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Original article

Phenotypic, transcriptomic and functional profiling reveal reduced activation thresholds of CD8$^+$ T cells in giant cell arteritis

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Abstract

Objectives. Evidence from temporal artery tissue and blood suggests involvement of CD8$^+$ T cells in the pathogenesis of GCA, but their exact role is poorly understood. Therefore, we performed a comprehensive analysis of circulating and lesional CD8$^+$ T cells in GCA patients.

Methods. Circulating CD8$^+$ T cells were analysed for differentiation status (CD45RO, CCR7), markers of activation (CD69 and CD25) and proliferation (Ki-67) in 14 newly diagnosed GCA patients and 18 healthy controls by flow cytometry. Proliferative capacity of CD8$^+$ T cells upon anti-CD3 and anti-CD3/28 *in vitro* stimulation was assessed. Single-cell RNA sequencing of peripheral blood mononuclear cells of patients and controls ($n = 3$ each) was performed for mechanistic insight. Immunohistochemistry was used to detect CD3, CD8, Ki-67, TNF-α and IFN-γ in GCA-affected tissues.

Results. GCA patients had decreased numbers of circulating effector memory CD8$^+$ T cells but the percentage of Ki-67-expressing effector memory CD8$^+$ T cells was increased. Circulating CD8$^+$ T cells from GCA patients demonstrated reduced T cell receptor activation thresholds and displayed a gene expression profile that is concurrent with increased proliferation. CD8$^+$ T cells were detected in GCA temporal arteries and aorta. These vascular CD8$^+$ T cells expressed IFN-γ but not Ki-67.

Conclusion. In GCA, circulating effector memory CD8$^+$ T cells demonstrate a proliferation-prone phenotype. The presence of CD8$^+$ T cells in inflamed arteries seems to reflect recruitment of circulating cells rather than local expansion. CD8$^+$ T cells in inflamed tissues produce IFN-γ, which is an important mediator of local inflammatory responses in GCA.

Key words: GCA, vasculitis, T cells, single-cell RNA sequencing, CD8$^+$ T cells

BASIC SCIENCE

Rheumatology key messages

- Circulating CD8$^+$ T cells of GCA patients show a proliferation-prone phenotype.
- Circulating CD8$^+$ T cells have reduced T cell receptor thresholds in GCA.
- Majority of CD8$^+$ T cells in GCA tissues produce pro-inflammatory cytokines.

Introduction

GCA is a form of vasculitis affecting the large- and medium-sized vessels. Patients experience symptoms such as headaches, visual disturbances, fever and weight loss. GCA is ageing-related as it only occurs in patients above 50 years of age [1, 2].

Several viruses have been suggested as potential triggers of GCA, but none of these could be verified [3, 4]. This might indicate that multiple viruses can

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trigger shared inflammatory pathways in GCA. In addition, HLA-class I genes have been linked to development of GCA [5].

$CD8^+$ T cells are multifunctional T cells that can kill infected, damaged and tumour cells. $CD8^+$ T cells can eliminate cells via the targeted release of perforin and granzymes and produce cytokines such as IFN-γ and TNF-α. Autoreactive $CD8^+$ T cells can produce pro-inflammatory cytokines in response to self-antigens [6]. In other vasculitic diseases such as Kawasaki disease and Takayasu arteritis, the influence of $CD8^+$ T cells in the disease pathogenesis has been studied more intensively than in GCA. Here, $CD8^+$ T cells are present in inflamed tissues of both diseases and have been suggested to participate in vascular injury [7–12].

In GCA, several studies indicate that $CD8^+$ T cells contribute to the immunopathogenesis of GCA especially in later stages of the vasculopathy (reviewed in [13]). $CD8^+$ T cells are present in temporal artery biopsies (TABs) of GCA patients, and the number of tissue-residing $CD8^+$ T cells correlates with disease activity [14]. However, compared with extensively studied $CD4^+$ T cells and macrophages, the role of $CD8^+$ T cells in the development of GCA is still poorly understood.

To gain more insight into the role of $CD8^+$ T cells in GCA pathogenesis, we used single-cell RNA sequencing, flow cytometry and *in vitro* T cell activation assays to comprehensively determine the phenotype and function of circulating $CD8^+$ T cells. In addition, we assessed the phenotype of $CD8^+$ T cells in GCA-affected tissues.

Methods

Study population

To assess the phenotype of $CD8^+$ T cells in peripheral blood, 14 newly diagnosed GCA patients before treatment and 18 age- and sex-matched healthy controls (HC) were included. Patients were diagnosed based on clinical signs and symptoms, together with a positive TAB and/or positive proof of GCA on imaging ([^{18}F]fluorodeoxyglucose-PET scan and/or an ultrasound). *In vitro* experiments were performed in the majority of these patients and controls (Supplementary Table S1, available at *Rheumatology* online). CMV serostatus was determined in 10 GCA baseline patients and 18 HCs (Supplementary Table S1, available at *Rheumatology* online). Absolute counts were assessed in 14 HCs and 14 GCA patients in EDTA blood according to the MultiTest TruCount method (BD Biosciences, San Jose, CA, USA). In addition, three newly diagnosed GCA patients before treatment and three age-matched healthy controls were selected for single-cell mRNA sequencing (scRNAseq) of peripheral blood mononuclear cells (PBMCs) (Supplementary Table S1, available at *Rheumatology* online). HCs received no immunosuppressive medication and had no morbidities as assessed by a clinician through physical examination. All patients and controls gave their written informed consent before participating in this study. The study was conducted in accordance with the Declaration of Helsinki and approved by the institutional review board of the University Medical Center Groningen (METc2010/222).

Biopsy studies were performed on TABs and aorta tissues retrospectively selected from GCA patients. TABs were initially taken for diagnostic purposes and aorta tissues from patients who underwent aortic aneurysm surgery. Three out of 11 GCA patients with a positive TAB and three out of 10 GCA patients with positive aorta tissue received prednisolone.

$CD8^+$ T cell phenotyping by flow cytometry

To determine the phenotype of circulating $CD8^+$ T cells, thawed cryopreserved PBMCs were first stained with Zombie Nir Fixable viability kit (Biolegend, San Diego, CA, USA) to exclude dead cells. To determine frequencies of $CD8^+$ T cell subsets and frequencies of activated $CD8^+$ T cells, cells were stained for surface markers with fluorochrome conjugated monoclonal antibodies detecting CD3, CD8, CD4, CCR7, CD45RO, CD25 and CD69 (Supplementary Table S2, available at *Rheumatology* online). We subsequently used the eBioscience Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific, Waltham, MA, USA) prior to staining for detection of intracellular Ki-67 and forkhead box P3 (FOXP3) expression (Supplementary Table S2, available at *Rheumatology* online). Samples were measured on a BD LSR-II flow cytometer. Appropriate isotype controls were used to set the gates, which were confirmed by fluorescence minus one controls. Flow cytometry data were analysed with Kaluza software v2.1 (Beckman Coulter, Indianapolis, IN, USA) (Supplementary Fig. S1, available at *Rheumatology* online).

In vitro proliferation assay

To assess the capacity of $CD8^+$ T cells to proliferate upon various stimuli, first $CD3^+$ T cells were isolated from thawed PBMCs by immunomagnetic negative enrichment according to the manufacturer's instructions (EasySep Human T cell isolation kit, Stemcell Technologies, Waterbeach, UK). Enriched T cell populations were labelled with cell proliferation dye by incubation at 37°C with a Cell Proliferation Dye eFluor 670 (1 μmol/ml, eBioscience) in PBS. To stop the labelling, cells were washed twice with RPMI + 10% fetal bovine serum culture medium. A 96-well flow-bottom plate was coated with purified anti-human CD3 (OKT3, 2.5 μg/ml, Biolegend) at 37°C for 2 h and at 4°C overnight. Wells were washed twice with PBS and once with culture medium before adding 2×10^5 labelled T cells to the wells. When indicated, soluble CD28 was added to the wells (FastImmune CD28/CD49d, clone L293, 125 ng/ml, BD Biosciences). Cells were cultured for 5 days at 37°C. Next, cells were stained with anti-CD8 (APC-H7, SK1, BD Biosciences) and Zombie UV Fixable viability kit (Biolegend). All samples were analysed on the BD LSR-

II flow cytometer. FCS express 6 (De Novo Software, CA, USA) was used to analyse the percentage of the original cells that divided at least once during the 5-day stimulation period, by measuring the dilution of the cell proliferation dye (Supplementary Fig. S1, available at *Rheumatology* online) [15].

Single-cell RNA sequencing

scRNAseq was performed on cryopreserved PBMCs by Single Cell Discoveries (https://www.scdiscoveries.com/, Utrecht, the Netherlands) according to standard 10× Genomics (Pleasanton, CA, USA) 3′ V3.1 chemistry protocols. Prior to loading the cells on the 10× Chromium controller, cells were counted to assess cell integrity and concentration. Cells were loaded and the resulting sequencing libraries were prepared following standard 10x Genomics protocols. The DNA libraries were paired end sequenced on an Illumina Novaseq S4, with a 2 × 150 bp Illumina kit. For scRNAseq data analysis methods, see Supplementary Data S1, available at *Rheumatology* online.

Immunohistochemistry

To confirm that CD8$^+$ T cells are present at the site of inflammation in GCA, immunohistochemistry (IHC) on formalin-fixed and paraffin-embedded tissues was used. We assessed the number of CD3 and CD8 positive cells per mm^2 in aorta tissues ($n = 8$ for CD3, $n = 9$ for CD8) and TABs ($n = 11$) from histologically proven GCA patients. To assess whether CD8$^+$ T cells were proliferating in GCA-affected tissues, six TABs were stained for Ki-67. Furthermore, to determine the expression of CD8$^+$ T cell-related cytokines, TAB tissues ($n = 11$) were stained for TNF-α and IFN-γ. Double-labelling of Ki-67 and CD8 was performed in three TABs of GCA patients to assess colocalization. For IHC staining methods, see Supplementary Data S2 and Supplementary Table S3, available at *Rheumatology* online.

Immunofluorescence

Double-labelling of CD8 and IFN-γ was performed on one representative TAB tissue. After deparaffinization and antigen retrieval, the tissue was incubated with primary antibodies followed by secondary and tertiary antibodies (Supplementary Table S4, available at *Rheumatology* online). Nuclei were stained with 4′,6-diamidino-2-phenylindole and images were taken with a Leica (Wetzlar, Germany) DFC345 FX. Image cubes were captured at ×40 magnification with Nuance Multispectral Imaging system (version 3.0.1, PerkinElmer, Waltham, MA, USA).

Statistical analysis

All graphs were generated using GraphPad Prism version 8 (GraphPad Software Inc., La Jolla, CA, USA). The same software was used for data analysis of flow cytometry and tissue staining experiments. Statistical significance between two groups was tested with a Mann–Whitney *U*-test. Spearman rank correlations were calculated when indicated in the text. *P*-values of <0.05 were considered statistically significant.

Results

CD8$^+$ T cells are readily detected in GCA-affected arteries

To confirm that CD8$^+$ T cells are present at the site of inflammation in GCA, we first stained TABs and aorta tissue for CD3 and CD8 expression (Fig. 1A–E). CD8$^+$ T cells were clearly present throughout the different layers of the vessel wall in TAB tissues. As expected, CD8$^+$ T cells were detected in CD3 rich areas. In aorta tissue, CD8$^+$ T cells were mainly located in the adventitia. Ratios of CD8/CD3 indicated that CD8$^+$ T cells are outnumbered by CD3$^+$CD8$^-$ T cells in all layers of the vessel wall in both TAB and aorta (Fig. 1E). Thus, our data confirm the presence of CD8$^+$ T cells in GCA-affected tissues and show a similar tissue dispersion to CD3$^+$CD8$^-$ T cells.

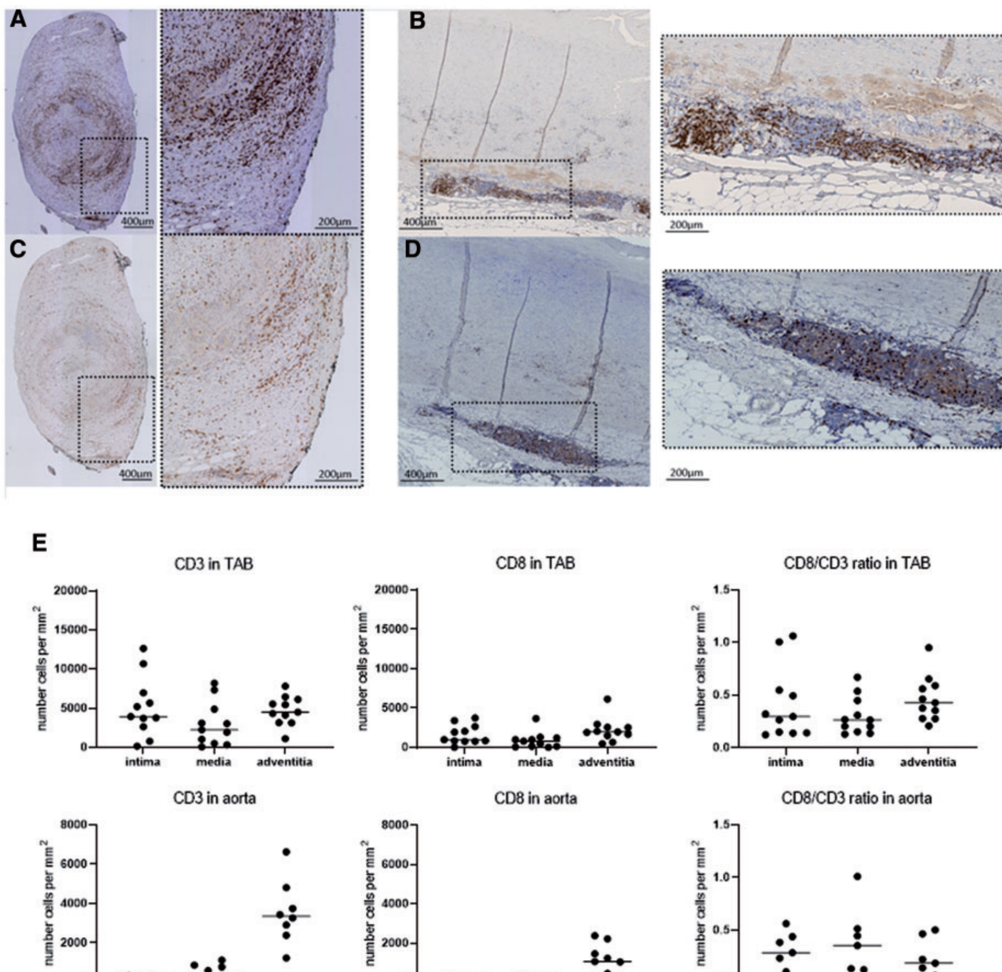
Increased expression of the Ki-67 proliferation marker in circulating CD8$^+$ T cells of GCA patients

As CD8$^+$ T cells were present in GCA-affected vessels, we next aimed to assess the possible role of CD8$^+$ T cells in GCA pathogenesis. To this end, we first investigated the differentiation, activation and proliferation status of circulating CD8$^+$ T cells. Here, we found a decrease in the frequencies of CD8$^+$ effector memory (T_{EM}) cells in GCA baseline patients when compared with healthy controls ($P = 0.025$, Fig. 2A). Absolute numbers of CD8 T_{EM} cells were lower in GCA baseline patients as well, despite equal counts of total CD8$^+$ T cells between the groups (Supplementary Table S5, available at *Rheumatology* online; $n = 14$ HCs, $n = 14$ GCA). No differences were found in frequencies and absolute numbers of naïve, central memory (T_{CM}) and effector memory T cells re-expressing CD45RA (T_{EMRA}) CD8$^+$ cells. However, a large individual variability was noted in all groups. Interestingly, the frequencies (data not shown) and absolute numbers of CD4$^+$ T cell differentiation subsets (Supplementary Table S5, $n = 14$ HCs, $n = 14$ GCA) did not differ between patients and controls.

Next, we assessed the activation status of T cells in GCA by analysis of CD69 and CD25. No differences were observed in frequencies of CD69$^+$ and CD25$^+$ T cells, both CD4$^+$ and CD8$^+$, between patients and controls (Supplementary Fig. S2A and B, available at *Rheumatology* online).

Lastly, we assessed the proliferative profile of CD8$^+$ T cells by analysis of Ki-67. A trend for higher frequencies of Ki-67$^+$CD8$^+$ T cells was noted in GCA when compared with healthy controls ($P = 0.066$) whereas frequencies of Ki-67$^+$CD4$^+$ were not different between patients and controls ($P = 0.296$, Supplementary Fig. S2C, available at *Rheumatology* online). As we found a trend for higher frequencies of Ki-67$^+$CD8$^+$ T cells in patients, we

Fig. 1 Detection of $CD3^+$ and $CD8^+$ cells in temporal artery biopsies (TAB) and aorta tissues of GCA patients



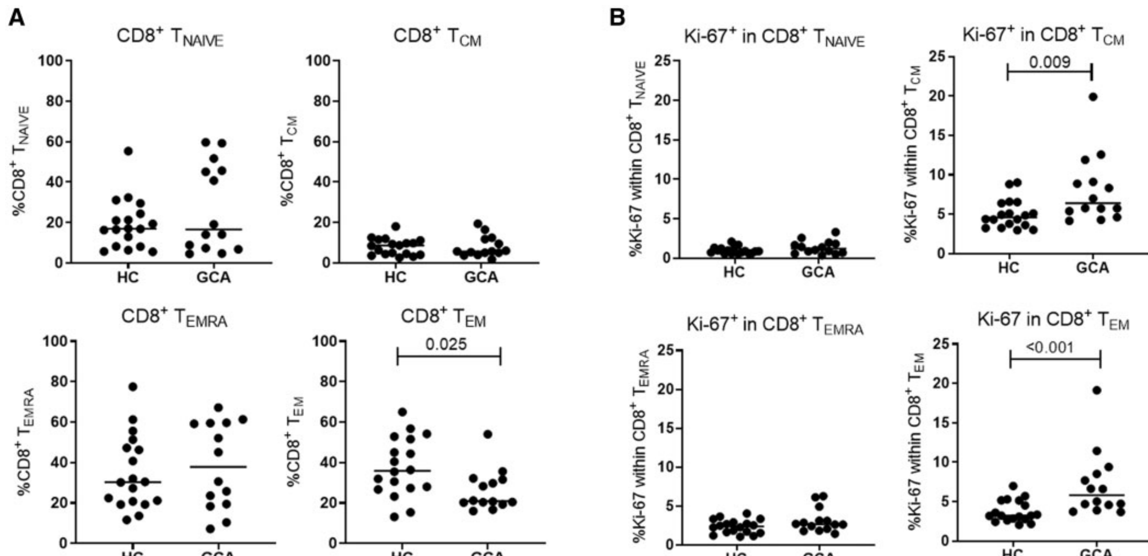
(**A**) CD3 expression in TAB, with delineated region shown enlarged on right. (**B**) CD3 expression in aorta, with delineated region shown enlarged on right. (**C**) CD8 expression in TAB, with delineated region shown enlarged on right. (**D**) CD8 expression in aorta, with delineated region shown enlarged on right. (**E**) Quantification of the number of CD3 and CD8 positive cells per mm^2 and the CD8/CD3 ratio as determined per cell layer by QuPath software. Expression of CD3 and CD8 was determined by immunohistochemistry in 11 TABs and eight or nine aorta tissues of biopsy-proven GCA patients.

next determined Ki-67 expression in the $CD8^+$ differentiation subsets. A higher frequency of Ki-67^+ cells within $CD8^+$ T_{CM} and $CD8^+$ T_{EM} cells in GCA patients was detected, indicating increased (*in vivo*) proliferation of these $CD8^+$ memory cells (Fig. 2B, $P = 0.009$ and $P < 0.001$, respectively).

Circulating $CD8^+$ T cells from GCA patients demonstrate reduced activation thresholds

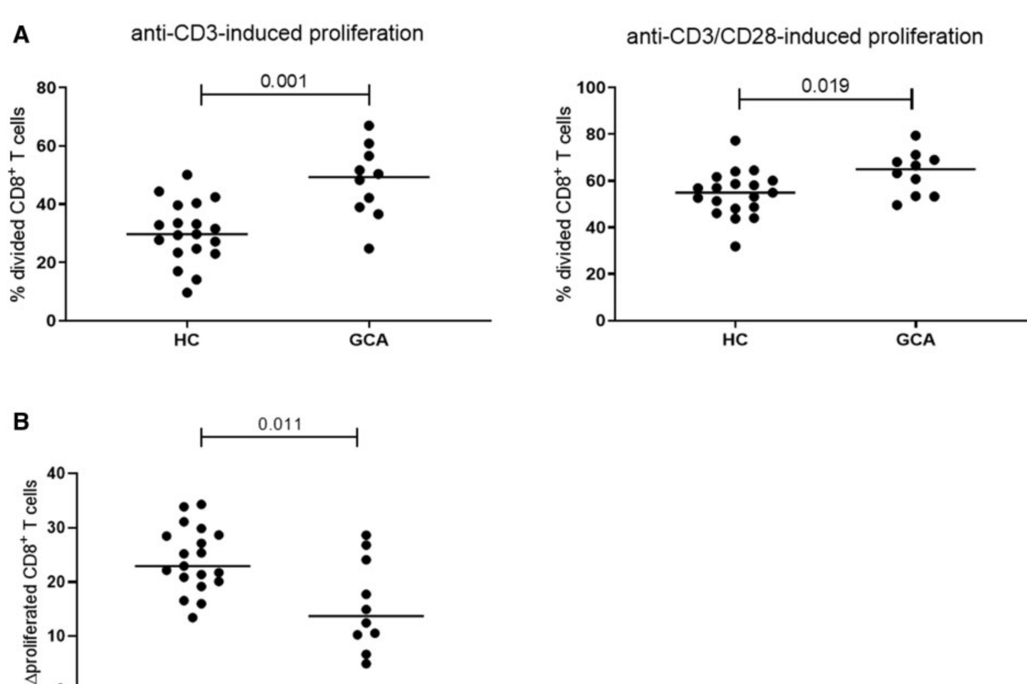
To further investigate the mechanism underlying the increased proliferative profile of $CD8^+$ T cells, we stimulated T cells with either anti-CD3 or anti-CD3 in combination with anti-CD28 (anti-CD3/28) to assess differences in proliferative capacity after TCR stimulation with or without co-stimulation. $CD8^+$ T cells of GCA patients showed an increased proliferative capacity as assessed by the percentage of divided cells upon anti-CD3 and anti-CD3/28 stimulation compared with HCs ($P < 0.05$, Fig. 3A). Furthermore, the proliferation index was higher in GCA patients upon anti-CD3 stimulation ($P < 0.001$) than in HCs as well (Supplementary Fig. S3, available at *Rheumatology* online). Interestingly, although both patients and controls responded to both anti-CD3 and anti-CD3/CD28 stimulation, the proliferative response of $CD8^+$ T cells of GCA patients to stimulation with anti-CD3 alone was more pronounced than seen in HC. Accordingly, the percentages of divided cells upon

Fig. 2 Altered distribution and proliferative status of CD8$^+$ T cell differentiation subsets in GCA



Frequencies of CD8$^+$ differentiation subsets (**A**) and Ki-67$^+$ cells (**B**) among CD8$^+$ subsets. Horizontal bars reflect median percentages. Mann–Whitney *U*-test was used to compare frequencies between HCs ($n = 18$) and GCA patients ($n = 14$). *P*-values are indicated in the graphs. T_{CM}: central memory; T_{EM}: effector memory; T_{EMRA}: effector memory cells re-expressing CD45RA.

Fig. 3 Enhanced proliferative capacity of CD8$^+$ T cells in GCA



(**A**) Percentages of divided CD8$^+$ T cells upon anti-CD3 and anti-CD3/CD28 stimulation. (**B**) Percentage of divided cells upon anti-CD3/CD28 stimulation minus the percentage of divided cells upon anti-CD3 stimulation indicated with delta proliferation. Horizontal bars reflect median percentages. Mann–Whitney *U*-test was used to compare frequencies between HCs ($n = 19$) and GCA patients ($n = 10$). *P*-values are indicated in the graphs.

anti-CD3/CD28 stimulation minus the percentage of divided cells upon anti-CD3 stimulation alone, the delta proliferative response, was significantly lower in GCA patients (Fig. 3B).

Next, we evaluated whether the proliferative capacity of $CD8^+$ T cells was related to disease state and/or patient characteristics. CRP levels did not correlate with the percentage of divided cells upon anti-CD3 or anti-CD3/CD28 stimulation. Age showed a positive correlation with the percentage of divided cells upon anti-CD3 stimulation in GCA patients, and a similar trend was found for HCs (GCA: $r = 0.66$, $P = 0.04$; HC: $r = 0.4$, $P = 0.078$).

Subsequently, we assessed whether the percentages of $CD8^+$ T cell differentiation subsets correlated with the proliferative capacity of $CD8^+$ T cells. In GCA, the percentage of $CD8^+$ $T_{NAÏVE}$ cells correlated inversely with the percentage of divided $CD8^+$ T cells upon anti-CD3 stimulation ($r = -0.84$, $P = 0.004$), whereas the percentage of $CD8^+$ T_{EMRA} cells showed a positive correlation ($r = 0.79$, $P = 0.009$). These correlations were not observed in HCs.

The data combined suggest that $CD8^+$ T cells from GCA patients rely less on co-stimulation, thereby demonstrating reduced TCR activation thresholds. The latter may underlie higher frequencies of circulating Ki-67^+CD8^+ T cells in GCA.

Increased proliferative capacity is not caused by decreased presence of regulatory cells

Next, we assessed whether the increased proliferative capacity of $CD8^+$ T cells can be explained by a decreased presence of regulatory $FOXP3^+CD4^+$ T cells in GCA patients. Here, we found no differences in frequencies of regulatory T cells between HCs and GCA patients (Supplementary Fig. S4, available at *Rheumatology* online). The data suggest that the proliferation-prone features of circulating $CD8^+$ T cells in GCA are not explained by reduced frequencies of regulatory cells.

Transcriptome profiling by scRNAseq confirms a proliferation-prone phenotype of peripheral $CD8^+$ T cells in GCA

To assess whether the increased proliferative capacity of $CD8^+$ T cells could be explained by differences in gene expression, single-cell RNA sequencing was performed. T cell subsets were assigned to cell clusters with the use of marker genes and the differentially expressed genes in each cluster (Fig. 4A–C**;** Supplementary Fig. S5, available at *Rheumatology* online). T cell subset distribution as assigned to cell clusters was compared with distribution of subsets as assessed by flow cytometry in GCA patients. $CD8^+$ naïve/memory subset distribution was comparable but memory subset distribution was not one-to-one comparable, possibly caused by the differences in techniques used and the fact that mucosal associated invariant T (MAIT) cells and the memory cluster 14 could not be distinguished by flow cytometry.

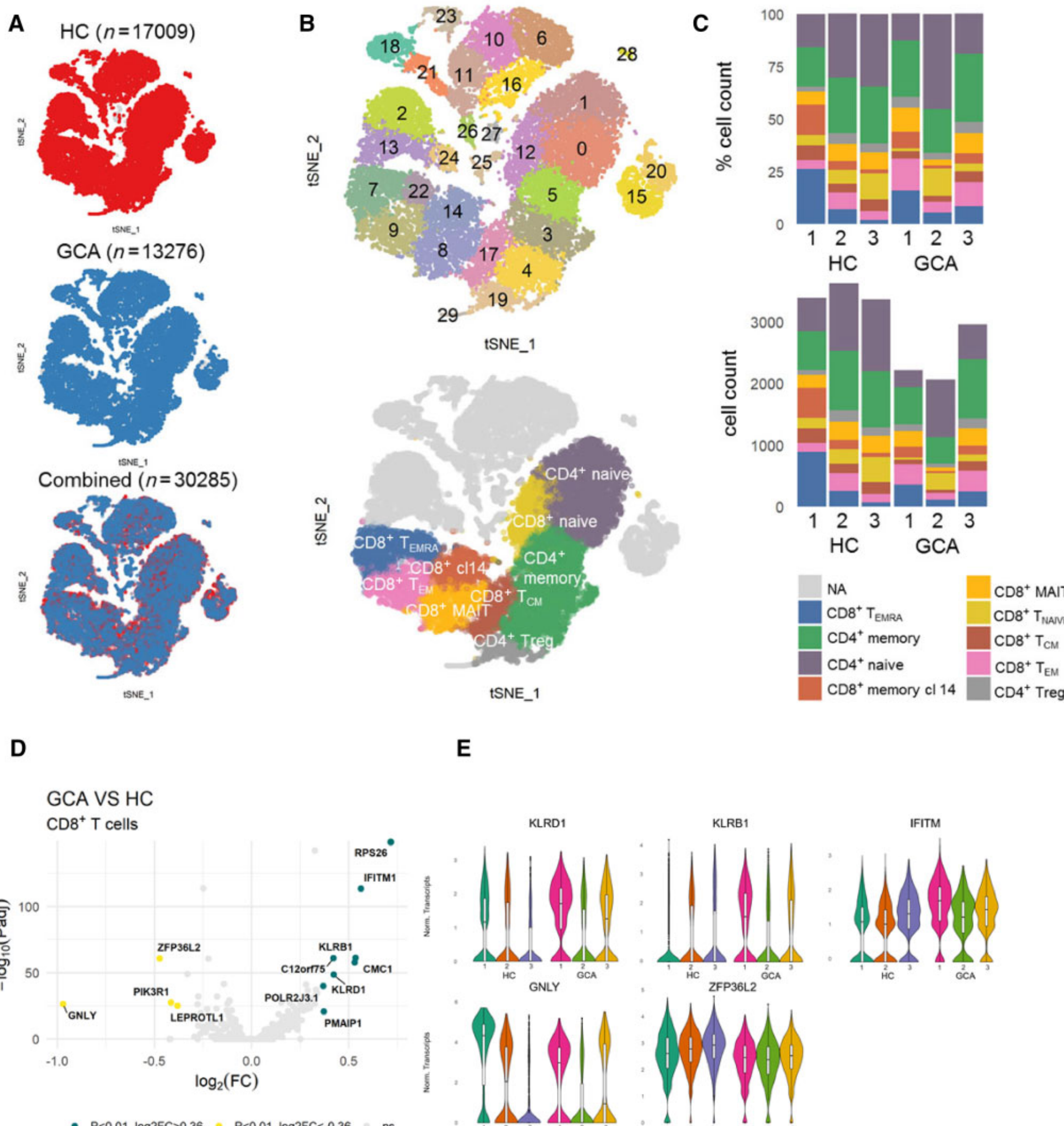
Next, differential expression tests were performed on the total $CD8^+$ T cell cluster to compare gene expression between GCA patients and HCs (Fig. 4D). Genes that were differentially expressed between GCA and HCs within $CD8^+$ T cells included *KLRD1*, *GNLY*, *LEPROTL1*, *PMAIP1*, *ZFP36L2*, *PIK3R1*, *RPS26*, *KLRB1*, *C12orf75*, *CMC1*, *IFITM1* and *POLR2J3.1*. Of these genes, the change in *RPS26* seems to be donor specific, as the difference was caused by a decrease in one HC rather than a decrease in the whole group (Supplementary Fig. S6, available at *Rheumatology* online).

Interestingly, transcripts of a number of genes linked to $CD8^+$ T cell function were upregulated in $CD8^+$ T cells in GCA patients compared with HCs (Fig. 4D and E). *KLRD1* encodes CD94, which can form dimers with NKG2A, NKG2E and other members of the C-type lectin-like NK cell receptor family. Dimerization results in transmission of either inhibitory or activation signals into the cell, depending on the specific dimer [16]. *KLRB1* encodes CD161, identifying $CD8^+$ T cells that may produce IL-17A, IFN-γ and/or TNF-α, although these gene transcripts were not upregulated in $CD8^+$ T cells of GCA patients [17]. *IFITM1* encodes interferon-induced transmembrane protein 1 (IFITM1), which can prevent infection by viruses by inhibiting viral crossing of the lipid bilayer of cells [18].

Other gene transcripts linked to $CD8^+$ T cell function were downregulated in total $CD8^+$ T cells in GCA patients (Fig. 4D and E). *GNLY* was downregulated in $CD8^+$ T cells of GCA patients, despite high donor variability (Fig. 4E). *GNLY* encodes granulysin, which is linked to cytotoxicity [19]. Interestingly, *ZFP36L2* was also downregulated in $CD8^+$ T cells of GCA patients. *ZFP36L2* encodes zinc finger protein 36-like 2 (ZFP36L2), which can inhibit proliferation by downregulating cyclin D expression [20]. As $CD8^+$ T cells from GCA patients showed reduced expression of *ZFP36L2*, this could underlie their increased proliferative capacity *in vitro* and *in vivo*.

Next, we assessed in which $CD8^+$ T cell subsets the above-mentioned genes were differentially expressed (Supplementary Fig. S7, available at *Rheumatology* online). *KLRD1* was upregulated in GCA patients in $CD8^+$ MAIT cells and $CD8^+$ memory cluster 14. *KLRB1*, on the other hand, was upregulated in GCA patients in $CD8^+$ T_{EMRA} cells and $CD8^+$ memory cluster 14. Interestingly, *IFITM1* was upregulated in GCA patients in all memory $CD8^+$ subsets, but not in $CD8^+$ $T_{NAÏVE}$ cells. *GNLY* was only downregulated in $CD8^+$ T_{EMRA} and $CD8^+$ memory cluster 14 cells in GCA patients. *ZFP36L2* was downregulated in all subsets ($CD8^+$ $T_{NAÏVE}$, $CD8^+$ T_{CM}, $CD8^+$ T_{EM}, $CD8^+$ MAIT and $CD8^+$ memory cluster 14 cells), except in $CD8^+$ T_{EMRA} cells. Thus, differential expression analysis reveals a gene-expression profile concurrent with a proliferation-prone, proinflammatory, anti-viral phenotype, with reduced cytotoxicity of $CD8^+$ memory cells.

Fig. 4 Single-cell RNA sequencing of peripheral blood mononuclear cells in GCA patients and healthy controls

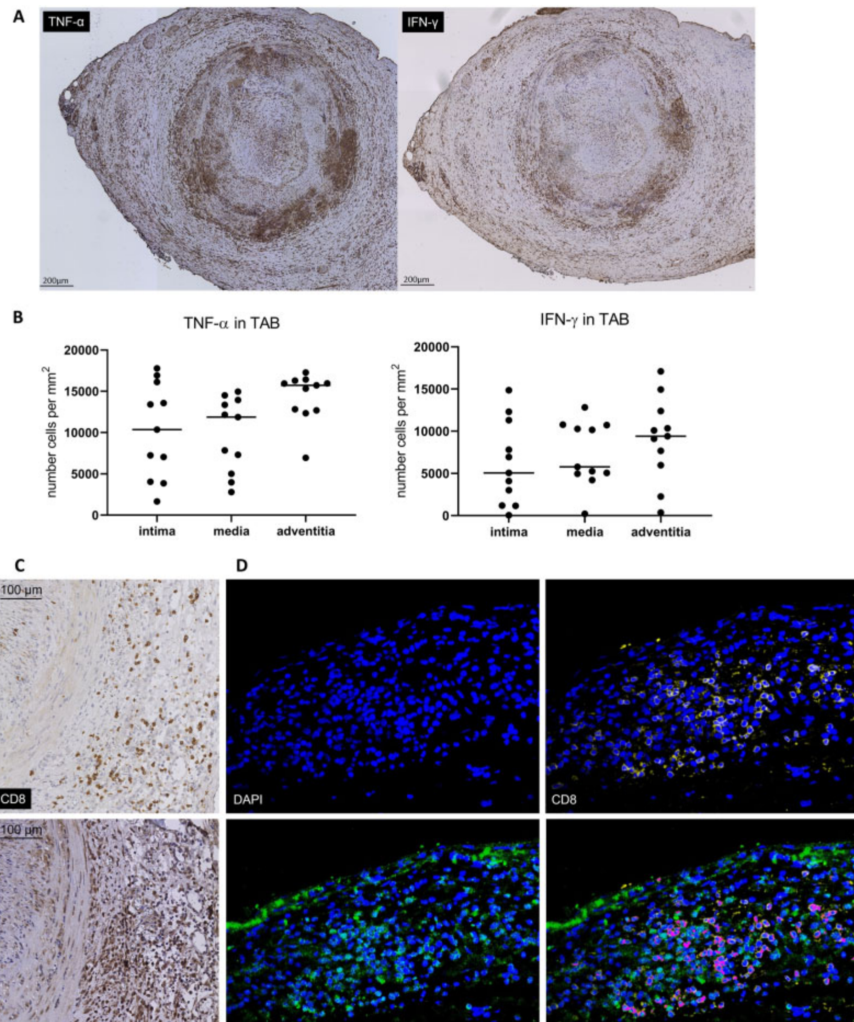


(**A**) tSNE representation of all immune cells from HCs ($n = 3$), GCA patients ($n = 3$) and all samples combined. (**B**) tSNE representation of all immune cell subsets (upper panel) and T cell subsets (lower panel). (**C**) Distribution of CD4^{+} and CD8^{+} T cell subsets per donor expressed as percentage of total T cells (upper panel) and absolute numbers (lower panel). (**D**) Volcano plot summarizing the differential expression results within total CD8^{+} T cells. The fold change of the genes is plotted against the log10 adjusted *P*-value. Yellow and green dots represent the genes with $P_{adj} < 0.01$ and absolute fold change >0.36. (**E**) Violin plots showing the expression of specific genes among all samples. MAIT: mucosal associated invariant T cells; T_{CM}: central memory; T_{EM}: effector memory; T_{EMRA}: effector memory cells re-expressing CD45RA; Treg: regulatory T cells; tSNE: t-distributed Stochastic Neighbor Embedding.

Tissue-infiltrated CD8^{+} T cells are strong producers of IFN-γ

As our phenotypical and functional analysis revealed reduced TCR activation thresholds and an associated proliferation-prone profile of circulating CD8^{+} T cells in GCA, we next aimed to elucidate the proliferative status of tissue-infiltrated CD8^{+} T cells. Both Ki-67^{+} and CD8^{+} cells were detected in tissues of GCA patients, but we found no co-expression of Ki-67 and CD8 in TAB tissues (Supplementary Fig. S8, available at *Rheumatology* online).

Fig. 5 TNF-α and IFN-γ expression in temporal artery biopsy (TAB) tissue of GCA patients



(**A**) Representative example of TNF-α and IFN-γ staining. (**B**) Quantification of total numbers of TNF-α^+ and IFN-γ^+ cells per mm^2 in each vessel layer. (**C**) Representative IHC staining of IFN-γ and CD8 in the same TAB tissue. (**D**) Immunofluorescence double staining of IFN-γ and CD8 in TAB tissue. DAPI is shown in blue, CD8 in yellow and IFN-γ in green. Overlapping pixels indicate the colocalization of CD8$^+$ and IFN-γ^+ cells, which is shown in pink. Expression of TNF-α and IFN-γ was determined by immunohistochemistry in 11 TABs from biopsy-proven GCA patients. DAPI: 4′,6-diamidino-2-phenylindole.

Subsequently, to assess the function of CD8$^+$ T cells in tissues, we assessed the expression of two key CD8$^+$ T cell-related cytokines, TNF-α and IFN-γ, in 11 TAB tissues from biopsy-proven GCA patients (Fig. 5A). TNF-α and IFN-γ were expressed throughout all the layers of the vessel wall (Fig. 5B). As especially IFN-γ is reported to be crucial in GCA pathogenesis and *in vitro* blockade of this pathway was found to be beneficial [21], we assessed whether CD8$^+$ T cells in tissues are IFN-γ producers. With immunofluorescence double staining we confirmed that the large majority of CD8$^+$ T cells indeed express IFN-γ (Fig. 5C and D).

Discussion

In this study, we provide evidence that circulating CD8$^+$ T cells in newly diagnosed, untreated GCA patients demonstrate a proliferation-prone phenotype probably caused by reduced TCR activation thresholds. In line with these observations, scRNAseq analysis of CD8$^+$ T cells revealed a differential expression profile of genes associated with regulation of proliferation, and indirectly with proinflammatory cytokine production, anti-viral activity and cytotoxicity. Also, we demonstrated reduced frequencies and numbers of CD8$^+$ T_{EM} cells in the circulation of GCA patients. CD8$^+$ T cells were detected in

both TAB and aorta tissues of GCA patients. The majority of CD8^{+} T cells in inflamed tissues were non-proliferative and produced IFN-γ consistent with an effector memory or T_{EMRA} phenotype.

In GCA, circulating CD8^{+} T cell subsets showed increased expression of Ki-67. Ki-67 is a protein expressed during all active cell cycle phases but not in resting cells [22]. Increased Ki-67 expression by T cells has been found in autoimmune diseases as well. For instance, T cells in psoriatic lesions were found to be Ki-67 positive, but it is unclear whether these were CD4^{+} or CD8^{+} [23]. Furthermore, CD8^{+} T_{EM} cells in synovial fluid of RA patients displayed an activated and proliferative phenotype defined by CD80, CD86, PD-1 and Ki-67 expression. RA patients had increased frequencies of circulating Ki-67-expressing CD8^{+} T_{EM} cells as well [24]. This matches with our findings, as especially the circulating CD8^{+} T_{EM} cells were more proliferative in GCA patients. The proliferative profile of circulating CD8^{+} T_{EM} cells may compensate for the reduced circulating CD8^{+} T_{EM} cell numbers, to maintain cellular homeostasis. Reduced numbers of circulating CD8^{+} T_{EM} cells may also indicate that these cells have migrated to tissues. Indeed, CD8^{+} T cells with effector functions such as IFN-γ production were detected in the GCA tissues. Furthermore, CD8^{+} T cells in tissues were Ki-67 negative, which implies that the presence of these cells in the tissues is a result of migration, rather than local expansion. Our data suggest that the increased Ki-67 expression is unique for CD8^{+} T cells as total CD4^{+}Ki-67^{+} percentages were similar between patients and controls.

The percentages of divided CD8^{+} T cells correlated inversely with CD8^{+} $T_{NAÏVE}$ cells and positively with CD8^{+} T_{EMRA} cells in GCA patients. This seems counter-intuitive as CD8^{+} T_{EMRA} cells are usually considered to have low proliferation rates upon TCR stimulation [25]. To investigate this further, we performed additional flow cytometry analyses in eight GCA patients and 15 HCs that were also included in the *in vitro* proliferation study. We stained for PD-1 to assess immune exhaustion and for CD28 and CD57 to investigate T cell senescence. These markers were not differently expressed in CD8^{+} $T_{NAÏVE}$ and T_{EMRA} cells in GCA patients compared with HCs (data not shown). An explanation for the correlations with CD8^{+} differentiation subsets and proliferative capacity in GCA is therefore still lacking and requires further investigation.

Reduced TCR activation thresholds in GCA patients may underlie the increase in Ki-67 expression frequencies *in vivo*. Interestingly, a recent study described increased frequencies of circulating Ki-67^{+} MAIT cells in GCA. MAIT cells, defined as CD3^{+}CD4^{-}TCR$\gamma\delta^{-}$TCRVα7.2^{+}CD161^{+}, had an increased proliferative capacity in response to IL-12 and IL-18 stimulation and showed a strong trend towards increased proliferative capacity after stimulation with anti-CD3/CD28 beads [26]. This study combined with our own data suggests that multiple CD8^{+} T cell subsets show an enhanced proliferative profile in GCA patients.

scRNAseq enabled more insight into the mechanisms underlying the altered phenotype of CD8^{+} T cells in GCA. *ZFPL36L* was downregulated in all subsets except CD8^{+} T_{EMRA} cells. ZFPL36L, encoded by the *ZFPL36L* gene, is a CCCH-type zinc finger protein. In a T cell line, T-Rex-394, and in three human colorectal cancer cell lines, ZFP36L2 overexpression was associated with decreased cell proliferation whereas knockdown resulted in increased cell proliferation [20]. These findings indicate that at the gene level CD8^{+} T cells have reduced regulation of proliferation, which together with reduced TCR activation thresholds could explain the observed proliferation-prone phenotype.

Analysis of scRNAseq data also showed a decrease in *GNLY* expression in CD8^{+}T_{EMRA} cells and cluster 14. These findings suggest that differentiated CD8^{+} T cells are not cytotoxic in the circulation [19]. At the same time, increased expression of *KLRB1* in these subsets suggests that these cells express more CD161, which is associated with IFN-γ and TNF-α production [17,27]. All memory CD8^{+} subsets appeared to have upregulated levels of *IFITM1*, which suggests increased anti-viral activity in GCA patients [18]. However, other genes related to cytotoxicity and cytokine production were not differentially expressed in CD8^{+} T cells of GCA patients. Therefore, expression of CD161, and the production of cytokines, granulysin or other cytotoxic proteins should be confirmed at the protein level before firm conclusions can be drawn.

CD8^{+} T cells in vascular tissues of GCA patients, appeared to be non-proliferating but strong producers of cytokines such as IFN-γ. IFN-γ has known implications in GCA pathogenesis, and IFN-γ-producing T cells seem to persist in tissues after glucocorticoid treatment [21]. Our study is the first to describe the involvement of CD8^{+}IFN-γ^{+}cells in tissues in GCA patients and could imply an active contribution of CD8^{+} T cells to the vascular inflammation.

This study has some limitations. First, due to the relatively small sample size ($n=3$), scRNAseq findings should be extended in a novel study involving a larger, independent cohort of GCA patients and controls. Second, we have not yet validated our scRNAseq findings at the protein level, which is necessary before firm conclusions can be drawn. Finally, next to scRNAseq analysis of circulating PBMCs, spatial transcriptomics in GCA-affected tissues would be of high interest as this can reveal more tissue specific functions of CD8^{+} T cells in GCA. Some studies have reported on a restricted TCR repertoire in CD8^{+} T cells of GCA patients [14]. It would therefore be interesting to study if certain TCR clonalities are associated with the proliferation-prone phenotype.

Conclusion

In conclusion, phenotypic, transcriptomic and functional analyses of peripheral and lesional CD8^{+} T cells indicate that these cells are active contributors to the vasculopathy in GCA. Lower activation thresholds render CD8^{+} T

cells more prone to react to antigens and to differentiate into pro-inflammatory memory cells contributing to the local inflammatory response.

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Data availability statement

Single-cell RNA sequencing data are uploaded to GEO: GSE198891. Other data supporting the findings of this study are available from the corresponding author on reasonable request.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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