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Cytomegalovirus Drives the Development of Cytotoxic CD4$^+$ T Cells in Patients With Multiple Sclerosis

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Abstract

Background and Objectives

Chronic immune activation is a hallmark of latent viral infections and autoimmune disorders, profoundly shaping immune cell phenotypes, including CD4$^+$ cytotoxic T lymphocytes (CD4 CTL). The mechanisms underlying CD4 CTL development remain elusive, although antigenic triggers and the local microenvironment are thought to influence their phenotype. In this study, it was investigated if CD4 CTL induced under different circumstances exhibit phenotypic differences.

Methods

Using single cell multiomics, we analyzed CD4 CTL from healthy cytomegalovirus (CMV)-seropositive donors, patients with CMV-seronegative relapsing-remitting multiple sclerosis (RR-MS) (autoimmune trigger), and patients with CMV-seropositive RR-MS (combination of viral and autoimmune trigger).

Results

Our findings reveal that the heterogeneous pool of CD4 CTL encompasses distinct subsets with divergent expression of proinflammatory, cytotoxic, and migratory markers. Moreover, we identified a pathogenic CD4 CTL subset coexpressing the MS-associated transcription factor eomesodermin (EOMES) and the migratory receptor class I–restricted T-cell–associated molecule, which accumulates in MS lesions and demonstrates resistance to natalizumab treatment.

Discussion

CMV was implicated as a dominant driver of development of highly cytotoxic CD4$^+$ T cells, as these cells were markedly enriched in CMV-seropositive individuals. This comprehensive phenotypic atlas of CD4 CTL advances our understanding of their development and highlights potential targets for diagnosing, treating, and preventing MS progression.

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Supplementary Material

Introduction

Once regarded an experimental artefact, CD4$^+$ cytotoxic T lymphocytes (CTL) are presently recognized as a biologically relevant T-cell subset with important functions in antiviral, anti-tumor, and autoimmune responses (see recent reviews[1,2]). These CD4 CTL are identified by loss of costimulatory receptors CD27 and CD28 and gain of natural killer (NK) receptors and cytotoxic molecules such as perforin and granzyme B.[3-5] Because CD4 CTL share oligoclonal antigen receptors with restricted antigen diversity, it has been suggested that repeated antigenic stimulation leads to expansion of CD4 CTL.[6,7] The β-herpesvirus cytomegalovirus (CMV),

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Glossary

ARRDC3 = arresting domain containing 3; **BBB** = blood-brain barrier; **CD4 CTL** = $CD4^+$ cytotoxic T lymphocyte; **CM** = central memory; **CMV** = cytomegalovirus; **CREM** = cAMP responsive element modulator; **CRTAM** = class I–restricted T-cell–associated molecule; **DEG** = differentially expressed gene; **EAE** = experimental autoimmune encephalomyelitis; **EOMES** = eomesodermin; **EZR** = Ezrin; **FOS** = Fos proto-oncogene, AP-1 transcription factor subunit; **GZMB** = granzyme B; **HC** = healthy donor; **HLA-DR** = histocompatibility complex class II DR; **HLA-DRB1** = major histocompatibility complex, class II, DR Bbeta 1; **IL7R** = interleukin-7 receptor; **LENG8** = leukocyte receptor cluster member 8; **MFI** = median fluorescent intensity; **MS** = multiple sclerosis; **NK** = natural killer; **NTZ** = natalizumab; **PBMC** = peripheral blood mononuclear cell; **PLEK** = pleckstrin; **RGCC** = regulator of cell cycle; **RR-MS** = relapsing-remitting MS; **SPOCK2** = SPARC ([Osteonectin)], cwcv and kazal like domains proteoglycan 2; **TCR** = T cell receptor; **TH** = T helper; **TXNIP** = thioredoxin interacting protein; **UMAP** = uniform manifold approximation and projection; **WTA** = whole transcriptome analysis; **ZC3HAV1** = zinc finger CCCH-type containing antiviral 1.

a latent virus with a global prevalence of 83%,[8,9] is one of the best described inducers of CD4 CTL development.[10-15] Donors seropositive for CMV are, therefore, ideal for studying CD4 CTL, although isolation of sufficient numbers of cells to study their phenotype and function in vitro remains challenging because of their low numbers in peripheral blood. Recently developed techniques such as single cell transcriptome analysis and spectral flow cytometry now allow for detailed analysis of rare cell subsets to identify markers that might otherwise be overlooked.[16,17]

Multiple sclerosis (MS) is the most prevalent chronic neurodegenerative disease in young adults,[18] and autoreactive T cells play an important role in the coordinated immune attack of the CNS in MS.[19] Several studies suggest a pathogenic role for CD4 CTL in MS, based on a correlation between circulating CD4 CTL and MS disease progression,[20-22] and the presence of CD4 CTL in the CSF[23,24,25] and CNS[26] of patients with MS. To date, there is no clear consensus on the defining markers to functionally phenotype CD4 CTL. It is, however, becoming increasingly clear that expansion of the CD4 CTL population can be induced by different antigenic triggers, which results in a variety of CD4 CTL phenotypes.[1] Not all CD4 CTL are necessarily disease-inducing or -worsening, as they can also contribute to antiviral and antitumor immune responses.[27] This suggests that different pathways can be employed by the $CD4^+$ T cell to acquire a cytotoxic profile. While chronic viral infections such as CMV are currently seen as the most important trigger for the development of CD4 CTL, the inflammatory micro-environment present in autoimmune pathology might also be sufficient to trigger differentiation into CD4 CTL.[28] In addition, the combination of a latent viral infection with an autoimmune pathology might have a synergistic effect on the pathogenicity of CD4 CTL.

In this study, we investigate whether differences exist between CD4 CTL induced under different circumstances using single cell multiomics analysis. We compare the phenotype of $CD4^+$ T cells derived from patients with relapsing-remitting multiple sclerosis (RR-MS) (i.e., $CD4^+$ T cells differentiated in an inflammatory micro-environment induced by autoimmunity[29]) with that of $CD4^+$ T cells derived from healthy donors (HCs) with a latent CMV infection (i.e., $CD4^+$ T cells differentiated in an inflammatory micro-environment induced by a persistent viral load[30]). To determine the synergistic effect of CMV and MS, RR-MS donors with and without a latent CMV infection are included. To the best of our knowledge, this study used sorted $CD4^+$ T cells enriched for CD4 CTL as source material for single cell sequencing, allowing for the in-depth characterization of human CD4 CTL subsets and the effect of their micro-environment on the resulting phenotype.

Methods

Statement Regarding Sex as a Biological Variable

Our study examined male and female human donors for most experiments, and similar findings are reported for both sexes. The only exemption was the single cell RNA/protein sequencing experiment, for which it was decided to include only female donors as MS predominantly affects women and to decrease donor-to-donor variability.

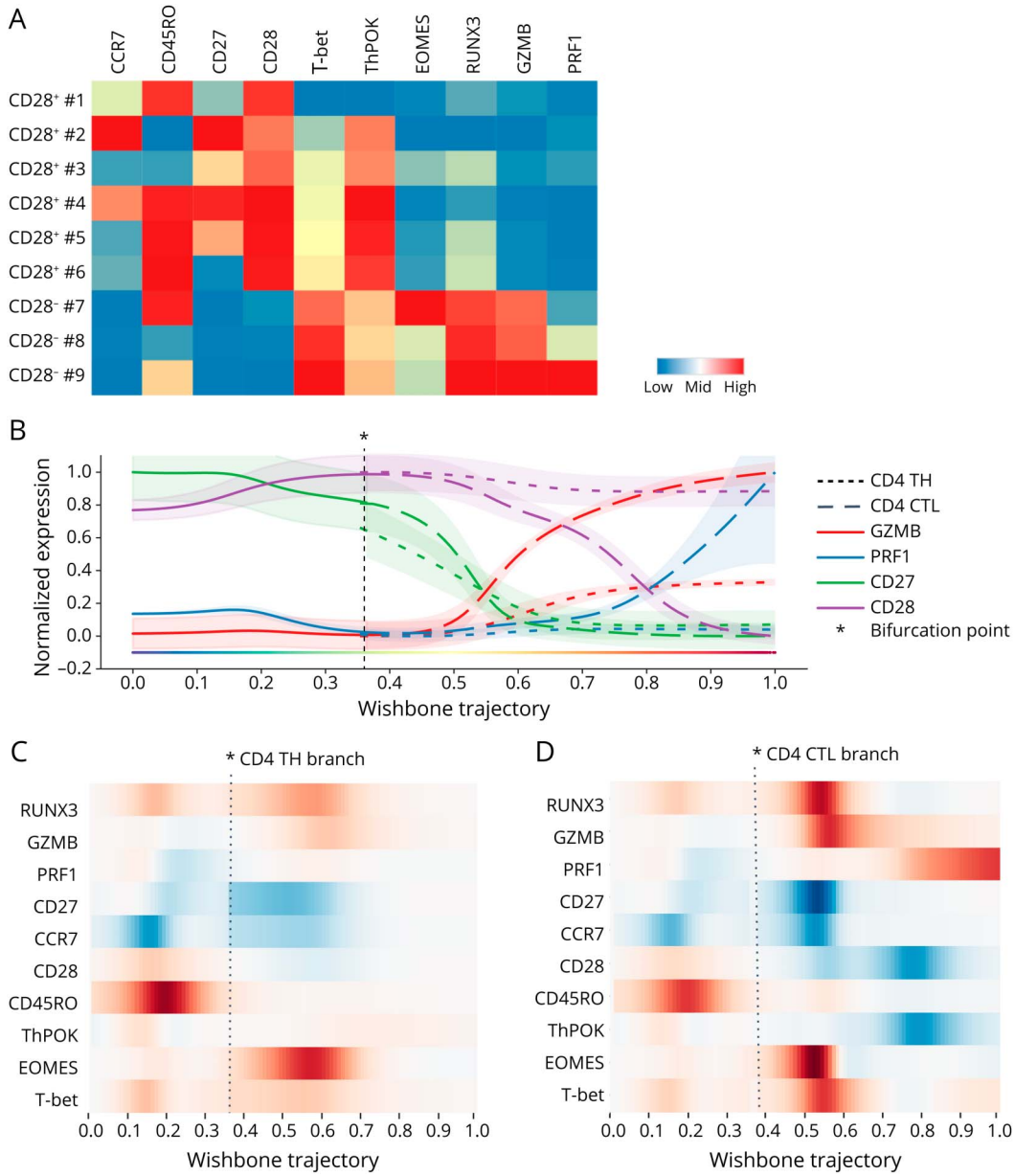
Sample Preparation and Experimental Assays

Details are provided in eMethods.

Standard Protocol Approvals, Registrations, and Patient Consents

Peripheral blood samples were obtained from HC and patients with MS, which were collected by Noorderhart hospital (Pelt, Belgium) and the University Biobank Limburg (UBiLim, Hasselt, Belgium).[31] Samples used for flow cytometry included samples from patients who received treatment, including IFN-β (Avonex, Rebif, Betaferon), glatiramer acetate (Copaxone), fingolimod (Gilenya), or natalizumab (NTZ) (Tysabri), at the time of sampling. Donor characteristics are given in eTable 1. For immunohistochemistry, brain material from 9 patients with chronic progressive MS was obtained from the Netherlands Brain Bank (NBB, Amsterdam, Netherlands). Further clinical details for

Figure 1 Loss of CD27 Precedes Acquisition of Cytotoxic Features During Differentiation of Conventional TH Cells Toward CD4 CTL



(A) Flow cytometry data of patients with CMV-positive RR-MS with known expansion of CD4 CTL (n = 7) were analyzed using the unsupervised automated clustering tool FlowSOM.[32] Live, single CD4$^+$ cells were categorized into 9 subpopulations based on their expression of canonical differentiation markers (CCR7, CD45RO, CD27, CD28), transcription factors (T-bet, ThPOK, EOMES, RUNX3), and cytotoxic molecules (PRF1, GZMB). The median expression of each marker within the subsets is displayed in the heatmap. (B) The Wishbone algorithm[33] was applied to flow cytometry data of live, single CD4$^+$ T cells acquired from a patient with RR-MS with known CD4 CTL expansion. Naive cells (CCR7$^+$CD45RO$^-$, representative dotplot in eFigure 1C) were used as starting gate. This algorithm predicts when 2 developmental paths start to digress from each other. After this bifurcation (indicated by an asterisk), expression pattern of known cytotoxic markers are shown in a dotted line for conventional TH cells, and a dashed line for CD4 CTL. (C and D) Derivative plot of the same sample, showing the changes in expression of markers in pseudotime along the trajectory displayed in (B) for conventional TH cells (C) and CD4 CTL (D). CD4 CTL = CD4$^+$ cytotoxic T lymphocyte; CMV = cytomegalovirus; RR-MS = relapsing-remitting multiple sclerosis.

these patients are summarized in eTable 2. This study was approved by the local ethical committees, and informed consent was obtained from all donors.

Data Availability

Data are available upon reasonable request by contacting the corresponding author.

Results

Loss of CD27 Precedes Acquisition of Cytotoxic Features by CD4 CTL

To gain an overall view on the ex vivo phenotype of CD4 CTL in a highly proinflammatory environment, circulating CD4$^+$ T cells from patients with untreated CMV-seropositive RR-

MS were analyzed for expression of selected canonical memory and cytotoxic markers using multiparameter flow cytometry. Using the unsupervised clustering approach of FlowSOM,[32] the $CD4^+$ T-cell population was subdivided into 9 discrete subpopulations (Figure 1A). Three of these 9 subsets exhibited loss of CD28 expression ($CD28^-$ subsets #7, #8, #9; eFigure 1A), which is a sign of terminal differentiation and gain of cytotoxicity in $CD4^+$ T cells. While all $CD28^-$ populations also lacked expression of CD27 and CCR7 (associated with early activation and homing to lymphoid tissues, respectively), they expressed T-bet (associated with IFN-γ production in Th1 and $CD8^+$ T cells), RUNX3 (transcription factor driving the $CD8^+$ tissue residency program) and granzyme B (GZMB; cytotoxic molecule) and showed a reduction in ThPOK levels (transcription factor responsible for maintaining Th cell identity). While subset #7 was the only $CD28^-$ subpopulation that expressed EOMES (transcription factor driving cytotoxicity and infiltration of inflamed tissues), subset #9 was the only $CD28^-$ population with a high expression of both GZMB and PRF1 (cytotoxic molecule), indicating a fully functional cytotoxic profile. Of note, 2 of the 6 $CD28^+$ subsets showed a decrease in CD27 expression (subset #1 and #6). Loss of CD27, therefore, appeared to precede loss of CD28, as can also be appreciated from the representative dotplot of CD27 and CD28 coexpression (eFigure 1B).

We next applied trajectory interference to model protein expression changes in pseudotime from naive $CD4^+$ T cells to CD4 CTL using the wishbone algorithm[33] (Figure 1, B–D, eFigure 1C). Naive $CD4^+$ T cells ($CD4^+CCR7^+CD45RO^-$) were used as starting point. We expected that not all naive $CD4^+$ T cells will eventually become CD4 CTL, as even in supercentenarians (≥110 years of age), the percentage of CD4 CTL is limited to 25% of the total $CD4^+$ population,[34] similar to autoimmune disease patients with CD4 CTL expansions.[35] After the bifurcation (indicated by an asterisk), expression patterns of the most relevant cytotoxic markers are shown in a dotted line for conventional $CD4^+$ T helper (TH) cells, and a dashed line for CD4 CTL (Figure 1B). When comparing median fluorescent intensity (MFI) of all markers in pseudotime for each branch separately (eFigure 1, C and D), it was evident that in the CD4 CTL branch, loss of CD27 and gain of RUNX3 and EOMES expression occurred shortly after bifurcation, which was rapidly followed by gain of GZMB expression. PRF1 expression was acquired at the final stage of CD4 CTL development.

To gain further insight into the events preceding CD4 CTL development and contribution of CMV infection thereof, the same flow cytometry panel was applied to 2 additional cohorts of patients with untreated RR-MS: 1) patients who were seronegative for CMV and 2) patients who were seropositive for CMV but did not exhibit expansion of CD4 CTL (<2% of $CD4^+CD28^-$ within their circulating $CD4^+$ population). To visualize the abundance of cellular subsets across groups, the FlowSOM clustering from Figure 1A was applied to an opt-SNE plot (eFigure 1D). Of interest, all 3 $CD28^-$ subsets (#7—red, #8—light pink, #9—purple) could be identified in all 3 cohorts, even in patients with RR-MS seronegative for CMV. Only $CD28^-$ subsets #8 and #9 were significantly increased in the group with preexisting CD4 CTL expansion (eFigure 1, E–G).

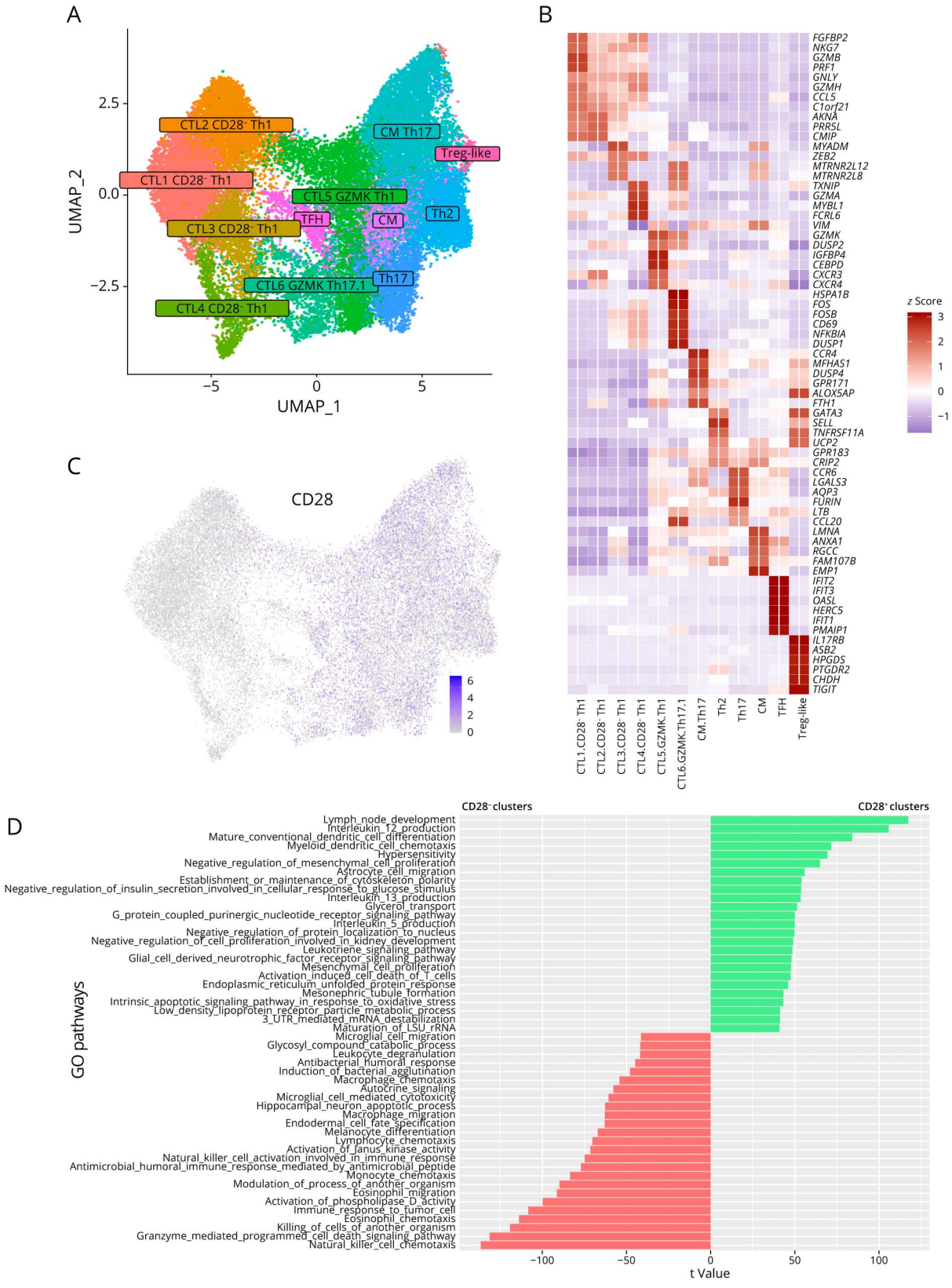
Taken together, these data show that loss of CD27 expression precedes the acquisition of cytotoxic features in CD4 CTL. It appears that during further differentiation, $CD4^+$ T cells start to lose CD28 expression and acquire increasing levels of cytotoxicity, as indicated by their gradual gain of PRF1 expression. Expansion of the CD4 CTL population, furthermore, appears to be driven mainly by an increase in $CD28^-$ cells positive for PRF1, while abundance of CD4 CTL with a less pronounced cytotoxic profile remains stable in different patient cohorts and could even be detected in CMV-seronegative patients.

CD4 CTL Are Heterogeneous and Composed of 4 Functionally Different Subsets

To further dissect the observed heterogeneity of CD4 CTL and to characterize these cells in depth beyond the commonly used cytotoxic markers, we next applied single cell multiomics analysis on CD4 CTL by combining single cell RNA sequencing with AbSeq technology. Herein, we included 4 age- and sex-matched donors per group. Given that MS predominantly affects females,[18] only female donors were included here to decrease donor-to-donor variability. To ensure that enough CD4 CTL were present in samples, we sorted $CD4^+CD27^-$ T cells prior to sequencing (eFigure 2). The clustering pattern of the resulting complete gene expression dataset of single cells was visualized with the uniform manifold approximation and projection (UMAP) dimension reduction technique (Figure 2A). Twelve clusters were identified and annotated based on gene expression of canonical markers (eTable 3), and relative expression patterns of the top 6 differentially expressed genes (DEGs) for each cluster are shown in Figure 2B. The cytotoxic genes *GZMB* and *PRF1* were found to be highly expressed in 4 out of the 12 clusters. These clusters also expressed the Th1 associated transcription factor T-bet (*Tbx21*, eTable 3) and were confirmed to be negative for CD28 at protein expression level (Figure 2C). Therefore, these clusters are referred to as CTL1 CD28neg Th1, CTL2 CD28neg Th1, CTL3 CD28neg Th1, and CTL4 CD28neg Th1. A different member of the granzyme family, granzyme K (*GZMK*), was not expressed in the CTL CD28neg Th1 clusters, but in contrast was found to be expressed in 2 clusters that had maintained CD28 expression. Based on CXCR3 and CCR6 expression, these clusters were annotated as CTL5 GZMK Th1 and CTL6 GZMK Th17.1, respectively. Gene ontology pathway analysis indicated an enrichment of cytotoxicity- and chemotaxis-associated pathways in the 4 CTL CD28neg Th1 clusters (Figure 2D), confirming their well-defined phenotype and function.[1,2,22,26,29,36-44]

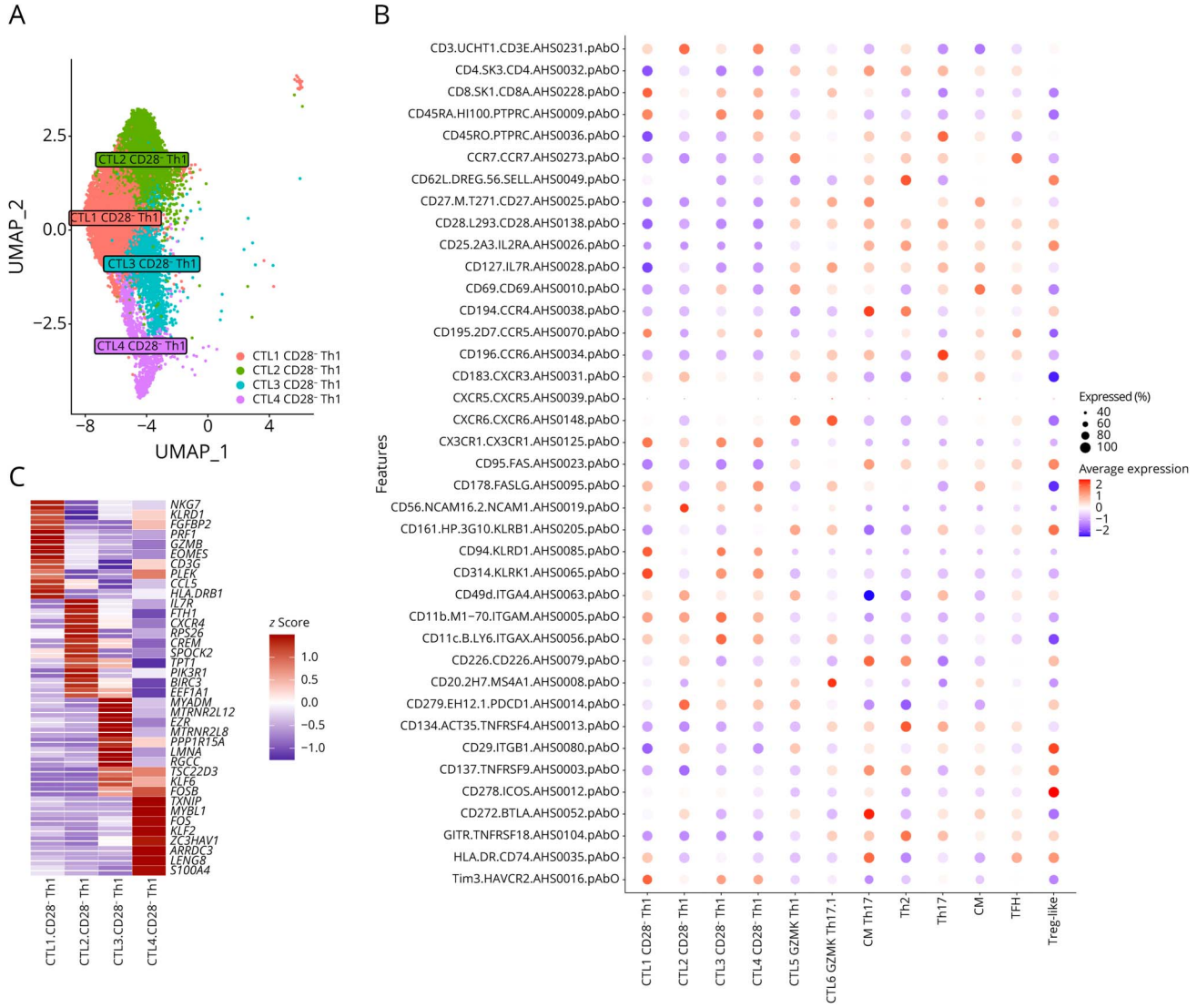
The transcriptomic dataset of the 4 CTL CD28neg Th1 clusters was mined further to identify DEGs for each cluster

Figure 2 Single Cell Multiomics Analysis of $CD3^+CD4^+CD27^-$ T Cells Reveals 6 Distinct Subpopulations of CD4 CTL



(A) Dimensionality reduction approach using the UMAP algorithm was applied to WTA sequencing results. Twelve clusters were identified and overlaid on UMAP plot. Clusters were annotated based on canonical markers listed in eTable 3. (B) Heatmap showing the top 6 differentially expressed genes for each cluster. (C) Protein expression of CD28 overlaid on UMAP plot. (D) Differences in gene set enrichment scores of the GO biological function pathways between the clusters expressing CD28 (clusters annotated as CTL5 GZMK Th1, CTL6 GZMK Th17.1, CM Th17, Th2, Th17, CM, TFH, Treg-like) compared with clusters lacking CD28 expression (clusters CTL1-4 CD28neg Th1). Differences are calculated using a Student *t* test. The *t* values of the 25 most significantly enriched pathways are shown for both CD28 positive clusters (green) and negative clusters (red). CD4 CTL = $CD4^+$ cytotoxic T lymphocyte; UMAP = uniform manifold approximation and projection.

Figure 3 CD4 CTL Clusters Have Distinct Coexpression Patterns of CD45RA/CD45RO, NK Receptors, and Adhesion Molecules at Protein Level



(A) UMAP plot showing the 4 CTL CD28neg Th1 clusters that were selected for further exploration. (B) The top 10 upregulated genes per cluster, when only transcriptome data from these clusters are used for analysis. (C) Dotmap showing the relative protein expression of selected surface markers identified using the BD AbSeq technology. The names on the X-axis indicate the corresponding clusters from the transcriptome analysis described in Figure 2 and eTable 3. Details on AbSeq antibody panel are given in eTable 4. CD4 CTL = $CD4^+$ cytotoxic T lymphocyte; UMAP = uniform manifold approximation and projection.

relative to all other CTL clusters (Figure 3A). For each cluster, 10 genes were identified that were differentially expressed compared with the other CTL clusters (Figure 3B). CTL1 CD28neg Th1 was characterized by NK receptors (*NKG7, KLRD1*), effector genes (*FGFBP2, CD3G, CCL5*), cytotoxicity-associated molecules (*PRF1, GZMB, EOMES*), cellular activation (major histocompatibility complex, class II, DR beta 1 (*HLA-DRB1*)), and migration (pleckstrin [*PLEK*]). CTL2 CD28neg Th1 was characterized by migration and proliferation (*CXCR4, RPS26, TPT1, PIK3R1, EEF1A1*), memory state (interleukin-7 receptor [*IL7R*]), antiviral function (SPARC [Osteonectin], cwcv and kazal like domains proteoglycan 2 [*SPOCK2*]), survival (*FTH1, BIRC3*), and cytokine production (cAMP responsive element modulator [*CREM*]). CTL3 CD28neg Th1 was characterized by migration (*MYADM, Ezrin* [*EZR*]), survival (*MTRNR2L8, MTRNR2L12*), cell cycle regulation (*PPP1R15A, regulator of cell cycle* [*RGCC*]), and activation (*LMNA, KLF6, TSC22D3*). CTL3 CD28neg Th1 was characterized by cell cycle regulation (*FOSB,* Fos proto-oncogene, AP-1 transcription factor subunit [*FOS*], *MYBL1*), glucose metabolism (thioredoxin interacting protein [*TXNIP*]), migration (*KLF2, S100A4*), antiviral function (zinc finger CCCH-type containing antiviral 1 [*ZC3HAV1*]), cellular signaling (arresting domain containing 3 [*ARRDC3*]), and mitochondrial activity (leukocyte receptor cluster member 8 [*LENG8*]). Oligonucleotide-conjugated antibodies directed against surface proteins were applied for single cell protein analysis in parallel with

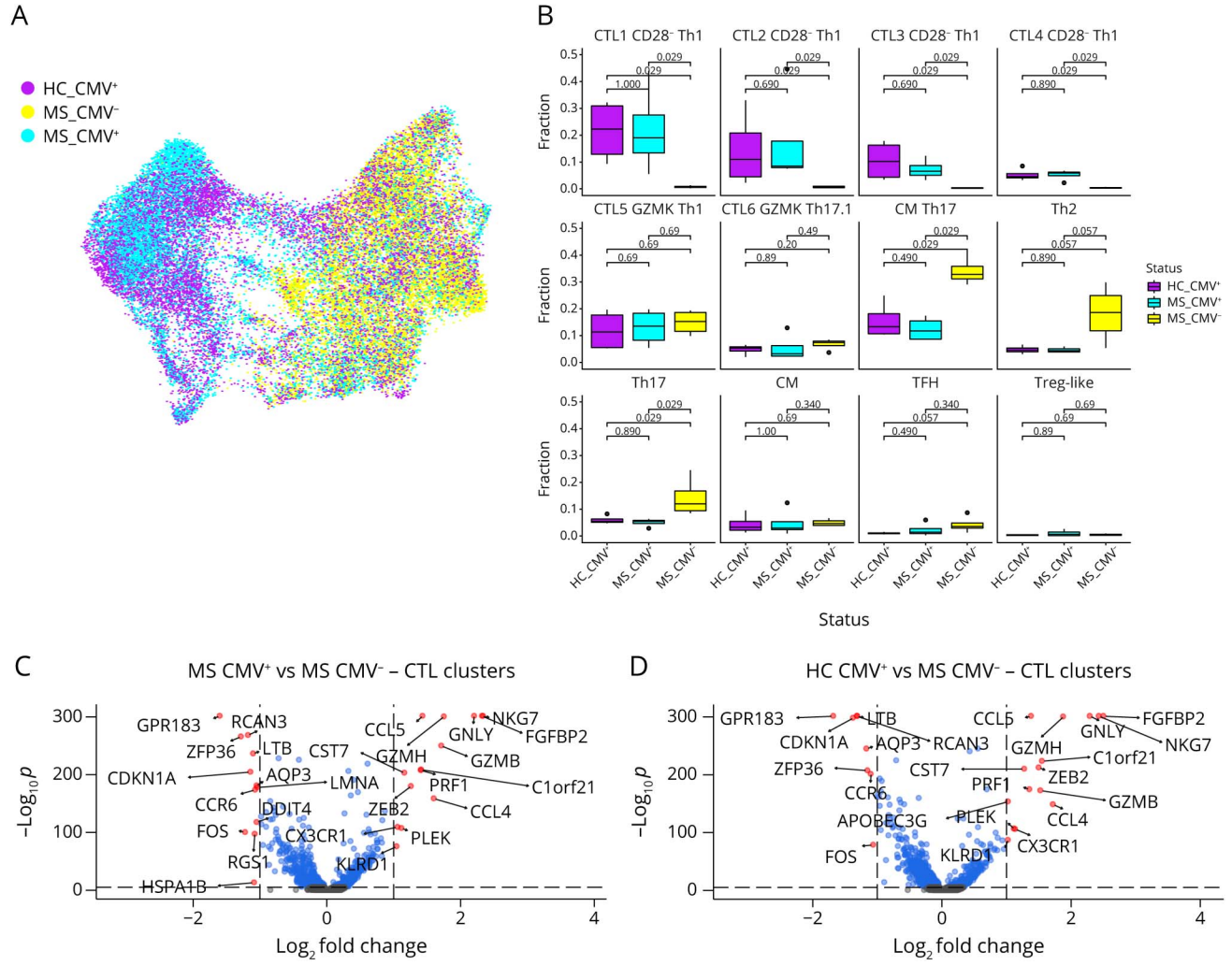
transcriptome analysis, to determine if CD4 CTL subsets could be identified based on protein expression of surface molecules. Using the same clustering pattern of Figure 2A, protein expression of selected surface markers (eTable 4) was analyzed across clusters (Figure 3C). The CTL1, CTL3, and CTL4 CD28neg Th1 clusters showed comparable patterns of protein expression, with high expression of CD45RA, CX3CR1, NK receptors KLRD1, NKG2D (encoded by the gene *KLRK1*), adhesion molecules CD11b and CD49d, and inhibitory receptor T-cell immunoglobulin and mucin domain 3. While expression of most of these markers was lower in CTL3 CD28neg Th1, this cluster exhibited high expression of CD56 and PD-1, and to a lesser extent also CD29 expression. Distinguishing features for other CD28neg CTL clusters were found in major histocompatibility complex class II DR (HLA-DR) expression (increased in CTL1 CD28neg Th1 only) and CD69 (increased in CTL3 CD28neg Th1 only). It should be noted that while from the relative expression differences shown in Figure 3C it might appear that CD8 is expressed in the CD28neg CTL clusters, absolute expression levels of CD8 are close to zero (eFigure 3), confirming the purity of the sorted $CD4^+CD27^-$ population and ruling out that contamination with $CD8^+$ cytotoxic T cells is driving the CTL clustering. Overall, these data reveal that CD4 CTL, defined as $CD4^+$ cells that have lost CD28 expression, are a heterogeneous population, but the differences in gene and protein expression between CD4 CTL subsets suggest that these are actually functionally different subsets rather than simply different phases of CD4 CTL development.

MS Pathology Without CMV Infection Is Not Sufficient to Induce Full-Blown Cytotoxicity in CD4 CTL

Next, we explored whether different antigenic triggers cause differences in CD4 CTL development. More specifically, we investigated which CD4 CTL subsets arise in patients with CMV-seronegative RR-MS, and to what extent these are different from CD4 CTL of patients with CMV-seropositive RR-MS and HC. First, by overlaying the different patient groups on the UMAP plot, it was evident that CMV-seropositivity dictates the loss of CD28 expression (Figure 4A; left: $CD28^-$, right: $CD28^+$; as compared to Figure 2C). CD28 protein expression across groups and clusters was analyzed in more detail to determine where $CD28^-$ T cells of CMV-seronegative RR-MS donors were clustered, given that all selected donors were confirmed to have >1% $CD4^+CD28^-$ expansion prior to inclusion. Although the absolute number of $CD28^-$ T cells was lower in the CMV-seronegative group, the expression pattern of CD28 was similar to that of the CMV-seropositive groups (eFigure 4A). To determine group-specific patterns of clustering, the abundance of each cluster was compared across groups (eTable 5 and Figure 4B). The distribution of cells across clusters did not differ between the 2 CMV-seropositive groups. The proportion of cells allocated to each of the 4 CTL CD28neg Th1 clusters was significantly lower in the CMV-seronegative RR-MS patient group, while this group exhibited an increased number of cells in the clusters central memory (CM) Th17 and Th17. The proportion of cells in CTL5 GZMK Th1 and CTL6 GZMK Th17.1 was not different between groups. To further visualize differences in gene expression provoked by CMV status or presence of MS, differential gene expression between the different groups is displayed in volcano plots (Figure 4, C and D, and eFigure 4, B–E). No significant differences were found between HC and MS CMV-seropositive donors, either when comparing all clusters (eFigure 4B) or when taking into account only the CTL clusters (eFigure 4C). In contrast, 13 genes, including *CCL5, NKG7,* and granulysin (*GNLY*), were significantly increased in CMV-seropositive donors compared with CMV-seronegative donors, regardless of presence or absence of MS disease (eFigure 4, D and E, eTables 6 and 7). When including only the CTL clusters, 26 DEG (12 downregulated, 14 upregulated) were identified when comparing patients with CMV-seronegative with CMV-seropositive MS (Figure 4C, eTable 8), and 24 DEG (8 downregulated, 16 upregulated) when comparing patients with CMV-seropositive HC with CMV-seronegative MS (Figure 4D, eTable 9). Again, CMV status was revealed as major driver of differences in gene expression of the CD4 CTL, as most DEG were shared across patients with CMV-seropositive MS and HC (8 out of, respectively, 12 and 8 downregulated genes, and 14 out of, respectively, 14 and 16 upregulated genes).

Finally, to explore differences in the cytotoxic profile of the 4 CTL CD28neg Th1 clusters between patient groups, we investigated the differential expression of selected cytotoxic or effector molecules and associated transcription factors between all clusters and across groups (Figure 5). While most cytotoxic genes were only expressed in the CTL CD28neg Th1 clusters (with exception of *GZMK*, which was exclusively found in clusters CTL5 GZMK Th1 and CTL6 GZMK Th17.1), differences in expression levels of these genes could be found between these 4 clusters (Figure 5A). When comparing gene expression of *PRF1, GZMB,* and *GZMK* between groups for the relevant clusters, the lowest numbers of cells expressing these genes were found in the CMV-seronegative MS group as expected (Figure 5B, eFigure 5, A and B). To validate our findings at protein level, peripheral blood mononuclear cells (PBMC) from an additional cohort of CMV-seropositive HC (n = 5), CMV-seronegative MS donors (n = 6), and CMV-seropositive MS donors (n = 11) were used to determine ex vivo PRF1, GZMB, and GZMK expression in their $CD4^+CD27^-CD28^+$ and $CD4^+CD27^-CD28^-$ populations by flow cytometry (Figure 5, C and D, eFigure 5C). Expression levels of PRF1 and GZMB were significantly increased in the $CD4^+CD27^-CD28^-$ fraction for both CMV-seropositive groups. For GZMK, it was confirmed that cells expressing GZMK were primarily $CD28^+$. In addition, the number of $CD28^+$ cells expressing GZMK was decreased in the MS donors regardless of their CMV status. Taken together, these data confirm that CMV has a greater effect on the level of cytotoxic molecules expressed by CD4 CTL than presence of an autoimmune disease such as MS. Although some CD4 CTL

Figure 4 CMV Status, and Not Presence of MS, Drives Abundance of CD28⁻ Clusters and Differences in Gene Expression Profiles of These Cytotoxic Clusters



(A) Distribution of sample groups overlaid on UMAP plot. Please refer to Figure 2 for an explanation of the UMAP clustering. (B) Boxplot comparison of relative distribution of sequenced cells across clusters for the 3 groups. The cluster names correspond to the UMAP shown in Figure 2A and the annotation in eTable 3. Data analyzed using Wilcoxon test. (C) Volcano plot illustrating DEGs between patients with CMV-seropositive RR-MS and patients with CMV-seronegative RR-MS across the CTL clusters only (CTL1-4 CD28neg Th1, CTL5 GZMK Th1, and CTL6 GZMK Th17.1). eTable 8 for details on DEG. (D) Volcano plot illustrating DEGs between patients with CMV-seropositive HC and CMV-seronegative RR-MS across the CTL clusters only (CTL1-4 CD28neg Th1, CTL5 GZMK Th1, and CTL6 GZMK Th17.1). eTable 9 for details on DEG. Statistical analyses for Volcano plots were performed using a Wilcoxon Rank Sum test. CD4 CTL = $CD4^+$ cytotoxic T lymphocyte; CMV = cytomegalovirus; DEG = differentially expressed gene; RR-MS = relapsing-remitting multiple sclerosis; UMAP = uniform manifold approximation and projection.

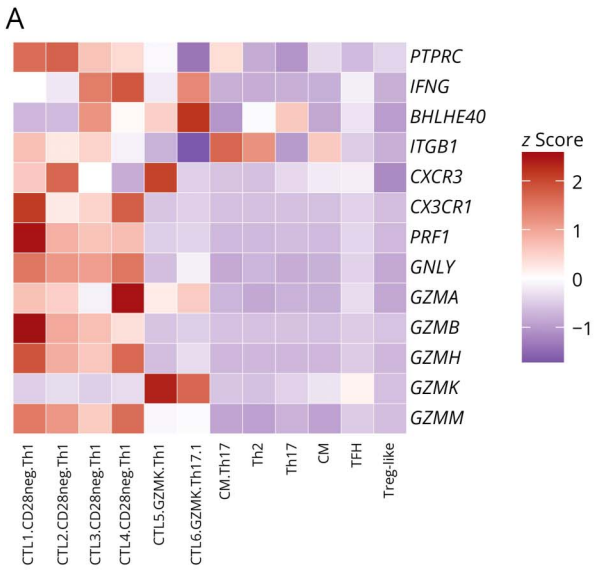
can be detected in the circulation of patients with CMV-seronegative MS, our data suggest that these are less cytotoxic than those found in patients with CMV-seropositive MS.

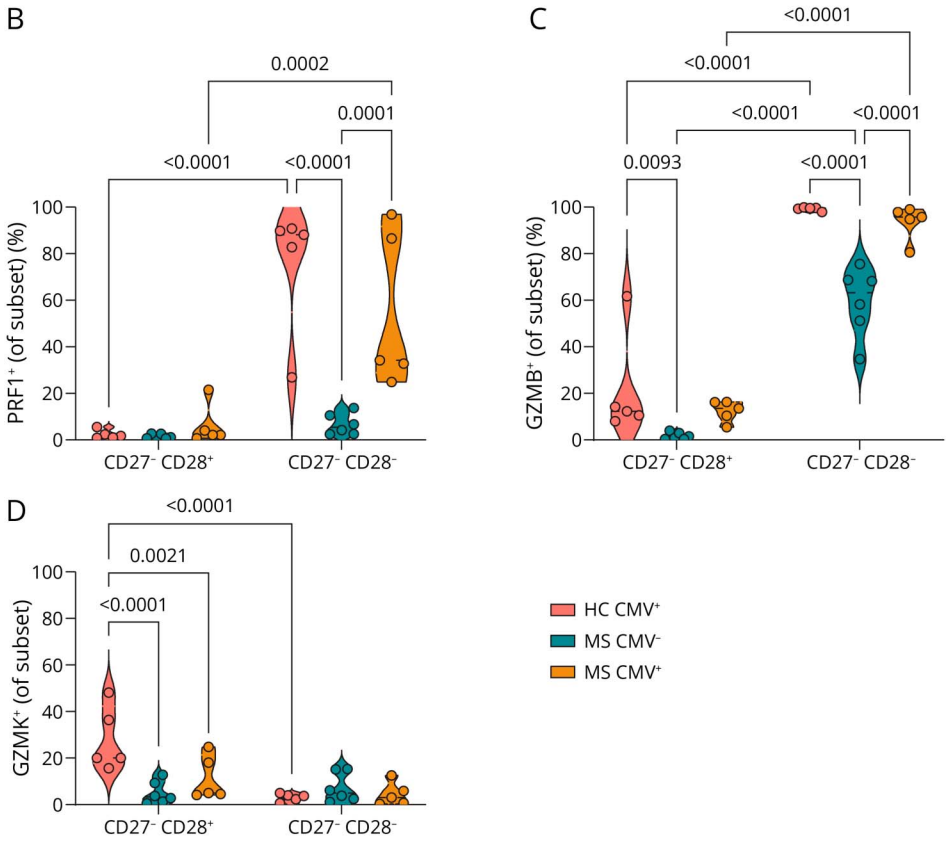
$CD4^+EOMES^+CRTAM^+$ T Cells Infiltrate MS Brain Lesions

Apart from the cytotoxic effector molecules, the transcription factor EOMES has been implicated in development of CD4 CTL[37,38] and is associated with MS severity.[23,30,45] Our initial findings show that a subset of CD4 CTL indeed express the transcription factor EOMES (Figure 1). When analyzing *EOMES* expression in our transcriptomic dataset, we found that its expression was upregulated in the CTL CD28neg Th1 clusters of both HC and MS CMV-seropositive compared with MS CMV-seronegative donors (eFigure 6A). At protein level, using the same cohort as described for Figure 5, C and D and eFigure 5C, expression of EOMES was increased in $CD4^+CD28^-$ T cells from patients with CMV-seropositive MS compared with their $CD28^+$ counterparts and compared with its expression levels in $CD4^+CD28^-$ cells from CMV-seropositive HC and patients with CMV-seronegative MS (Figure 6A).

To date, therapeutic approaches that can specifically target EOMES-expressing CD4 CTL are lacking. We, therefore, next sought for surface markers that were coexpressed with *EOMES* to identify potential therapeutic candidates. We found that gene expression of class I–restricted T-cell–associated molecule

Figure 5 Subpopulations of CD4 CTL Are Distinguished by Differential Expression of Cytotoxic Molecules

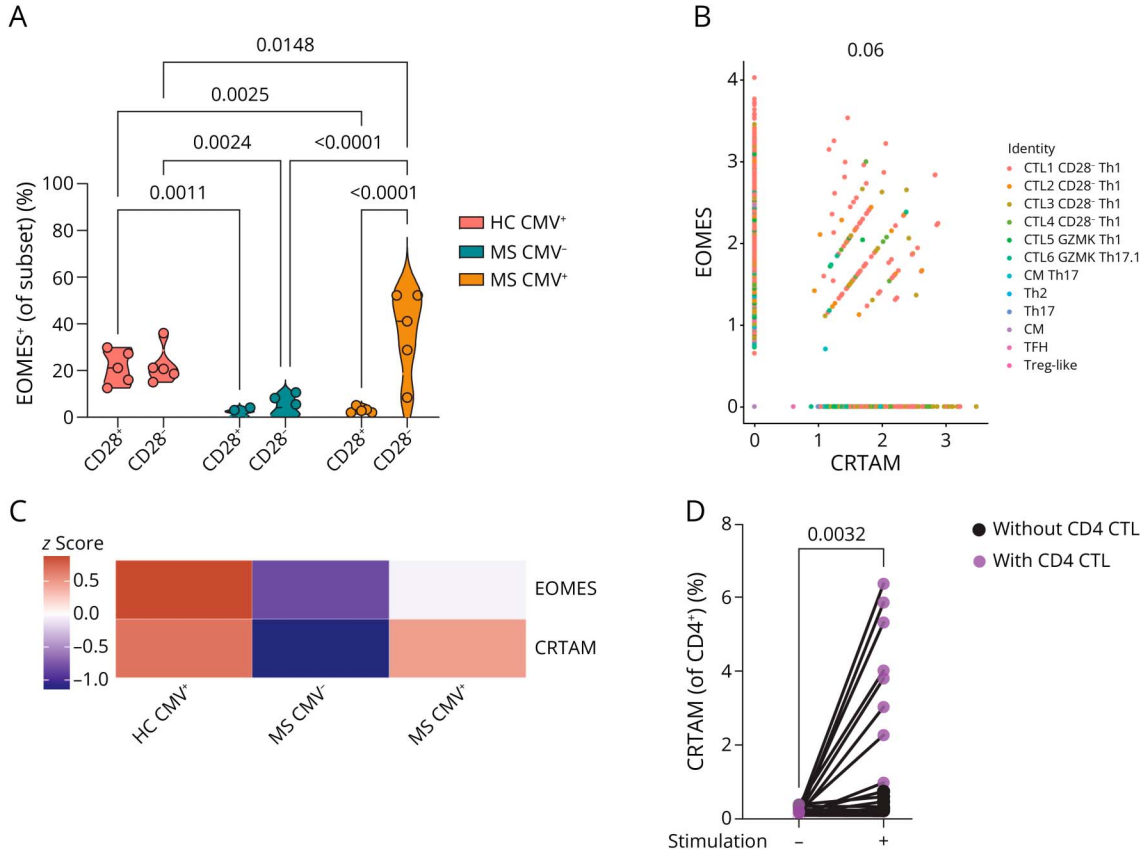




(A) Heatmap showing the gene expression patterns of selected cytotoxic molecules, proinflammatory cytokines, and surface receptors associated with a cytotoxic profile. Relative expression of each marker is shown for each cluster when expression levels are compared across the selected clusters. (B–D) Flow cytometric analysis of ex vivo protein expression of PRF1 (B), GZMB (C), and GZMK (D) in unstimulated PBMC isolated from CMV-seropositive HC donors (n = 5), patients with CMV-seronegative RR-MS (n = 6), or patients with CMV-seropositive RR-MS (n = 5), gated on single, live $CD4^+CD27^-CD28^+$ or $CD4^+CD27^-CD28^-$ cells. Data analyzed with two-way ANOVA and post-hoc Fisher LSD test. CD4 CTL = $CD4^+$ cytotoxic T lymphocyte; GZM = granzyme; RR-MS = relapsing-remitting multiple sclerosis.

(*CRTAM*) was correlated with *EOMES* expression across clusters (Figure 6B) and was restricted to the CTL CD28neg Th1 clusters (eFigure 6C). Expression of *CRTAM* was detected mainly in the CMV-seropositive HC and MS donors (Figure 6C, eFigure 6B). CRTAM is an attractive candidate to target pathogenic EOMES-expressing T cells, as CRTAM is known to be involved in cell-cell adhesion and trafficking of T cells to sites of inflammation,[46,47] and expression of CRTAM is controlled by EOMES in $CD8^+$ T cells.[48,49] In addition, CRTAM is described to interact with its ligand Necl2 and trigger a cytotoxic response in $CD8^+$ T cells and NK cells.[e1-e5] To validate CRTAM functionality in human CD4 CTL further, we first sought to confirm

Figure 6 Expression of the Pathogenic Transcription Factor EOMES Is Increased in CD28⁻ CTL Subsets of CMV-Seropositive MS Donors, and Its Expression Correlates With Expression of the Migratory Receptor CRTAM



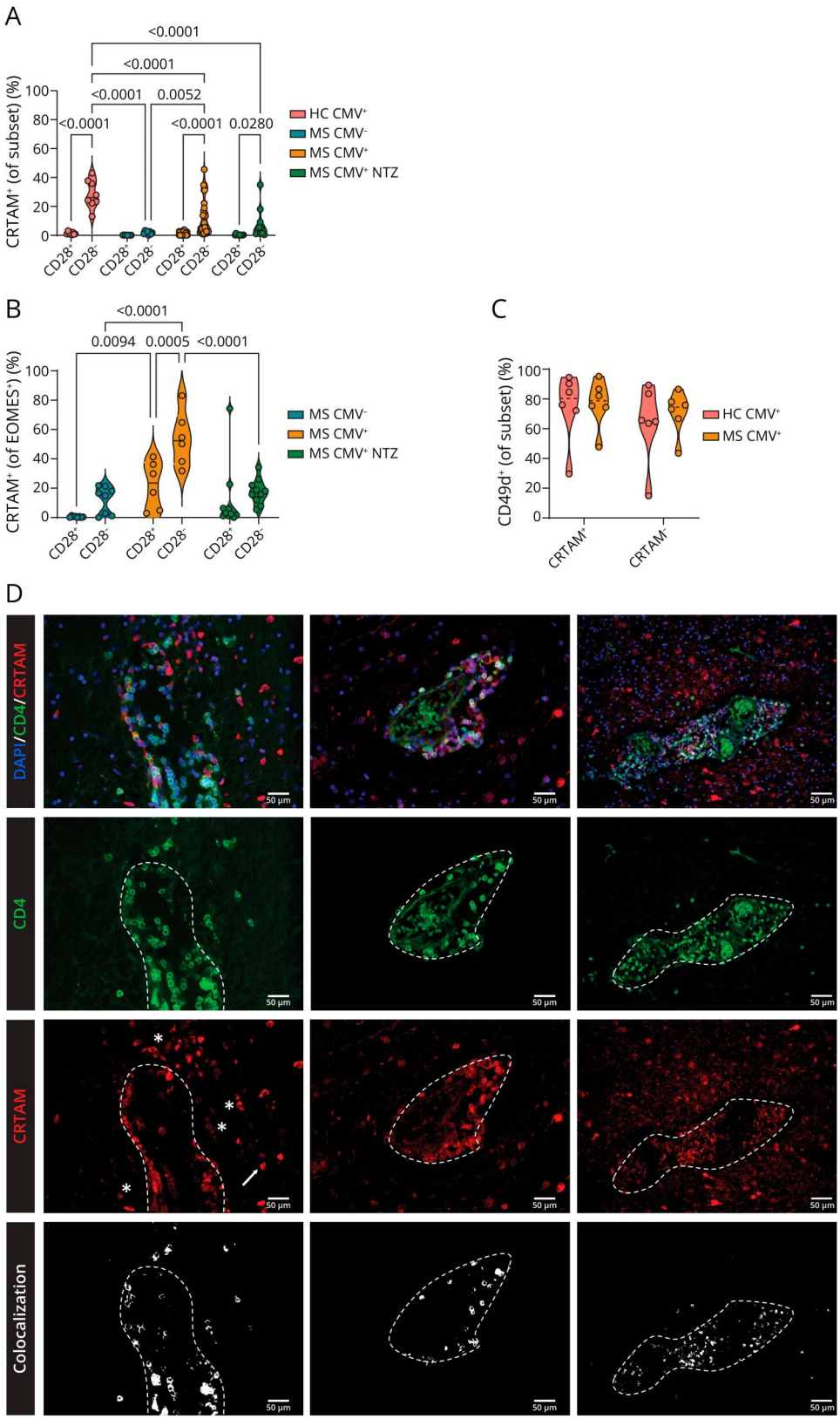
(A) Flow cytometric analysis of ex vivo protein expression of EOMES in unstimulated PBMC isolated from CMV-seropositive HC donors (n = 5), patients with CMV-seronegative RR-MS (n = 6), and patients with CMV-seropositive RR-MS (n = 5), gated on single, live $CD4^+CD27^-CD28^+$ or $CD4^+CD27^-CD28^-$ cells. Data analyzed with two-way ANOVA and post-hoc Fisher LSD test. (B) Scatterplot showing the Pearson correlation between EOMES and CRTAM coexpression within a cell ($r = 0.0558$, $p < 0.0001$). Each dot represents a cell color-coded for each cluster. (C) Heat map visualizing the relative coexpression of EOMES and CRTAM compared between groups. (D) Paired flow cytometry measurements of CRTAM expression on surface of unstimulated and stimulated $CD4^+$ T cells for HC without CD4 CTL expansion (black dots, n = 10) and HC with CD4 CTL expansion (purple dots; n = 8). Data analyzed by Wilcoxon test. CD4 CTL = $CD4^+$ cytotoxic T lymphocyte; CMV = cytomegalovirus; CRTAM = class I–restricted T-cell–associated molecule.

its expression on resting and stimulated $CD4^+$ T cells, as CRTAM is only described in murine $CD4^+$ T cells, where it is transiently upregulated quickly after T cell receptor (TCR) stimulation.[47] Upon 14 hours of stimulation, CRTAM expression was significantly upregulated within the total $CD4^+$ population of HC. As expected, this increase was the highest in CMV-seropositive donors with a preexisting CD4 CTL expansion (Figure 6D). To rule out differences in kinetics of CRTAM upregulation after stimulation between HC and patients with MS, we confirmed that CRTAM expression reaches its peak at 14 hours after treatment in HC as well as MS donors (eFigure 6D).

Next, we investigated differences in CRTAM expression after stimulation in $CD4^+CD28^+$ and $CD4^+CD28^-$ T-cell populations to gain more insight in its pattern of expression. Increased CRTAM expression after stimulation was restricted to the $CD4^+CD28^-$ population of all groups of CMV-seropositive donors (Figure 7A). In this study, we included an additional MS patient cohort of patients with CMV-seropositive MS treated with NTZ. NTZ is a therapy that should confine brain-homing T cells in the peripheral circulation via blockade of CD49d, as was demonstrated for Th17.1 cells.[23,e6] Of interest, in both patients with untreated and NTZ-treated CMV-seropositive MS, the percentage of stimulated $CD4^+CD28^-$ cells expressing CRTAM was lower compared with HC. This suggests that these $CD4^+CD28^-CRTAM^+$ cells have migrated out of the peripheral circulation in patients with CMV-seropositive MS, in spite of NTZ treatment.

To further validate the correlation between *EOMES* and *CRTAM* expression we observed in our transcriptome data, coexpression of CRTAM and EOMES at protein level was determined in stimulated PBMC. In MS donors seropositive for CMV, a large proportion of the $CD4^+CD28^-EOMES^+$ population was found to coexpress CRTAM. Strikingly, the $CD4^+CD28^-EOMES^+CRTAM^+$ population was significantly decreased in patients with CMV-seropositive MS treated with NTZ compared with patients with untreated CMV-seropositive MS (Figure 7B and eFigure 7). This decrease,

Figure 7 CRTAM Is Expressed on a Proportion of Stimulated CD4$^+$CD28-EOMES + Cells, and CD4+CRTAM + Cells Are Found Infiltrating Active MS Lesions



(A) Paired flow cytometry measurements of CRTAM expression on surface of stimulated CD4$^+$CD28$^+$ and CD4$^+$CD28$^-$ T cells for HC (n = 8), patients with CMV-seronegative RR-MS (n = 8), patients with CMV-seropositive RR-MS (n = 30), and patients with CMV-seropositive RR-MS treated with NTZ (n = 10). Data analyzed by two-way ANOVA, post hoc Tukey. (B) Paired flow cytometry measurements of coexpression patterns of EOMES and CRTAM compared between CD4$^+$CD28$^+$ and CD4$^+$CD28$^-$ cells from patients with CMV-seronegative RR-MS (n = 8), patients with CMV-seropositive RR-MS (n = 6), and patients with CMV-seropositive RR-MS that received NTZ treatment (n = 10). Data analyzed by two-way ANOVA, post-hoc Fisher LSD. Representative dot plots for CD4$^+$CD28$^+$ and CD4$^+$CD28$^-$ cells for each group are shown in eFigure 7. (C) Paired flow cytometry measurements of CD49d expression on surface of stimulated CD4$^+$CD28$^-$CRTAM and CD4$^+$CD28$^-$CRTAM$^+$ T cells for CMV-seropositive HC (n = 6) and patients with CMV-seropositive RR-MS (n = 6). Data analyzed by two-way ANOVA, post hoc Fisher LSD. (D) Immunofluorescent stainings of CD4 (green), CRTAM (red), and nuclei (DAPI, blue) in 3 representative active MS lesions are shown. The estimated perivascular cuffs are outlined with a white dotted line, based on morphology. The white asterisk in the CRTAM panel of the first lesion indicates a CD4$^+$CRTAM$^+$ cell, the white arrow indicates a CD4$^-$CRTAM$^+$ cell, likely a CD8$^+$ T cell or NK cell. Colocalization of CD4 and CRTAM was analyzed using ImageJ. Scale bars represent 50 µm. CMV = cytomegalovirus; CRTAM = class I–restricted T-cell–associated molecule; NK = natural killer; NTZ = natalizumab; RR-MS = relapsing-remitting multiple sclerosis.

again suggesting a lack of NTZ effectiveness, could not be explained by an altered expression of CD49d in $CD4^+CRTAM^+$ cells compared with $CD4^+CRTAM^-$ cells (Figure 7C).

Finally, to examine the tissue-infiltrating capacity of $CD4^+CRTAM^+$ T cells, we evaluated their expression in active MS brain lesions (Figure 7D). $CD4^+CRTAM^+$ T cells were detected in 3 out of 9 analyzed lesions. Notably, $CD4^+CRTAM^+$ T cells were detected inside as well as outside perivascular cuffs, supporting their ability to cross the different layers of the blood-brain barrier. Taken together, our data suggest that CD4 CTL expressing EOMES use the migratory receptor CRTAM to escape the peripheral circulation and enter the inflamed CNS of patients with MS, where their cytotoxic cargo can potentially elicit local damage and aggravate MS symptoms.

Discussion

In this study, we established that circulating CD4 CTL are a heterogeneous population comprising of at least 4 functionally different subsets. We further showed that the occurrence of CMV infection is crucial to induce a full-blown cytotoxic CD4 CTL, while the presence of MS disease only partly induced this phenotype. Notably, we identified a particularly pathogenic CD4 CTL subset that is characterized by expression of EOMES and CRTAM, as we found evidence of these cells migrating into the CNS of patients with MS and resisting NTZ treatment.

Circulating CD4 CTL displayed a high ex vivo expression of RUNX3 and T-bet similar to murine CD4 CTL[43,e7] and in vitro differentiated human CD4 CTL.[30] Unsupervised clustering analysis, however, revealed heterogeneity within CD4 CTL, which was confirmed using single cell whole transcriptome analysis (WTA) analysis resulting in 6 CD4 CTL subsets, of which 2 were atypical as these retained CD28 expression and expressed GZMK as main cytotoxic molecule. Two recent studies found an enrichment of GZMK-expressing T follicular helper cells in the CSF of patients with MS.[24, e8] The CTL GZMK clusters from our dataset resemble the pathogenic Th17.1 subset that is prone to migrate to the CNS of patients with MS.[23] Until recently, the main function of granzymes was thought to induce cell death in target cells (such as oligodendrocytes[26]) and that their entry into the target cells is mediated by PRF1.[e9, e10] Recent reports now show that GZMB can enter the target cell independent of PRF1,[e11, e12] and that $CD4^+$ T-cell–derived GZMB induces sublethal injury in astrocytes via caspase activation.[e13] In addition, evidence for cytotoxic and proinflammatory extracellular effects of granzymes is expanding,[e14-e21] suggesting that granzymes secreted by infiltrated CD4 CTL in the CNS of patients with MS contribute to blood-brain barrier (BBB) damage, demyelination, and axonal loss via multiple pathways.

Although it is tempting to assume that the clusters identified in this study each represent a distinct subset of $CD4^+$ T cells, it is likely that at least some of these clusters represent the same functional subset of cells at different stages in differentiation. Given that $CD4^+$ T cells are known to exhibit plasticity to respond appropriately to diverse antigenic threats,[e22] cells might switch between clusters upon encountering specific cues. The difference in expression level of the effector molecule IFN-γ as well as the diverse cytotoxic molecules might indicate that CTL2 CD28neg Th1 and CTL3 CD28neg Th1 are less differentiated CD4 CTL. Several other molecules associated with cytotoxicity were enriched in the CD4 CTL subsets, although not all of our findings are in accordance with literature. Expression of CD56 e.g.,[26, e23] was mostly restricted to CTL2 CD28neg Th1, while CD29 was mostly expressed by non-CTL clusters in contrast with.[e27] Overall, the CD4 CTL clusters showed enrichment of proteins associated with transmigration, such as CD11b,[e28] CX3CR1,[26] and CD49d,[29] while at transcriptome level, especially CTL2 CD28neg Th1 was characterized by a highly active and migratory phenotype, including upregulation of CD69.[e24-e26]

The increase in EOMES expression in CD4 CTL from the MS patient group is of special interest, as EOMES is confirmed to be involved in the pathogenesis of MS as transfer of $CD4^+$ $EOMES^+$ T cells in the chronic phase of experimental autoimmune encephalomyelitis (EAE) exacerbates disease[21,45] and EOMES-expressing $CD4^+$ T cells migrate toward the inflamed CNS of patients with MS.[23,24] This predisposition for migration in EOMES-expressing CD4 CTL is further supported by our findings on the correlation between EOMES and CRTAM expression, previously regarded as a precursor marker for mouse CD4 CTL,[47,e29] and the presence of $CD4^+CRTAM^+$ T cells in active MS lesions. Of interest, $CD4^+EOMES^+CRTAM^+$ T cells were not enriched in the blood of patients with NTZ-treated CMV-seropositive MS, suggesting that these cells employ an alternative way of CNS infiltration, possibly through CRTAM. In line with our findings, a recent study showed the abundance of terminally differentiated $CD4^+$ T cells in the CSF of patients with MS despite NTZ treatment.[25] This subset was characterized by high CD45RA and CX3CR1 expression,[25] similar to the CTL1 CD28neg Th1 cluster we identified here, supporting our hypothesis that $CD4^+EOMES^+CRTAM^+$ T cells escape NTZ treatment. To further provide direct evidence for this, in vitro and in vivo blocking studies are warranted. Taken together, our data suggest that CRTAM is an interesting therapeutic target to specifically block migration of EOMES-expressing CD4 CTL and thereby prevent CD4 CTL–mediated disease progression in patients with CMV-seropositive MS.

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Author Contributions

C Hoeks: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. B. Boeckx: drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data. P. Baeten: major role in the acquisition of data; analysis or interpretation of data. D. Hermans: major role in the acquisition of data; analysis or interpretation of data. J. Randall: major role in the acquisition of data. B. Van Wijmeersch: drafting/revision of the manuscript for content, including medical writing for content. V. Popescu: drafting/revision of the manuscript for content, including medical writing for content. V. Somers: drafting/revision of the manuscript for content, including medical writing for content. P. Stinissen: drafting/revision of the manuscript for content, including medical writing for content. D. Lambrechts: drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data. B. Broux: drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data N. Hellings: drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data.

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Disclosure

The authors declare no competing interests. Go to Neurology.org/NN for full disclosures.

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