy controls. The study also evaluated potential impacts of DNA methylation on gene expression alterations and assessed cross-talk with CD14$^+$ monocytes.

Results: This study has uncovered a substantial number of genes and pathways potentially contributing to the pathogenicity of CD4$^+$ T cells in GCA. Specifically, CD4$^+$ T cells from GCA patients with active disease exhibited altered expression levels of genes involved in multiple immune-related processes, including various interleukins (IL) signaling pathways. Notably, IL-2, a decisive interleukin for regulatory T cells homeostasis, was among the most significant. Additionally, impaired apoptotic pathways appear crucial in GCA development. Our findings also suggest that histone-related epigenetic pathways may be implicated in promoting an inflammatory phenotype in GCA active patients. Finally, our study observed altered signaling communication, such as the Jagged-Notch signaling, between CD4$^+$ T cells and monocytes that could have pathogenic relevance in GCA.

Conclusions: Our study suggests the participation of novel cytokines and pathways and the occurrence of a disruption of monocyte-T cell crosstalk driving GCA pathogenesis.

1. Introduction

Giant cell arteritis (GCA) is an immune-mediated vasculitis primarily affecting the elderly, with a peak of incidence in people over 70 years [1]. It is characterized by granulomatous inflammation of medium and large-sized blood vessels, which lead to heterogeneous clinical manifestations, ranging from systemic symptoms, such as fever and weight loss, to more severe complications, such as blindness and aneurysm development [2]. The current management of this vasculitis remains suboptimal, with common adverse effects secondary to glucocorticoid (GC) therapy and a considerable rate of relapses [3].

While the pathogenesis of GCA remains incompletely understood, there is a consensus regarding the crucial role of aberrant CD4$^+$ T cell responses [4]. It is now accepted that chemotactic factors produced by

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https://doi.org/10.1016/j.jaut.2024.103240
Received 5 February 2024; Received in revised form 16 April 2024; Accepted 3 May 2024
Available online 15 May 2024

activated resident vascular dendritic cells promote the migration and activation of macrophages and $CD4^+$ T cells. These activated $CD4^+$ T cells release pro-inflammatory cytokines and infiltrate the arterial wall, where they interact with macrophages, causing tissue damage and arterial remodeling [5,6].

Understanding the transcriptomic reprogramming of dysfunctional cell populations is essential for elucidating disease mechanisms. In GCA, transcriptomic studies on $CD4^+$ T cells are scarce. The first study, consisting in a longitudinal gene expression analysis on $CD4^+$ and $CD8^+$ T cells, revealed novel genes potentially implicated in GCA pathogenesis. Nonetheless, the findings obtained were limited due to the relatively modest sample size analyzed [7]. A more recent study identified alterations in the transcriptomic profile of regulatory $CD4^+$ T cells (Treg) from GCA patients, including changes in transcription factors (TF), glycolytic enzymes and IL-2 signaling mediators [8]. Despite the progress achieved, the majority of the GCA patients included in these studies were undergoing treatment, which may obscure the interpretation of the alterations occurring only due to pathogenic processes.

Improvements in the comprehension of GCA pathogenesis will pave the way for new therapeutic opportunities. Therefore, we aimed to understand transcriptomic dysregulation of $CD4^+$ T cells of active GCA patients without the influence of medications. In addition, we evaluated the way these cells are affected by remission and by the effect of GC treatment. We also investigated whether both changes in the DNA methylation patterns and alterations in cross-talk with monocytes could be influencing the observed gene expression changes in $CD4^+$ T cells.

2. Material and methods

2.1. Study cohort

A total of 70 individuals diagnosed with GCA and 28 healthy controls, matched by sex and age, were recruited for this cross-sectional study. All individuals had European ancestry and were collected at Hospital Clinic of Barcelona. The ethics committee of the hospital approved the study, and all participants signed an informed consent form in accordance with the ethical guidelines of the 1975 declaration of Helsinki. GCA patients fulfilled the 1990 American College of Rheumatology classification criteria [9] and were consecutively selected among newly diagnosed cases and those monitored at the outpatient facility of Hospital Clinic of Barcelona. In addition, for all GCA patients, the diagnosis was confirmed by a positive temporal artery biopsy. Clinical and laboratory features at disease onset are listed in Additional file 1: Table S1. Samples from controls were collected from healthy individuals accompanying the patients to the clinic.

At the time of sample collection, all 70 GCA patients were further categorized into three groups based on their clinical disease activity status as follows: a) active ($n = 17$): newly diagnosed patients before starting GC treatment; b) in remission with treatment ($n = 26$): patients in disease remission while receiving low doses of prednisone ($\leq$10 mg/day) for a minimum of 1 month; c) in remission without treatment ($n = 27$): patients in remission without any treatment for at least 1 month. Remission was considered when individuals presented absence of GCA-related symptoms along with normal acute phase reactants.

2.2. Sample collection

First, peripheral blood mononuclear cells were acquired from whole blood by density gradient centrifugation using Ficoll-Paque (Rafer, Zaragoza, Spain). These cells were then incubated with CD4-APC conjugated antibodies (MiltenyiBiotec, Germany) in staining buffer (PBS with 2 mM of EDTA and 4 % FBS) for 20 min. Next, flow cytometry sorting was used to isolate $CD4^+$ T cells through positive selection. Finally, both genomic DNA and total RNA were extracted from the same cell pellet by using AllPrep DNA/RNA/miRNA Universal kit (Qiagen, Hilden, Germany).

2.3. RNA-seq and data processing

Total RNA quality was first determined by the 2100 Bioanalyzer System (Agilent, CA, USA), and 1 μg of those samples with excellent quality RNA (RNA integrity number >7) was used for library synthesis with the TruSeq Stranded mRNA Library Prep Kit (Illumina, CA, USA) according to the manufacturer's protocol. Subsequently, paired-end sequencing was performed on a HiSeq sequencer (Illumina, CA, USA) producing 34.6 × 2 M raw paired reads sample on average.

RNA-seq data were processed using miARma-Seq pipeline which includes FastQC software for quality evaluation of the raw data, and STAR for sequence alignment to the GRCh38 reference genome [10]. First, counts per million (CPM) per gene were calculated for each sample. For identifying outliers exhibiting significant deviations from the overall trend in our sample set, we used the R package bigutilsr following the code deposited in GitHub (https://github.com/privefl/bigutilsr). Principal component analysis (PCA) and hierarchical clustering of normalized samples were performed to check the similarity of RNA-sequencing samples (Additional file 2: Supplementary Figure 1-2). Samples identified as outliers were filtered out from further analyses. We also evaluated the contribution of potential confounding factors, such as sex and age, by performing PCA as well as principal coordinate analysis.

2.4. Gene expression and enrichment analyses

Differential expression analyses were conducted using the limma package [11]. First, we removed the low expressed genes (CPM< 1) and calculated reads per kilobase per million mapped reads (RPKM) for downstream analyses. Then, we performed a linear model using the limma voom function [12] including sex as a covariate. Finally, we carried out an eBayes test in order to identify genes that are differentially expressed between the subgroups in our study. We considered statistically differentially expressed genes (DEGs) those with a false discovery rate (FDR) < 0.05. Log2 fold changes (logFC) was used to assess expression changes among groups of comparison. We also calculated TMM (trimmed mean of M-values) values for graphical representations using ggplot2 R package [13].

Subsequently, we used the online tool Enrichr [14] to perform enrichment analysis of DEGs based on the following databases: BioPlanet 2019 and gene ontology (GO) for biological processes, cellular component, and molecular function. Significant enrichments were considered when $p < 0.05$ and a minimum count of 3 genes. Finally, we performed TF binding motif enrichment analysis of DEGs by applying the DoRothEA v2 tool [15]. We restricted our analyses to the most reliable interactions (A, B and C levels), and a $p < 0.05$ and normalized enrichment score (NES) of ± 2 were considered significantly enriched.

2.5. DNA methylation profiling

Bisulfite-converted DNA with the EZ DNA MethylationTM kit (Zymo Research, Irvine, CA, USA) was hybridized onto the Infinium MethylationEPIC Bead Chip array (Illumina, Inc., San Diego, CA, USA) following manufacturer's instructions. This platform allows to interrogate >850,000 methylation sites at single-nucleotide resolution, covering 99 % of reference sequence (RefSeq). Raw methylation data were processed with the ShinyÉPICo pipeline [16] and probes were annotated using IlluminaHumanMethylationEPICmanifest v0.4.0. Probes containing either a single nucleotide polymorphism (SNP) at the CpG interrogation site or at the single base extension site as well as probes within sex chromosomes were excluded. Probes with a detection p-value <0.01 were also removed. After Noob + Quantile normalization, beta values used for visual representation and M values used for statistical analyses were calculated. Data were analyzed using eBayes moderate *t*-test from the limma package [11]. CpG sites showing signs of changes in methylation levels ($p < 0.05$) were used for the integration

analysis.

2.6. Integration of gene expression with DNA methylation

First, we carried out a global correlation of gene expression levels of DEGs and DNA methylation levels of those CpG sites annotated to these genes by applying a Spearman's correlation test. We also used the MatrixEQTL R package [17] to examine specific gene expression-CpG interactions that could be relevant in the pathophysiology of GCA and/or in the clinical status, selecting DEGs and CpG sites with $p < 0.05$. A maximum distance of 1 Mb between CpG sites and genes was defined. Gene expression-CpG interactions with FDR <0.05 were considered significant.

2.7. Cell-cell communication

We used NicheNet, a computation tool designed to infer how the gene expression profile of a cell is influenced by interacting cells [18]. Briefly, this tool predicts ligand-target pairs between interacting cells by integrating their expression data with existing data sources of ligand-receptor, signaling, and gene regulation. Here, we applied this method to evaluate putative altered cell-cell communications between $CD4^+$ T cells and $CD14^+$ monocytes, two crucial cell types in GCA pathogenesis. For that, we used transcriptome data of $CD14^+$ monocytes from a previous work of our group [19]. Noteworthy, both $CD14^+$ and $CD4^+$ transcriptome datasets were obtained from the same patients at the same time point. We assessed the potential cross-talk in both directions. We first designated $CD14^+$ monocytes as the receiver cells, and then $CD4^+$ T cells. Only DEGs were employed to select interactions, highlighting significant interactions where both the ligand and target gene exhibited differential expression within each cell type. We applied Pearson correlation coefficient to measure ligand activities, which represent how well the ligand predicts the observed changes in gene expression in receiver cells. NicheNet analyses were performed following the code deposited in GitHub (https://github.com/saeyslab/nichenetr/blob/master/vignettes/seurat_steps.md). Ligand activity and ligand-target network were visualized as heatmap by using NicheNet R package. In addition, prioritized ligand-targets pairs were visualized as Circos plots by using circlize R package [20].

3. Results

To shed light into the molecular alterations of $CD4^+$ T cells in the context of GCA pathogenesis and the way these cells are affected by remission and by the effect of GCs, we conducted transcriptome profiling using RNAseq. After stringent quality controls, 81 samples were analyzed, including 23 healthy controls and 58 GCA patients grouped in three clinical status: patients with active disease before starting GC treatment [n = 13], patients in remission with GC treatment [n = 22], and patients in remission without GC treatment [n = 23]. In addition, we evaluated the potential correlation between the transcriptional reprogramming of $CD4^+$ T cells with changes in both the DNA methylation profiles and the cross-talk with $CD14^+$ monocytes.

3.1. Gene expression deregulation in GCA $CD4^+$ T cells

First, the comparison of gene expression patterns between all GCA patients and healthy controls did not reveal any significant DEGs (FDR <0.05), possibly due to the heterogeneity within GCA patients. Subsequently, we carried out additional comparisons by stratifying GCA patients according to their clinical status, as explained above. Consistent with findings from a previous study analyzing $CD14^+$ monocytes from GCA patients [19], the comparison between healthy controls and untreated patients in remission did not show any DEGs.

To evaluate the gene alterations underlying the pathogenic role of the $CD4^+$ T cells in the active state of GCA, and considering that healthy controls and patients in remission without treatment exhibited similar gene-expression profiles, we compared patients with an active disease state with both healthy controls and patients in remission without treatment collectively. We identified a total of 242 DEGs, of which 163 were over-expressed and 79 under-expressed in $CD4^+$ T cells from active patients (Fig. 1A and Additional file 1: Table S2). These genes were found to be enriched in a large number of gene ontology terms and molecular pathways (Fig. 1C and Additional file 1: Table S3). Notably, the IL-2 signaling pathway represented the most significantly enriched pathway (p = 4.55E-06). Of note, numerous pathways related with apoptotic processes were identified, including IL-5 regulation of apoptosis (p = 8.23E-03), IL-4 regulation of apoptosis (p = 1.60E-03), positive regulation of apoptotic process (p = 4.65E-03), and T cell receptor regulation of apoptosis (p = 1.55E-02). A total of 29 DEG, the majority of them under-expressed in the subgroup of patients with active disease, were included in these pathways (Table 1 and Fig. 1C). Consistent with GCA pathogenesis, we also detected crucial molecular processes such as integrin signaling pathway (p = 4.05E-02), regulation of angiogenesis (p = 1.20E-02), IL-1 signaling pathway (p = 1.81E-02), Notch signaling pathway (p = 1.59E-02), and signaling events mediated by VEGFR1 and VEGFR2 (p = 1.03E-02). Remarkably, enrichment analyses also pointed out additional molecular mechanisms that could be relevant in GCA pathogenesis, such as RXR/VDR pathway (p = 2.54E-04), and AP-1 transcription factor network (p = 1.59E-03). It is also interesting the enrichment observed in histone methyltransferase activity (p = 5.76E-04), including four under-expressed genes, which could suggest that this epigenetic mechanism might be playing an important role in the remodeling of the transcriptome landscape of this cell type in the active state of the disease. Furthermore, among the DEGs, we identified known autoimmunity-related genes such as: *CXCL13* (p = 1.02E-06; logFC = 2.63), *TNIP2* (p = 2.31E-06; logFC = 0.57), *VDR* (p = 6.20E-05; logFC = 0.88), *IRF3* (p = 7.09E-05; logFC = −0.56) and *MICA* (p = 4.95E-05; logFC = −0.49).

Subsequently, we observed substantial differences in the gene expression profiles between active patients and patients in remission receiving GC treatment, with a total of 632 DEGs identified (Fig. 1D and Additional file 1: Table S4). Remarkably, 119 of these DEGs were also found to be deregulated in the previous comparison, of which 86 were over-expressed in the subgroup of patients with active disease. These common DEGs encompass genes involved in apoptosis, angiogenesis, activation of the AP-1 transcription factor family, and epigenetic processes among others (Table 1 and Additional file 1: Table S4). Aside from the similarities, enrichment in signaling pathways of multiple ILs, including IL-1 (p = 2.06E-03), IL-2 (p = 6.59E-08), IL-4 (p = 2.42E-05), IL-5 (p = 5.25E-05), IL-6 (p = 1.73E-03), IL-9 (p = 8.49E-03), IL-11 (p = 6.50E-04), IL-12 (p = 8.81E-03), IL-22 (p = 7.09E-03), IL-23 (p = 2.86E-02), IL-27 (p = 8.49E-03), and IL-35 (p = 4.29E-03), were also detected. Additional biological processes potentially implicated in GCA immunopathogenicity, such as CD40/CD40L signaling pathway (p = 3.65E-04), T cell receptor signaling pathway (p = 4.72 E−04), regulation of receptor signaling pathway via JAK-STAT (p = 4.86E-04), regulation of I-kappaB kinase/NF-kappaB signaling (p = 3.58E-04), EGF/EGFR signaling pathway (p = 1.42E-02) were significantly enriched. Finally, we also observed enrichment in pathways involved in epigenetic processes, such as mechanisms of transcriptional repression by DNA methylation (p = 1.08E-02), regulation of histone H3–K4 methylation (p = 2.09E-02), MLL1/2 complex (p = 4.76E-02) which are members of the histone methyltransferase family, signaling events mediated by histone deacetylase (HDAC) class I (p = 6.52E-03) and class II (p = 3.12E-02), and nucleosome disassembly (p = 1.80E-02) (Fig. 1F and Additional file 1: Table S5).

Next, we inspected TFs potentially involved in the transcriptomic alterations observed in $CD4^+$ T cells from GCA patients with an active state of the disease (Fig. 1B and E). In line with the results obtained from transcriptional analyses, which revealed enrichments in epigenetic pathways and chromatin remodeling processes, a chromatin-level

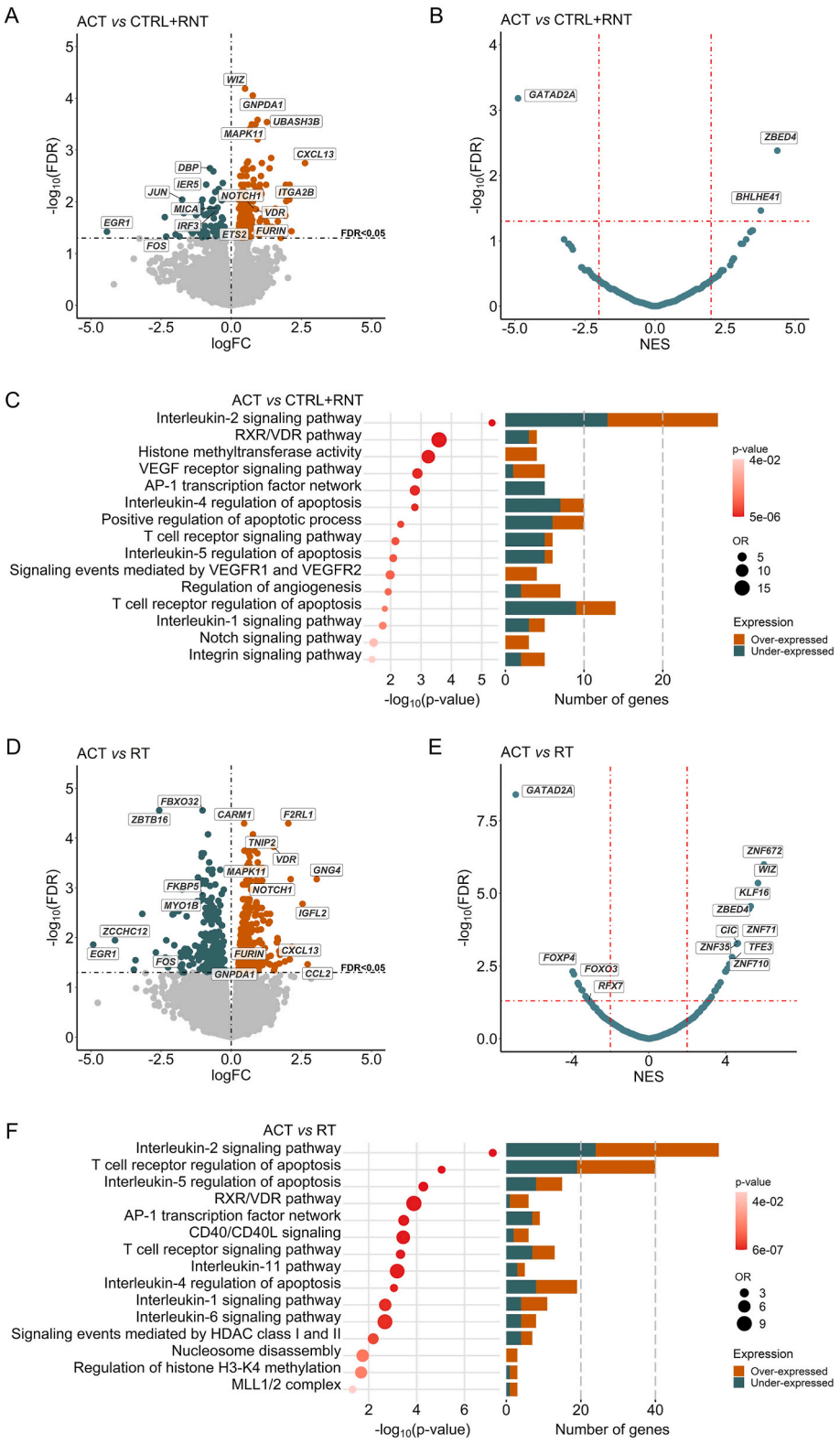

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Fig. 1. Results of gene expression analysis of CD4$^+$ T cells from GCA patients with active disease. (A–C) Results from the comparative between GCA active patients versus healthy controls and patients in remission without treatment. A) Volcano plot showing the results of gene expression analysis. False discovery rate (FDR) values are plotted on the -log$_{10}$ scale on the y-axis. Dashed lines mark the significant threshold (FDR<0.05). The effect size and direction obtained for each gene are plotted on the x-axis. The green and orange dots represent the under- and over-regulated differentially expressed genes (DEGs), respectively. (B) Volcano plot showing the normalized enrichment score (NES) from the transcription factor (TF) enrichment analysis. Dotted lines represent the cutoffs for statistical significance (FDR <0.05 and NES ± 2). (C) Scheme summarizing the most interesting results from the GO enrichment analysis. Red gradient represents the statistical significance and circle size indicates the Odds ratio (OR) of enrichment. Bar plot represent the number of under- and over-expressed genes, respectively, in each molecular pathway displayed. (D–F) Results from the comparative between GCA active patients versus patients in remission with treatment. (D) Volcano plot showing the results of gene expression analysis. (E) Volcano plot showing the NES from the TF enrichment analysis. (F) Scheme summarizing the most interesting results from the GO enrichment analysis.

Table 1
List of selected common differentially expressed genes.

Gene	ACT *vs* CTRL + RNT		ACT *vs* RT	
	logFC	FDR	logFC	FDR
Interleukin-2 signaling pathway				
BYSL	0.79	4.70E-03	1.03	4.34E-04
CITED2	−0.73	2.92E-02	−1.08	8.29E-04
DUSP1	−1.69	1.65E-02	−2.06	3.33E-03
HNRNPAB	0.33	6.45E-03	0.33	8.90E-03
MICA	−0.49	1.30E-02	−0.49	1.67E-02
MMD	0.47	2.23E-02	0.52	1.32E-02
NOLC1	0.60	1.67E-03	0.56	3.99E-03
PPRC1	0.47	6.45E-03	0.58	8.59E-04
SSBP2	−0.57	4.02E-02	−0.56	4.83E-02
TXNRD1	0.48	2.92E-02	0.50	2.31E-02
VWA5A	0.73	3.19E-02	0.71	1.09E-03
Activation of the AP-1 family of transcription factors				
JUN	−1.75	9.07E-03	−1.61	2.36E-02
MAPK11	0.86	3.19E-04	0.94	3.07E-04
FOS	−2.32	4.70E-02	−2.69	1.98E-02
Epigenetic processes				
DOT1L	0.40	1.08E-02	0.46	4.37E-03
SMARCC1	0.32	7.58E-03	0.27	4.01E-02
CARM1	0.32	3.19E-03	0.46	5.08E-05
Apoptosis				
EGR1	−4.43	3.77E-03	−4.92	1.38E-02
CREB1	−0.24	3.06E-02	−0.25	2.94E-02
IER2	−1.08	1.13E-03	−1.03	2.27E-02
PRRC2A	0.28	2.63E-02	0.29	2.73E-02
F2RL1	1.37	2.26E-03	2.03	5.08E-05
TNF	−2.37	1.98E-02	−2.24	2.65E-02
WIZ	0.49	6.51E-05	0.44	4.30E-04
Hypoxia and angiogenesis				
FLT1	1.24	3.74E-02	1.63	1.24E-02
CXCL13	2.63	1.79E-03	2.09	2.73E-02
VASH1	−0.44	4.33E-02	0.55	1.21E-02
RHOB	−1.98	4.26E-02	−2.32	1.13E-02
Notch signaling pathway				
NOTCH1	0.81	1.35E-02	1.11	7.14E-04
FURIN	1.01	2.40E-02	0.98	3.21E-02

ACT, active disease; CRTL, controls; RT, remission with treatment; RNT, remission without treatment; logFC = log-two fold change; FDR= False discovery rate.

regulator namely GATAD2A stands out. GATAD2A is a subunit of the nucleosome remodeling and histone deacetylase (NuRD) complex. It is also remarkable that from the total of 39 TF regulons enriched, 3 were associated with concomitant upregulation of their coding genes: WIZ, CIC and TFE3. In particular, TFE3 has been identified as a master regulator of the autophagy-lysosomal pathways [21]. Additionally, it has been described that TEF3 is critical for T cell function through their direct control of CD40L expression (Additional file 1: Table S8) [22].

3.2. GC treatment modulates the transcriptome of CD4$^+$ T cells from GCA patients

To investigate the transcriptional remodeling derived by the effect of GCs, we compared the gene expression profiles of GCA patients undergoing GC treatment with healthy controls and patients in remission without treatment. Predictably, the results of this comparison exhibited that both subgroups were notably dissimilar. Particularly, a total of 632 DEGs were identified, of which 309 genes were induced and 323 genes were repressed in GCA patients with GC treatment (Fig. 2A and Additional file 1: Table S6). Among the most significant DEGs, we observed the upregulation of genes mediating the molecular response to GCs such as *ZBTB16* (p = 4.64E-16; logFC = 2.68), *TXNIP* (p = 1.79E-12; logFC = 1.11) and *FKBP5* (p = 5.39E-12; logFC = 1.23), as well as the downregulation of genes involved in the immune response such as *IL27RA* (p = 1.63E-10; logFC = −053), *IRF1* (p = 1.88E-07; logFC = −066), *IL2RB* (p = 8.23E-07; logFC = −068) and *TNIP1* (p = 1.67E-06; logFC = −045). Consistently, pathways analyses revealed enrichment for genes related to the immune response, such as IL-6 signaling pathway (p = 7.52E-05), positive regulation of type I IFN production (p = 7.06E-04), IL2/STAT5 pathway (p = 1.41E0-2), NF-kappaB signaling (p = 1.55E-08), CD40/CD40L signaling (p = 4.13E-05), and TNFR2 signaling pathway (p = 7.15E-07), amongst others. In addition, it is also noted the enrichment in molecular processes such as apoptosis, including IL-4 and IL-5 regulation of apoptosis (p = 1.39E-06 and p = 5.25E-05, respectively), and epigenetic mechanisms such as, mechanisms of transcriptional repression by DNA methylation (p = 1.08E-02), regulation of histone H3–K4 methylation (p = 2.09E-02) and histone deacetylase binding (p = 2.79E02) (Fig. 2C and Additional file 1: Table S7).

Moreover, our results indicate that GC treatment might shift the altered expression profile of CD4$^+$ T cells from GCA patients with an active disease towards the one observed in patients in remission without treatment and controls, potentially reflecting the anti-inflammatory effect of the GC treatment. Specifically, it is noteworthy that of the 242 DEGs identified in the active state of the disease, 228 of them (94 %) present similar levels of expression in patients in remission treatment compared with patients in remission without treatment and controls. Of special relevance are the 14 genes that maintain altered expression levels after GC treatment (Fig. 2D), among which, we identified genes involved in the Wnt signaling pathway (*RARG* and *WNT10A*).

Subsequently, TF enrichment analyses revealed overrepresentation of TFs with marked relevance to the immune response, including BATF2 which is part of the AP-1 TF family [23], and NFKB2 which is a subunit of the NF-kB TF complex with a key role in the regulation of many inflammatory processes. Of note, *NFKB2* was downregulated in treated GCA patients in comparison to controls and patients in remission without treatment (Fig. 2B and Additional file 1: Table S8).

3.3. Modest effects of DNA methylation on gene expression alterations

We next interrogated whether DNA methylation dynamics might be contributing to the identified gene expression changes. To inspect this potential relationship, we obtained global DNA methylation data from CD4$^+$ T cells of the same individuals enrolled in this study. We observed an overall negative correlation between the expression levels of the DEGs and those CpGs annotated to these genes (r = −0.52, p = 4.4E-14) (Fig. 3A). In particular, from the total of 1168 DEGs previously identified in at least one of the comparisons performed, 8 % were significantly correlated (FDR <0.05) with at least one CpG. Specifically, 166 unique gene expression-CpG interactions were significant, composed of 97 genes and 157 CpGs (Additional file 1: Table S9). We also observed that 73,49 % of the interactions were negative correlations.

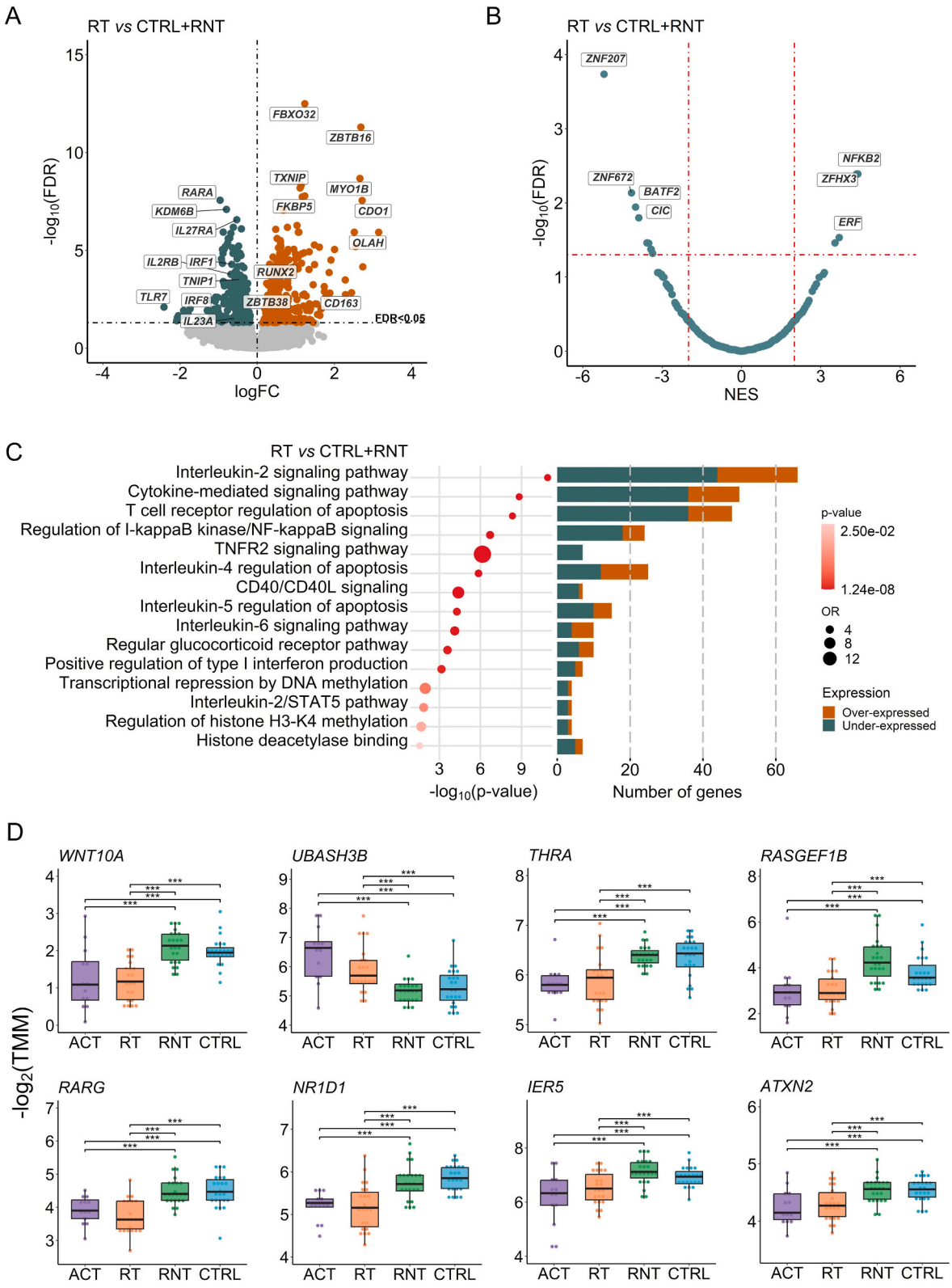

Fig. 2. Results of gene expression analysis of CD4^{+} T cells from GCA patients in remission with glucocorticoid (GC) treatment. A) Volcano plot showing the results of gene expression analysis. False discovery rate (FDR) values are plotted on the -log$_{10}$ scale on the y-axis. Dashed lines mark the significant threshold (FDR<0.05). The effect size and direction obtained for each gene are plotted on the x-axis. The green and orange dots represent the under- and over-regulated differentially expressed genes (DEGs), respectively. (B) Volcano plot showing the normalized enrichment score (NES) from the transcription factor (TF) enrichment analysis. Dotted lines represent the cutoffs for statistical significance (FDR <0.05 and NES ± 2). (C) Scheme summarizing the most interesting results from the GO enrichment analysis. Red gradient represents the statistical significance and circle size indicates the Odds ratio (OR) of enrichment. Bar plot represent the number of under- and over-expressed genes, respectively, in each molecular pathway displayed. (D) Box plots representing selected DEGs that maintain altered expression levels after GC treatment. *** marks significant differences (FDR<0.05). ACT, active disease; CRTL, controls; RT, remission with treatment; RNT, remission without treatment.

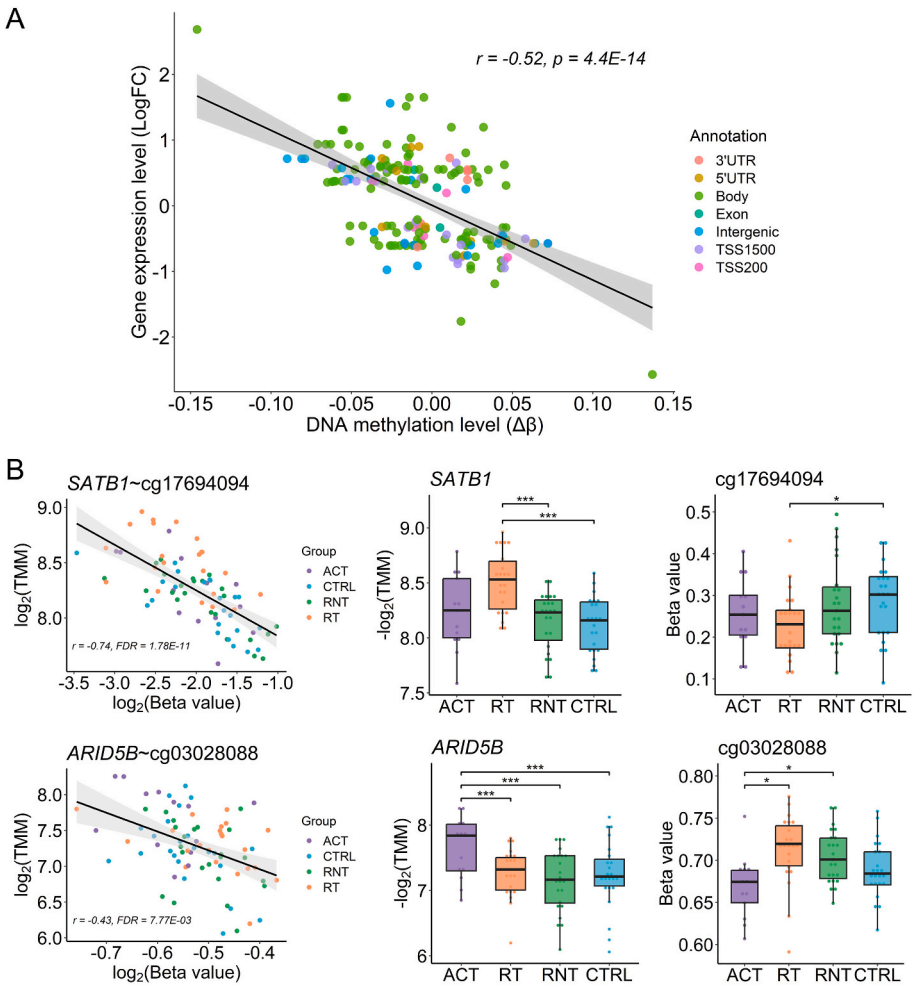

Fig. 3. Integrative analysis of gene expression and DNA methylation. (A) Overall correlation of gene expression levels with DNA methylation levels. LogFC of the gene expression is plotted on the y-axis, and DNA methylation levels are depicted on the x-axis. Points are coloured according to their genomic context. (B) Selected examples of specific CpG-gene expression interactions. Box plots representing CpG sites and differentially expressed genes (DEGs). Significant differences are marked (***FDR<0.05 and *p-value<0.05). Graphical representation of the correlation of DNA methylation and gene expression of CpG site-DEG pairs is also shown. DNA methylation and gene expression levels are illustrated in each subset of individuals. ACT, active disease; CRTL, controls; RT, remission with treatment; RNT, remission without treatment.

Among the strongest interactions, we observed that the upregulation of *SATB1* in patients in remission with treatment negatively correlated with nine different CpGs: cg17694094 (r = −0.74; FDR = 1.78E-11), cg16489771 (r = −0.74; FDR = 2.47E-11), cg00641002 (r = −0.73; FDR = 4.48E-11), cg26797786 (r = -0.72; FDR = 3.25E-10), cg22336648 (r = -0.71; FDR = 4.47E-10), cg10407807 (r = 0.68; FDR = 8.54E-09), cg25904398 (r = −0.67; FDR = 1.84E-08), cg17694094 (r = −0.66; FDR = 2.93E-08), cg19818826 (r = −0.65; FDR = 8.50E-08). This gene encodes a matrix-associated protein involved in chromatin organization and tissue-specific gene expression regulation [24]. In this line, we also detected a negative correlation between DNA methylation and gene expression levels of *ARID5B*, which encodes a member of the ARID family of DNA binding proteins part of a histone H3K9Me2 demethylase complex (cg03028088, r = −0.43; FDR = 7.77E-03). We also detected interactions between genes that might play important roles in the pathophysiology of GCA. One example was *VASH1,* which encodes a tyrosine carboxypeptidase involved in angiogenesis [25]. This gene, upregulated in GCA patients with active disease, was correlated with three different CpGs: cg20881054 (r = −0.41; FDR = 1.52E-02), cg17441584 (r = 0.39; FDR = 2.35E-02), cg23436960 (r = 0.42; FDR = 9.94E-03). Finally, and in line with previous observations [19], we detected negative correlations for the transcription factor *ZBTB16,* which contributes to energy homeostasis after GC receptors activation [26] (cg25345365, r = −0.51; FDR = 5.31E-04) (Fig. 3B and Additional file 1: Table S9).

3.4. Potential altered cell-cell communication between $CD4^+$ T cells and $CD14^+$ monocytes in GCA pathogenesis

Given the significant alterations revealed by the transcriptome analysis, including genes associated with cytokine and interleukin activity, we aimed to explore whether these changes might be modulating the communication with other immune cell types. To investigate intercellular cross-talk, we applied NicheNet, a computational method that predicts active ligands and their gene regulatory effects on interacting cells. In particular, we inspected cell-cell communication alterations between $CD4^+$ T cells and $CD14^+$ monocytes in the context of GCA. Gene expression data from $CD14^+$ monocytes were obtained from a previous study [19]. Remarkably, both transcriptome datasets, $CD4^+$ and $CD14^+$, were obtained from the same individuals at the same time point. Only DEGs were considered, highlighting significant interactions in which both the ligand and the target gene was differentially expressed in each cell type.

First, when considering $CD4^+$ T cells as the sender cell, our analysis inferred a total of 26 altered ligands. These were predicted to influence the transcriptome of $CD14^+$ monocytes by affecting a total of 463 genes

(Additional file 1: Table S10). Noteworthy, among the top ranked predicted ligands, we observed CD40L and HLA-A, both of them found to be deregulated in active patients. Furthermore, CCL2 and CXCL13, two chemokines upregulated in GCA patients with active disease, were also predicted to affect the gene expression levels of 151 genes in $CD14^+$ monocytes. These genes include relevant genes for the pathogenesis of GCA, such as inflammatory cytokines like IL-6, IL-10, genes encoding diverse integrins, such as MMP9 and VEGFA, among others (Fig. 4A and Additional file 2: Supplementary Figure 3-4).

Next, we also wanted to characterize the potential impact of $CD14^+$ monocytes in $CD4^+$ T cells (Fig. 4B). NicheNet analysis confirmed a notable increase in ligand-to-target signaling when monocytes act as sender cells, detecting a total of 3073 interactions. Specifically, a total of 66 altered ligands were predicted to regulate 197 genes (Additional file 1: Table S11). Remarkably, we identified the JAGG-NOTCH signaling pathway among the most interesting pairs of ligand-receptors with altered expression levels in our data. In addition, IL-18 ligand, a pro-inflammatory cytokine of the IL-1 family, also stood out. This analysis inferred that IL18 may alter 57 genes found to be deregulated in $CD4^+$ T cells in the context of GCA, including multiple chemokines and ILs, IRF and NFKB family members, CD40L, and relevant TFs such as FOS, JUN and ETS1. Finally, we also observed altered ligands known to be crucial in the pathophysiology of GCA, such as IL-6, MMP9 and multiple chemokines (Fig. 4B and Additional file 2: Supplementary Figure 5-6).

4. Discussion

In this study, we have investigated the transcriptome dysregulation in $CD4^+$ T cells in GCA patients with different disease activity and treatment status, including active patients before starting GC treatment and patients in remission with and without treatment. The findings of this work have revealed more than 1000 DEGs, providing new evidence of genes and pathways contributing to the pathogenic role of this cell type in GCA. We have also observed that only a small proportion of the gene expression alterations identified correlated with DNA methylation changes. Finally, we have also detected significant changes in ligand-receptor pairs which suggest alterations in the cross-talk between $CD14^+$ monocytes and $CD4^+$ T cells.

The transcriptomic profile of circulating $CD4^+$ T cells from GCA patients with active disease reflected the crucial role that this immune cell type plays in the pathophysiology of GCA. As expected, our results revealed an enrichment of genes related to multiple immune-related processes, such as Notch and CD40/CD40L signaling, and also signaling pathways of multiple interleukins, including IL-6 and IL-1, among others. In this context, one of the most significantly enriched pathways detected was the IL-2 signaling pathway, a key cytokine known to promote the differentiation, homeostasis and survival of Treg cells [27]. Treg cells are a subset of $CD4^+$ T cells crucial for maintaining immune tolerance and immune balance [28]. It has been well-established that dysfunctions in Treg are associated with various autoimmune diseases, including GCA [4]. Of note, disturbances of Treg homeostasis in systemic lupus erythematosus (SLE) have been linked to deficiency of IL-2 [29,30]. While our results did not show low expression levels of *IL-2*, it is possible that an impairment of the IL-2 signaling pathway might still be affecting Treg homeostasis and survival in GCA. Indeed, dysregulation of several genes downstream of IL-2 signaling in GCA Tregs has been recently described [8]. Our findings strengthen the potential role of IL-2 in GCA pathogenesis, highlighting the need for further studies. Understanding the impact of IL-2 signaling could provide valuable insights into disease mechanisms and potentially lead to new therapeutic approaches targeting this pathway. In this regard, early clinical trials performed in autoimmune diseases have shown promising results with the use of low-dose of IL-2 for Treg expansion and/or restoring cell fitness [31,32].

It is also worth mentioning the enrichment identified in the IL-11 signaling pathway. Although *IL11* was not differentially expressed, these observations are in line with our previous study on monocytes which pointed to the response to IL-11 as a new molecular mechanism potentially implicated in GCA [19]. Thus, further investigations are required to confirm and determine the role of this cytokine in GCA.

Interestingly, our findings indicate that $CD4^+$ T cells from GCA patients with an active disease showed altered expression levels of genes involved in signaling pathways of interleukins that have been also mapped to vasculitic lesions, including TNF, IL1- β, IL-2, IL-6, IL-9 and IL-22 [5,33]. Furthermore, several chemokines, such as CCL2 and CXCL3 among others, are of central relevance for cellular communication and recruitment in GCA [34]. In this sense, our findings revealed an overexpression of *CCL2* and *CXCL13* in circulating $CD4^+$ T cells from

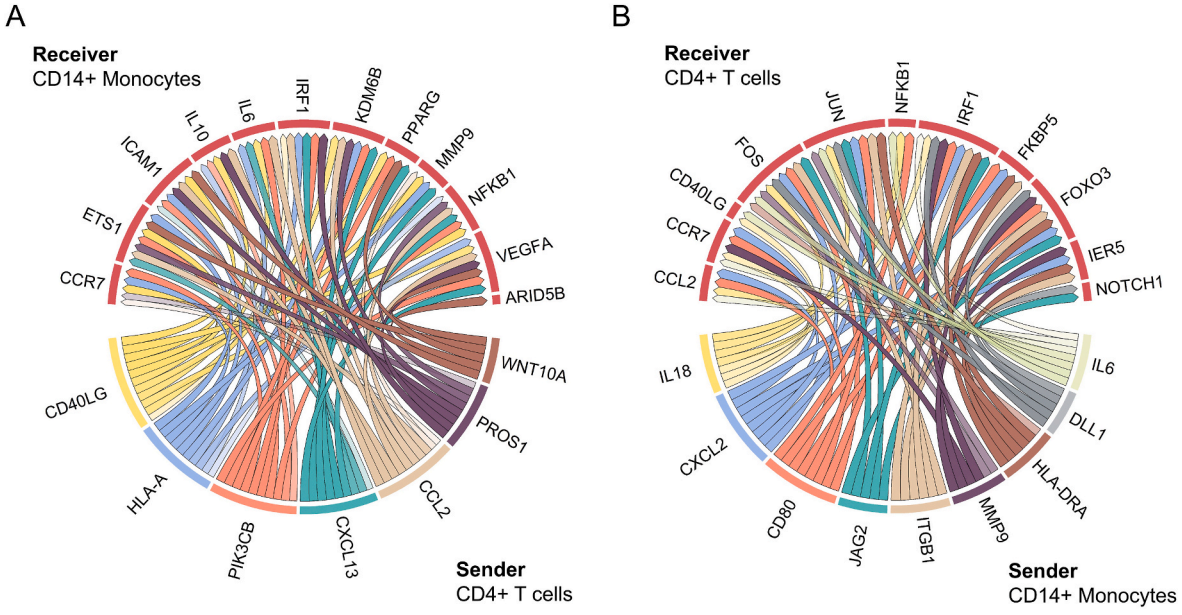

Fig. 4. Cell-cell communication between $CD4^+$ T cells and $CD14^+$ monocytes in GCA pathogenesis. (A) Circle plot showing links between predicted ligands from $CD4^+$ T cells (sender) with potentially targeted genes in $CD14^+$ monocytes (receiver). (B) Circle plot showing links between predicted ligands from $CD14^+$ monocytes (sender) with potentially targeted genes in $CD4^+$ T cells (receiver).

GCA active patients. Notably, previous studies have shown *CCL2* overexpression in monocytes from GCA active patients, as well as in temporal artery biopsies from GCA patients with relapsing disease [19,35]. These observations reinforce the implication of these cytokines and chemokines in driving GCA-related inflammation both at systemic and vascular levels, and highlight their potential use as biomarkers.

On the other hand, we can also observe disparities between the transcriptome profile of circulating $CD4^+$ T cells and the gene expression alterations described in positive temporal artery biopsies from GCA patients. For instance, dysregulation of senescence associated genes, such as *HES1*, *SIN3A* or *ADAMTS1*, has been observed in postmortem GCA arteries bearing an inflammatory phenotype [36]. However, our results do not show expression alterations in genes and molecular pathways related to cellular senescence. In addition, we also observed differences between the transcriptome profile of circulating $CD4^+$ T cells and lesion-residing $CD4^+$ T cells. It has been described that $CD4^+$ T cells in inflamed arteries strongly express program cell death protein 1 (PD-1), which upon interaction with its ligands can result in T cell apoptosis or exhaustion [37,38]. However, the increase of $CD4^+$ PD-1+ T cells has not been detected in peripheral blood [37]. In this regard, our results demonstrated no significant alterations in *PD-1* expression levels in $CD4^+$ T cells from GCA patients with active disease when compared with patients in remission or controls. Taken together, these observations suggest that the impairment of this immunomodulatory checkpoint is specific to vasculitic tissue and is not manifested in peripheral blood of GCA patients. In this sense, it should be mentioned that our results revealed enrichment in other apoptotic processes such as IL-4 and IL-5 regulation of apoptosis. Although there are scarce studies investigating apoptosis in GCA, our findings suggest that the impairment of apoptotic pathways could be crucial for the development of this condition.

In line with our previous study on $CD14^+$ monocytes, we noted that patients undergoing GC therapy exhibited a substantial remodeling of the transcriptome of $CD4^+$ T cells, which might reflect the anti-inflammatory effect of GC. Particularly, among the most overexpressed genes in GCA patients in remission with treatment when compared with controls and patients in remission without treatment, we detected a common upregulation of genes encoding GC receptors, such as *ZBTB16* and *FKBP5* [19]. Consequently, the upregulation of these two GC receptors involved in controlling inflammation [26,39], was also observed in an independent longitudinal expression profiling of $CD4^+$ and $CD8^+$ T cells in GCA patients, the majority of whom were receiving steroid treatment (Additional file 1: Table S12) [7]. Furthermore, our findings suggest that GC treatment might be restoring most altered gene expression levels observed in patients with active disease. Remarkably, 14 genes maintained altered expression levels even being on GC treatment, appearing to be less amenable to treatment. Among these, *RARG* and *WNT10A* are involved in the Wnt canonical signaling pathway. Notably, the downregulation of WNT10A was also identified in the previous longitudinal study in most of the time points analyzed (Additional file 1: Table S12) [7]. Wnt signaling is considered a key regulator of a variety of biological processes, including a regulatory role in the homeostasis of the immune system [40,41]. Interestingly, it has been recently described that upregulation of β-catenin, an essential component of the Wnt signaling pathway, induces pro-inflammatory properties in human Treg cells [42]. The potential role of the Wnt signaling pathway in GCA warrants further investigation. Indeed, there is evidence supporting the hypothesis of a potential involvement of the dysregulation of Wnt signaling in the pathogenesis of many autoimmune diseases, including rheumatoid arthritis (RA) and inflammatory bowel disease [42] [–] [44].

Despite that DNA methylation has been established as an important potential regulator of gene expression, our results indicate a moderate contribution to the gene expression alterations identified in $CD4^+$ T cells from GCA patients. Specifically, we have detected only 166 significant gene expression-CpG interactions. Among these, we identified a novel link between upregulated *ARID5B* expression levels and GCA patients with active disease, which was inversely correlated with DNA methylation levels of a CpG site within the gene body. Furthermore, the upregulation of *ARID5B* was also identified in the previous longitudinal study in the first time point corresponding with the acute phase of the disease (Additional file 1: Table S12) [7]. *ARID5B* encodes a component of a histone-demethylase complex that removes the repressive H3K9Me2 mark and activates its target genes [45]. This transcription coactivator has shown pleiotropic effects in chondrogenesis, lipid metabolism, and autoimmune diseases [46,47]. Indeed, it has been previously associated with SLE [48] and RA [49]. Of note, a recent study reported that *ARID5B* knockdown on lipopolysaccharide-stimulated THP1 monocytes reduced the expression of pro-inflammatory cytokines, activator and effector cytokines from the type I interferon signaling pathway, and antigen processing and presentation genes, such as *HLA-DRA* and *HLA-DRB* [50]. Taken together, these observations suggest that increased *ARID5B* expression in $CD4^+$ T cells, and thus histone methylation alterations, might be dysregulating immune processes towards an inflammatory phenotype in GCA patients with an active state of the disease. Likewise, we observed that DEGs identified in this study exhibited enrichment in multiple histone-related epigenetic pathways, further supporting the importance of investigations focused on histone modifications in the context of this vasculitis.

In GCA, the interplay between $CD4^+$ T cells and resident cells of the arterial wall, such as endothelial cells or macrophages, has been well-established [51,52]. However, less is known about the potential cross-talk between circulating immune cells. In this study, we employed novel computational methods developed to inspect intercellular communication based on gene expression profiles in order to evaluate the cross-talk between circulating $CD4^+$ T cells and $CD14^+$ monocytes in the context of GCA. Providing strength to our results, the transcriptome datasets used for this analyses were obtained from the same patients at the same time point. The results of this analysis were broadly consistent with previous knowledge, highlighting the robustness of the approach. Notably, key players in GCA pathogenesis, such as *IL-6* and *MMP9* [5,6,53], stood out as altered ligands in $CD14^+$ monocytes from GCA patients. These ligands were predicted to influence the gene expression of numerous genes in $CD4^+$ T cells. We also identified relevant chemokines, such as *CCL2* and *CXCL3* as altered ligands in $CD4^+$ T cells. Among the most interesting results, we observed the implication of the NOTCH signaling pathway in the cross-talk between these two immune cell types. We found that increased expression of Notch ligands, including *DLL1*, *JAG1* and *JAG2*, in $CD14^+$ monocytes are related to increased *NOTCH1* and *NOTCH2* levels in $CD4^+$ T cells in GCA patients with active disease. Accordingly, a previous study reported the ability of endothelial cells expressing Jagged-1 to lead $CD4^+$ T cells activation, infiltration and differentiation through Notch interactions [52]. These observations suggest that the altered Jagged-Notch signaling communication between $CD4^+$ T cells and $CD14^+$ monocytes could also have pathogenic relevance, and support the notion that targeting the Jagged-Notch axis could lead to new therapeutic opportunities for GCA. Furthermore, our findings also emphasize that the cross-talk communication in GCA pathogenesis is not only critical within the arterial wall tissue but also occurs at the circulating level.

The strengths of our work include the analysis of a representative study collection that comprised GCA patients with different activity states, thus better reflecting the clinical landscape. Of special relevance, the inclusion of treatment-naive patients provided a unique insight into the baseline molecular events driving the disease occurring prior to any therapeutic intervention. Nevertheless, it should be acknowledged that despite our relevant findings, $CD4^+$ T cells consist of several subpopulations differing in functionality. This cellular heterogeneity could potentially hinder the detection of subpopulation specific DEGs.

5. Conclusions

In summary, this work advances our understanding of GCA

pathogenesis in relation to dysregulation of $CD4^+$ T cells, a major cell type in this condition. In addition to contributing to a better comprehension of the pathogenic mechanisms involved in GCA, our study has yielded a significant number of molecules that could be investigated in further functional studies for evaluation as biomarkers or novel therapeutic approaches.

Funding

This work was supported by the HELICAL Innovative Training Network, a European Commission funded project under the Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 813545, Redes de Investigación Cooperativa Orientadas a Resultados en Salud (RICORS) (RD21/0002/0039). L.O.F. was supported by Juan de la Cierva Incorporación fellowship (IJC2019-040746-I) funded by MCIN/AEI /10.13039/501100011033

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona and all participants signed an informed consent form in accordance with the ethical guidelines of the declaration of Helsinki.

CRediT authorship contribution statement

Elkyn Estupiñán-Moreno: Data curation, Formal analysis, Writing – original draft. **José Hernández-Rodríguez:** Conceptualization, Data curation, Resources, Writing – review & editing. **Tianlu Li:** Resources, Writing – review & editing. **Laura Ciudad:** Resources, Writing – review & editing. **Eduardo Andrés-León:** Formal analysis, Methodology, Writing – review & editing. **Laura Carmen Terron-Camero:** Formal analysis, Methodology, Writing – review & editing. **Sergio Prieto-González:** Resources, Writing – review & editing. **Georgina Espígol-Frigolé:** Resources, Writing – review & editing. **Maria C. Cid:** Conceptualization, Data curation, Writing – review & editing. **Ana Márquez:** Data curation, Writing – review & editing. **Javier Martin:** Conceptualization, Data curation, Writing – review & editing. **Esteban Ballestar:** Conceptualization, Data curation, Writing – review & editing. **Lourdes Ortiz-Fernández:** Conceptualization, Data curation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

Data availability

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository, through a SuperSerie with the following accession number: GSE252024.

Acknowledgements

We thank all the patients and control donors who participated in this study and Sofia Vargas and Gema Robledo for their excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2024.103240.

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