Brain-homing CD4⁺ T cells display glucocorticoid-resistant features in MS

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Abstract

Objective

To study whether glucocorticoid (GC) resistance delineates disease-relevant T helper (Th) subsets that home to the CNS of patients with early MS.

Methods

The expression of key determinants of GC sensitivity, multidrug resistance protein 1 (MDR1/*ABCB1*) and glucocorticoid receptor (GR/*NR3C1*), was investigated in proinflammatory Th subsets and compared between natalizumab-treated patients with MS and healthy individuals. Blood, CSF, and brain compartments from patients with MS were assessed for the recruitment of GC-resistant Th subsets using fluorescence-activated cell sorting (FACS), quantitative polymerase chain reaction (qPCR), immunohistochemistry, and immunofluorescence.

Results

An MS-associated Th subset termed Th17.1 showed a distinct GC-resistant phenotype as reflected by high MDR1 and low GR expression. This expression ratio was further elevated in Th17.1 cells that accumulated in the blood of patients with MS treated with natalizumab, a drug that prevents their entry into the CNS. Proinflammatory markers C-C chemokine receptor 6, IL-23R, IFN- γ , and GM-CSF were increased in MDR1-expressing Th17.1 cells. This subset predominated the CSF of patients with early MS, which was not seen in the paired blood or in the CSF from patients with other inflammatory and noninflammatory neurologic disorders. The potential of MDR1-expressing Th17.1 cells to infiltrate brain tissue was confirmed by their presence in MS white matter lesions.

Conclusion

This study reveals that GC resistance coincides with preferential CNS recruitment of pathogenic Th17.1 cells, which may hamper the long-term efficacy of GCs in early MS.

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Glossary

CCR6 = C-C chemokine receptor 6; **EAE** = experimental autoimmune encephalitis; **FACS** = fluorescence-activated cell sorting; **GC** = glucocorticoid; **GM-CSF** = granulocyte-macrophage colony-stimulating factor; **GR** = glucocorticoid receptor; **HPA** = hypothalamus-pituitary-adrenal; **IFN** = interferon; **IL** = interleukin; **MDR1** = multidrug resistance protein 1; **qPCR** = quantitative polymerase chain reaction; **Rh123** = rhodamine 123; **ROR γ t** = RAR-related orphan nuclear receptor γ t; **RPMI 1640** = Roswell Park Memorial Institute 1640 medium; **RRMS** = relapsing-remitting MS; **STAT3** = signal transducer and activator of transcription 3; **T-bet** = T-box transcription factor; **Th** = T helper.

Early MS is characterized by waves of brain-infiltrating immune cells that drive inflammation within the CNS, resulting in demyelination and eventually neurodegeneration.¹ Glucocorticoids (GCs) are broad-spectrum immunosuppressive drugs that are used as standard regimen to dampen acute exacerbations in MS.² Although GCs effectively relieve clinical symptoms, these drugs do not halt subsequent exacerbations or disease progression.³ Of interest, blood cells of patients with relapsing-remitting MS (RRMS) show reduced sensitivity to GCs,^{4,5} indicating a limited window of opportunity for inducing long-term efficacy of this MS treatment. To achieve this, more insights into the underlying mechanisms of GC insensitivity are required in early MS.

On binding its ligand, the glucocorticoid receptor (GR/*NR3C1*) hyperphosphorylates, forms dimers with other nuclear receptors, and translocates into the nucleus.² Within the nucleus, these dimers recognize GR elements in promoter regions or interact with transcription factors to transactivate or repress proinflammatory gene expression.² Apart from binding to the GR, GCs can be excreted out of the cell by the multidrug resistance protein 1 (*MDR1/ABCB1*).⁶

In experimental autoimmune encephalitis (EAE) mice, the therapeutic efficacy of GCs depends on the suppression of CNS recruitment of proinflammatory CD4⁺ T helper (Th) cells.^{7,8} Because GCs are unable to induce long-term protective effects in MS, it is tempting to speculate that certain pathogenic Th cells avoid GC-mediated suppression and thereby contribute to recurrent disease activity.⁹ C-C chemokine receptor 6 (CCR6) expression on memory Th cells promotes their recruitment into the CNS and is essential for the induction of EAE.^{10,11} In patients with RRMS, blood-derived CCR6⁺ and not CCR6⁻ memory Th cells show increased proinflammatory capacity in response to myelin peptides.¹² Previously, our group revealed that a human pathogenic CCR6⁺ Th subset commonly termed Th17.1 (interleukin (IL)-17^{low}; interferon (IFN)- γ ^{high} granulocyte-macrophage colony-stimulating factor (GM-CSF)^{high}) is associated with early MS.^{13–17} Of interest, we and others found that Th17.1 cells from healthy individuals express high levels of *ABCB1*,^{13,15} which encodes for MDR1 and links to the reduced GC sensitivity found in patients with RRMS.⁴

In this study, we addressed how MDR1 and GR are expressed among memory Th subsets including Th17.1 and whether this coincides with their potential to infiltrate the CNS of patients with early MS.

Methods

Patients and sampling

Treatment-naïve patients with clinically isolated syndrome and RRMS were diagnosed based on the McDonald 2017 criteria and included at the MS Center ErasMS, Erasmus MC. We collected fresh CSF and blood from these patients and patients with noninflammatory and other inflammatory neurologic diseases (table 1). Postmortem blood, meninges, and white matter tissues were obtained from autopsied MS donors (Netherlands Brain Bank, Amsterdam; table 1) and freshly processed as previously described.¹⁸ Furthermore, blood samples were collected from patients with RRMS treated with natalizumab for 12 months and frozen down until further use. The use of primary material and experimental procedures were approved by the medical ethics committee of each respective center.

Antibodies and flow cytometry

The anti-human monoclonal antibodies used for flow cytometry are described in supplementary table 1A (links.lww.com/NXI/A325). For MDR1 surface staining, cells were taken up in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific) containing 2% fetal calf serum and 25 μ M cyclosporin A (Sigma-Aldrich, St Louis, MO), which allows for a conformational change exposing the epitope¹⁹ or absolute ethanol (Merck, Schiphol-Rijk, The Netherlands) as a vehicle control. Subsequently, MDR1 (Biolegend, London, UK) antibody was added, and cells were incubated for 20 minutes at 37°C and 5% CO₂. Cells were stained using Fixable Viability (L/D) Stain 700 (BD Biosciences, Erembodegem, Belgium), eFluor 506, or eFluor 520 (Thermo Fisher Scientific, Landsmeer, The Netherlands) in the dark for 15 minutes at 4°C. Other surface markers were stained in the dark for 30 minutes at 4°C. Cy5.5-Annexin V (BD Biosciences) was added in the presence of calcium chloride to define early apoptotic (Annexin V⁺Live/Dead⁻) and late apoptotic (Annexin V⁺Live/Dead⁺) memory CD4⁺ Th cells. Cells were measured using the LSRII-Fortessa or FACSARIA-III flow cytometer and analyzed using FACSDiva software (Version 8.0.1; BD Biosciences).

Cell sorting

CD4⁺ cells were isolated from fresh healthy blood donors (Sanquin, Amsterdam, The Netherlands) using CD4 microbeads and the autoMACS Pro Separator (both Miltenyi Biotec, Bergisch Gladbach, Germany) and were frozen down until further use. After thawing, CCR6⁺ memory Th populations (CD3⁺CD4⁺CD8⁻CD25^{low}CD45RA⁻CD45RO⁺) were isolated using a FACSARIA-III machine. CCR6⁺ memory Th

Table 1 Patient characteristics

Peripheral blood	Ex vivo analysis			In vitro analysis ^a	
Cohort	HC	RRMS, NTZ treated ^{b,c}		HC	RRMS, NTZ treated ^{b,c}
Individuals, n	9	18		5	11
Females, n (%)	4 (44)	13 (72)		3 (60)	8 (72)
Age in years, median (range) ^d	47 (23–53)	36 (19–53)		46 (29–62)	36 (19–49)
Disease duration in months, median (range) ^e	NA	100 (12–202)		NA	59 (12–151)
CNS compartment	CSF, ex vivo			Brain/meninges, ex vivo	Brain tissue, in situ
Cohort	CIS ^{b,f}	RRMS ^{b,f}	NIND/OIND ^{g,f}	MS (postmortem)	MS (postmortem)
Patients, n (paired blood)	4 (4)	11 (10)	8 (4)	8 (5)	3
Females, n (%)	2 (50)	5 (45)	5 (63)	6 (86)	2 (67)
Age in years, median (range) ^d	38 (25–42)	32 (18–40)	57 (26–68)	65 (51–70)	49 (48–58)
Follow-up time in months, median (range)	11 (1–22)	6 (2–56)	NA	NA	NA
Disease duration in months, median (range) ^e	3 (0–4)	3 (0–55)	NA	NA	NA
PMD in hours, median (range) ^h	NA	NA	NA	7:08 (5:10–8:20)	09:20 (08:30–10:45)

Abbreviations: CIS = clinically isolated syndrome; HC = healthy control; MDR1 = multidrug resistance protein 1; NA = not applicable or available; NIND = noninflammatory neurologic disease; NTZ = natalizumab; OIND = other inflammatory neurologic diseases; PMD = postmortem delay; RRMS = relapsing-remitting MS.

^a Rhodamine efflux and MDR1 shift assay.

^b Diagnosis according to the McDonald 2017 criteria.

^c Ex vivo analyses: 11 clinical responders and 7 clinical nonresponders. In vitro analyses: 5 clinical responders and 6 clinical nonresponders.

^d At the time of sampling.

^e Time from CIS or if applicable RRMS diagnosis to sampling.

^f Patients did not receive glucocorticoids before sampling.

^g Diagnosis: myelopathy, ulnar neuropathy, neuro-Behçet disease, neurosarcoidosis, B-cell lymphoma, vasculitis, capillary telangiectasia, and neurosyphilis.

^h Depicted as hour:minutes.

subsets were further defined based on differential expression of CXCR3 and CCR4; Th17 (CCR6⁺CXCR3⁺CCR4⁺), Th17 double-positive (“DP”; CCR6⁺CXCR3⁺CCR4⁺) and Th17.1 (CCR6⁺CXCR3⁺CCR4^{−/dim}).¹⁴ Freeze-thawing effects on CCR6 and CXCR3 expression are displayed in supplementary figure 1A (links.lww.com/NXI/A323).

RNA isolation and quantitative PCR

RNA isolation, complementary DNA synthesis, and real-time quantitative PCRs were performed as previously described.¹³ Primer-probe sets were designed using the Universal ProbeLibrary (Roche Applied Science, Penzberg, Germany). Apart from *IFNG* and *CSF2*, which were measured after Phorbol 12-myristate 13-acetate and ionomycin stimulation, gene expression was measured in unstimulated cells.¹³ An overview of all used primer sequences can be found in supplementary table 1B (links.lww.com/NXI/A325). RNA samples containing less than 75 ng total RNA were excluded from the analysis.

In vitro proliferation and apoptosis assay

CD4⁺ cells from healthy blood donors were thawed and labeled with 0.075 μM CellTrace carboxyfluorescein succinimidyl ester according to the manufacturer’s instructions (Thermo Fisher Scientific). After washing, Th17 and Th17.1 memory cells were purified using FACS, as described above. Sorted Th17 and Th17.1 cells were plated at 2.5 × 10⁵ cells/mL

and activated with aCD3/CD28 dynabeads (1:5; Thermo Fisher Scientific) for 3 days. Cells were cultured in RPMI 1640 supplemented with 1% penicillin/streptomycin (Lonza, Verviers, Belgium), 5% human AB serum (Sanquin), and 75 μM methylprednisolone sodium succinate (Pfizer, Capelle a/d IJssel, The Netherlands) or a vehicle control. A similar concentration is given as IV pulse therapy to dampen acute MS relapses.²⁰ To compare differences in proliferation, the frequencies of viable, carboxyfluorescein succinimidyl ester-negative cells were analyzed using flow cytometry. For the apoptosis assay, CD4⁺ cells were plated out, activated, and exposed to methylprednisolone in a similar manner as described above. As a positive control for apoptosis induction, 1 × 10⁶ healthy donor peripheral blood mononuclear cells were exposed to a temperature of 65°C for 20 minutes, put on ice, and mixed with the same amount of nonexposed cells.

Rhodamine efflux and MDR1 shift assay

We combined a rhodamine 123 (Rh123) efflux and MDR1 shift assay to measure both the efflux potential and MDR1 expression of each Th17 subset.^{15,19} Peripheral blood mononuclear cells were thawed and resuspended in RPMI 1640 containing 2% fetal calf serum. Cells were resuspended at a concentration of 1 × 10⁷ cells/mL and were incubated with Rh123 (0.5 μg/mL) or a vehicle control (ethanol; both Sigma-Aldrich) in the dark and on ice for 30 minutes. Cells were

washed and supplemented with and without cyclosporin A (25 μ M). Next, MDR1 antibody (supplementary table 1A, links.lww.com/NXI/A325) was added to all tubes for 2 hours at 37°C and 5% CO₂, after which cells were stained with Th17 subset-defining antibodies as described above. In this way, we could analyze the efflux potential in one tube (Rh123 without cyclosporin A) and both the blocking of this efflux and MDR1 surface expression in another tube (Rh123 with cyclosporin A) for each subset.

In vitro transmigration assay

Memory Th cells (CD3⁺CD8⁻CD25^{low}CD45RA⁻) were separated from fresh, MACS-sorted CD4⁺ cells using FACS. Trans-endothelial migration was assessed using 5 × 10⁵ memory Th cells and transwell plates coated with a confluent monolayers of human brain endothelial cells (hCMEC/D3 cell line) as previously described.^{13,21,22} Migration was performed for 4 hours at 37°C and 5% CO₂. All experiments were performed in duplicate. The average percentages of CCR6⁺ Th subsets were compared before and after migration.

Immunohistochemistry and multiplex immunofluorescence

Immunohistochemistry was performed with an automated, validated, and accredited staining system Ventana Benchmark ULTRA using the OptiView Universal DAB Detection Kit (both Ventana Medical Systems, Oro Valley, AZ). In brief, after deparaffinization and heat-induced antigen retrieval, 7- μ m-thick brain sections were incubated with the antibody of interest for 32 minutes. This was followed by a hematoxylin II counter staining for 12 minutes and the addition of a blue coloring reagent for 8 minutes according to the manufacturer's instructions (both Ventana Medical Systems). To assess MDR1 expression on Th cells, immunofluorescent staining for MDR1 and CD4 was performed using the automated multiplex platform Benchmark Discovery (Ventana Medical Systems). In short, brain sections were deparaffinized and antigen retrieved with cell conditioning 1 (Ventana Medical Systems) for 32 minutes. Tissue slides were incubated with MDR1 antibody for 32 minutes at 37°C, followed by detection with fluorescein amidite (Roche Applied Science). Antibody denaturation was performed using CC2 (Ventana Medical Systems) for 8 minutes at 100°C. Subsequently, CD4 antibody was incubated for 32 minutes at 37°C, followed by detection with Cy5 (Roche Applied Science). Finally, slides were washed in phosphate-buffered saline and mounted with Vectashield containing 4',6-diamidino-2-phenylindole (Vector laboratories, Peterborough, UK). For detailed information of the used antibodies, see supplementary table 1A (links.lww.com/NXI/A325).

Statistical analysis

Statistical tests were performed using GraphPad Prism 5 software and are described in each figure legend. Results are displayed as individual data points with/without the standard error of the mean or as a box and whiskers plot. For all tests, a *p* value of <0.05 (*) was considered significant.

Data availability

On request from qualified investigators, any acquired data not published within this article are available. If desired, please contact the corresponding author of this article.

Results

High *ABCB1* and low *NR3C1* expression defines GC-resistant Th17.1 cells with enhanced proinflammatory capacity

GC sensitivity is determined by the expression of *ABCB1* (MDR1) and *NR3C1* (GR) (figure 1A). Using an MDR1 shift assay, we found that MDR1 was predominantly expressed on CCR6⁺ vs CCR6⁻ memory Th cells within the blood of healthy individuals (*p* < 0.001; figure 1, B and C), indicating that high MDR1 expression is not associated with Th1 cells. After subdivision of CCR6⁺ Th cells into functionally distinct subsets based on CXCR3 and CCR4 expression, MDR1 was abundant on Th17.1 (CCR6⁺CXCR3⁺CCR4^{-dim}; IL-17^{low}IFN- γ ^{high}GM-CSF^{high}) compared with Th17 (CCR6⁺CXCR3⁻CCR4⁺; IL-17^{high}IFN- γ ^{neg}GM-CSF^{dim}) and Th17 DP (CCR6⁺CXCR3⁺CCR4⁺; IL-17^{dim}IFN- γ ^{low}GM-CSF^{dim}) cells^{13,14} from the same blood donors (*p* < 0.001 and *p* < 0.01; figure 1, D and E). Subsequently, we sorted these populations and analyzed coexpression of MDR1 (*ABCB1*) with GR (*NR3C1*). *NR3C1* was selectively down-regulated in Th17.1 cells (*p* < 0.05; figure 1F), resulting in strongly elevated *ABCB1*/*NR3C1* expression ratios (*p* < 0.001; figure 1G). In vitro experiments confirmed that proliferating Th17.1 cells and MDR1⁺ fractions in particular were less sensitive to methylprednisolone compared with paired Th17 cells (figure 1H). This is probably not related to apoptotic effects because methylprednisolone hardly induced early and late apoptosis of memory Th cells under similar conditions (supplementary figure 2A, links.lww.com/NXI/A323).^{23,24} Th17.1-associated genes IL-23 receptor (*IL23R*), IFN- γ (*IFNG*), and GM-CSF (*CSF2*)^{13,14} displayed elevated expression in MDR1⁺ vs MDR1⁻ fractions of Th17.1 (*p* < 0.01 vs *p* < 0.05, respectively; figure 1I). In contrast to DNAX accessory molecule 1, expression levels of adhesion molecules P-selectin glycoprotein ligand 1 and very late antigen 4 were increased on MDR1⁺ vs MDR1⁻ Th17.1 cells (see supplementary figure 1B, links.lww.com/NXI/A323). These findings show that Th17.1 cells have a distinctive GC-resistant phenotype, which probably contributes to their role in MS disease activity.¹³

Th17.1 cells trapped in the blood of natalizumab-treated patients with MS show increased *ABCB1* and reduced *NR3C1* expression

In our previous study, Th17.1 cells were found to selectively accumulate in the blood from patients with MS who clinically responded to natalizumab treatment.¹³ This peripheral entrapment makes it possible to analyze the GC resistance profile of Th17.1 cells that infiltrate the CNS during early MS. After sorting of these and other CCR6⁺ memory Th cells from the blood, we found selectively increased *ABCB1* expression in Th17.1 cells from 11 patients with RRMS who clinically responded to natalizumab treatment vs 9 age- and sex-matched

Figure 1 High *ABCB1* and low *NR3C1* expression in Th17.1 cells from healthy blood donors

(A) Simplistic illustration of glucocorticoid regulation within an immune cell. GCs diffuse through the plasma membrane and bind to GR (*NR3C1*) within the cytoplasm. On binding, GRs form dimers and translocate into the nucleus to regulate proinflammatory gene expression. However, GCs can also be transported out of the cell by MDR1 (*ABCB1*). (B) Representative FACS dot plots with the gating strategy and MDR1 surface expression for thawed CCR6⁺ and CCR6⁺ memory Th (CD3⁺CD4⁺CD8⁺CD25⁺CD45RA⁺) cells. (C) Frequencies of MDR1-expressing cells within paired CCR6⁺ and CCR6⁺ memory Th cells from healthy blood donors (n = 6). Data were compared using paired t tests. (D and E) Representative gating, percentages, and median fluorescence intensity (MFI) of MDR1 expression for MDR1-expressing cells within each CCR6⁺ Th subset. Cells were obtained from 6 healthy blood donors and analyzed using a 1-way analysis of variance (ANOVA) with a Newman-Keuls multiple comparison test. Relative expression of *NR3C1* (F) and their *ABCB1/NR3C1* ratios (G) were analyzed for paired Th17, Th17 DP, and Th17.1 cells using qPCR (n = 7–8). Data were compared using a repeated measurement 1-way ANOVA with a Newman-Keuls multiple comparison test. (H) In vitro effects of methylprednisolone (MP; 75 μM) on the proliferation of Th17 and Th17.1 cells (left) and MDR1⁺ and MDR1⁺ fractions of Th17.1 (right) of 6 healthy blood donors. The percentage of CFSE-labeled cells was compared with vehicle controls after anti-CD3/CD28 stimulation for 3 days. Data were compared using paired t tests. (I) *IL23R* (IL-23 receptor), *IFNG* (IFN-γ), and *CSF2* (GM-CSF) expression relative to 18S in paired MDR1⁺ vs MDR1⁺ Th17.1 cells from 6 to 8 healthy donors. Data were analyzed using Wilcoxon and paired t tests. *p < 0.05, **p < 0.01, ***p < 0.001. CCR6 = C-C chemokine receptor 6; GC = glucocorticoid; GR = glucocorticoid receptor; MDR1 = multidrug resistance protein 1; Th = T helper.

healthy controls ($p < 0.05$; figure 2A). This was not found in patients who experienced clinical relapses despite natalizumab therapy (nonresponders; n = 6; figure 2A). Despite the fact that all nonresponders were female, sex did not affect expression profiles within the whole group of patients and controls (data not shown). *NR3C1* was reduced in all CCR6⁺ Th subsets

analyzed from these patients, which was only significant in nonresponders and mainly found in Th17.1 (figure 2A). As a result, *ABCB1/NR3C1* expression ratios were enhanced especially in natalizumab responders compared with healthy controls (figure 2A). Although the frequencies of MDR1⁺ Th17.1 cells were elevated in the responders ($p < 0.05$), we did not find

differences in MDR1 surface expression (supplementary figure 1C, links.lww.com/NXI/A323) or Rh123 dye efflux (figure 2, B and C) for Th17.1 cells between these groups. CSF-homing marker CCR6 was higher expressed on MDR1⁺ vs MDR1⁻ Th17.1 cells from the blood of natalizumab-treated patients with MS ($p < 0.0001$), which was not seen for CXCR3 (figure 2D). These data show that GC-resistant Th17.1 cells have a phenotype associated with preferential recruitment to the CSF in early MS.

MDR1⁺ Th17.1 cells are enriched in the CSF of patients with early MS and not in patients with other neurologic disorders

Ex vivo flow cytometric analysis of paired blood and CSF samples from treatment-naïve patients with early MS ($n = 9$, table 1) revealed a significant rise in MDR1-expressing CD4⁺ ($p < 0.0001$) and not CD8⁺ T memory cells, resulting in increased CD4/CD8 ratios ($p < 0.001$) within the CSF (figure 3A). CCR6-expressing memory Th cells were increased in paired CSF vs blood samples from patients with early MS ($p < 0.0001$; supplementary figure 1D, links.lww.com/NXI/A323) and expressed higher surface levels of MDR1 than CCR6⁻ memory fractions in MS CSF ($p < 0.0001$; figure 3B). Th17.1 predominated the CSF of patients with early MS compared with Th17 and Th17 DP cells ($n = 15$, table 1 and $p < 0.001$, figure 3C). This selective enrichment was not found in paired blood samples or in the CSF of 8 patients with other inflammatory or noninflammatory neurologic disorders (table 1 and figure 3C). The predominance of Th17.1 cells in early MS CSF was even more apparent when analyzing MDR1-expressing proportions ($p < 0.001$; figure 3D). Within the blood of these patients, CCR6 and not CXCR3 was enriched on MDR1⁺ compared with MDR1⁻ Th17.1 cells ($p < 0.0001$ and $p < 0.01$; figure 3E). MDR1⁺ Th17.1 cells in the CSF showed higher CXCR3 levels than their counterparts in the blood ($p < 0.05$; figure 3E). The enhanced recruitment of MDR1⁺ Th17.1 cells to early MS CSF shows that this subset preferentially migrates across blood-CNS barriers, which was confirmed in vitro (supplementary figure 2B and C, links.lww.com/NXI/A323).

MDR1-expressing CD4⁺ T cells are recruited to MS brain tissue and show a Th17.1 phenotype

To study whether MDR1^{high} Th17.1 cells also infiltrate the inflamed MS brain, we first performed in situ analyses of postmortem white matter tissues from 3 late-stage MS donors showing a high number of perivascular infiltrates. Immunohistochemical analysis for MDR1 and CD4 revealed their coexistence in perivascular areas of active lesions (figure 4, A and B). Besides the expected presence of MDR1 in other CNS-resident cells,²⁵ we were able to validate the coexpression of MDR1 with CD4 using confocal microscopy (figure 4C). To address how MDR1 was expressed among MS brain-infiltrating CCR6⁺ Th subsets, we analyzed ex vivo single-cell suspensions of postmortem blood, meninges, and white matter tissues from 8 late-stage MS donors using flow cytometry (figure 4, D and E). Although a

trend increase was seen for the amount of CCR6⁺ memory Th cells in the brain and meninges compared with blood, this was not significant (Supplementary figure 1E, links.lww.com/NXI/A323). CXCR3⁺ memory Th cells were enriched in brain vs meningeal and blood samples and showed highest MDR1 expression in brain tissues ($p < 0.05$; Supplementary figure 1F, links.lww.com/NXI/A323). CD8⁺ memory T cells showed a similar trend, yet not significant (Supplementary figure 1G, links.lww.com/NXI/A323). In contrast to Th17 and Th17 DP cells, frequencies of MDR1⁺ Th17.1 cells were significantly increased in brain tissue compared with meninges ($p < 0.05$) and blood ($p < 0.0001$; both figure 4E). Although not significant, Th17.1 also seemed to be more present in the meninges than in the blood and were more abundant than Th17 DP cells in brain tissues. Overall, these findings support the use of MDR1 as a marker to delineate CNS-homing, potentially pathogenic Th cells in patients with early MS.

Discussion

CSF from patients with early MS is characterized by increased numbers of CD4⁺ and not CD8⁺ T cells.^{18,26} Because GCs are used to dampen acute MS relapses, we examined GC resistance features among different pathogenic CD4⁺ Th cell subsets during early MS. Our results demonstrate that Th17.1 cells display a unique GC-resistant (*ABCB1*^{high}*NR3C1*^{low}) phenotype, which is accompanied by a selective enrichment in the CSF from patients with early MS and not in patients with other neurologic diseases. The fact that this potentially GC-resistant Th subset is also localized in MS white matter lesions puts Th17.1 cells and particularly MDR1 forward as a promising target for predicting and boosting GC treatment efficacy in early MS.

Several factors may be responsible for the selective upregulation of *ABCB1* (MDR1) and downregulation of *NR3C1* (GR) in Th17.1 cells. A pathogenic feature of MS is chronic activation of the hypothalamus-pituitary-adrenal (HPA) axis, resulting in elevated levels of the natural GC cortisol.⁵ Because GCs can also upregulate MDR1²⁷ and downregulate GR,^{28–30} such increased levels may trigger GC-resistant, brain-homing Th17.1 cells in MS. Chronic inflammation was also found to upregulate MDR1 expression on lymphocytes via activation of signal transducer and activator of transcription 3 (STAT3) protein.³¹ Consistently, STAT3 drives the expression of RAR-related orphan nuclear receptor γ t (ROR γ t), which inhibits GC action³² and is highly abundant in Th17.1 cells.¹³ This probably also explains our finding that the MDR1⁺ subset of Th17.1 expresses high levels of GM-CSF, which is directly controlled by ROR γ t.³³ Of note, steroid receptor coactivators, which control GR responses,³⁴ have been recently linked to ROR γ t-dependent pathogenic Th17 differentiation.³⁵ In addition, IL-2 is not only an important trigger of GM-CSF in Th memory cells from patients with MS³⁶ but is also able to reduce GR expression.³⁷ In EAE,

Figure 2 Increased frequencies of MDR1⁺Th17.1 cells in the blood of natalizumab-treated patients with MS compared with healthy controls

(A) *ABCB1*, *NR3C1*, and *ABCB1*/*NR3C1* expression ratios for Th17, Th17 DP, and Th17.1 cells from thawed peripheral blood mononuclear cells of patients with MS who clinically responded ($n = 10-11$) or did not respond ($n = 5-7$) to natalizumab treatment and age- and sex-matched healthy controls ($n = 6-9$). Data were analyzed using Kruskal-Wallis tests. (B) Representative FACS plot showing Rh123 efflux in Th17, Th17 DP, and Th17.1 cells. (C) Frequencies of MDR1⁺ Th17, Th17 DP, and Th17.1 cells within Th cells (top) and percentages of Rh123⁻ cells within each subset (bottom) for 11 natalizumab-treated patients with MS and 5 healthy controls. Data were compared using Kruskal-Wallis tests. (D) CCR6 and CXCR3 expression (MFI) on MDR1⁻ vs MDR1⁺ Th17.1 cells from 11 natalizumab-treated patients with MS. Data were analyzed using paired t tests. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. HC = healthy control; MDR1 = multidrug resistance protein 1; NR = did not respond; NTZ = natalizumab; R = responded; Rh123 = rhodamine 123; Th = T helper.

Figure 3 Selective enrichment of MDR1+Th17.1 cells in the CSF of treatment-naïve patients with early MS

(A) Percentages and ratios of MDR1-expressing CD4⁺ and CD8⁺ memory fractions within CD3⁺ T cells in fresh blood and CSF samples from patients with early MS (n = 9). Data were analyzed using paired *t* tests. (B) Percentages of MDR1-expressing cells within paired CCR6⁻ and CCR6⁺ memory Th cells from early MS CSF samples (n = 9). Data were analyzed using paired *t* tests. (C) Top panel: percentages of CCR6⁺ subsets within the memory Th pool in the blood (left panel, n = 14) and CSF (right panel, n = 15) of patients with early MS. Bottom panel: same items displayed for patients with NIND or OIND (see table 1; n = 4 for blood and n = 8 for CSF). Data were compared by a repeated measurement 1-way ANOVA with a Newman-Keuls multiple comparison test. (D) Percentages of MDR1⁺ CCR6⁺ Th subsets in the CSF (left) and MDR1⁺ Th17.1 frequencies in the CSF vs blood (right) from patients with early MS (n = 9–10). Data sets were analyzed using a 1-way ANOVA with a Newman-Keuls multiple comparison test and a paired *t* test, respectively. (E) CXCR3 expression (MFI) on CSF- and blood-derived MDR1⁺ Th17.1 cells from patients with early MS (n = 7). (E) Data were analyzed using paired *t* tests. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. CCR6 = C-C chemokine receptor 6; MDR1 = multidrug resistance protein 1; NIND = noninflammatory neurologic disease; OIND = other inflammatory neurologic disease; Th = T helper.

macrophage migration inhibitor factor induced GC resistance of Th cells via upregulation of T-box transcription factor (T-bet),³⁸ another Th17.1-associated transcription factor.¹³ In parallel with this, we found that T-bet-dependent IFN- γ was upregulated in MDR1⁺ Th17.1 cells. Together, these studies indicate that the GC-resistant phenotype of Th17.1 may be induced by combined effects of a hyperactive HPA axis and chronic inflammation in patients with MS.

For MDR1, transcript levels were selectively increased in Th17.1 cells that accumulated in the blood from clinical responders to natalizumab. This also corresponds to the increased very late antigen 4 expression on MDR1⁺ vs MDR1⁻ Th17.1 cells. MDR1 surface expression was not increased on Th17.1 cells from responders. One limitation of this study is that we did not analyze total protein levels of MDR1, which could explain this discrepancy. Furthermore, posttranslational

Figure 4 Presence of MDR1+Th17.1 cells in postmortem white matter tissue of patients with MS

Representative immunohistochemical staining for CD4 and MDR1 (A) as well as human leukocyte antigen II (HLA-II), myelin oligodendrocyte glycoprotein (MOG), microtubule-associated protein 2 (MAP2) (B) in formalin-fixed, paraffin-embedded white matter lesions of 3 MS donors. Images were taken with a $\times 20$ digital magnification, and lesions were classified as active and postdemyelinating (MS-1), active and demyelinating (MS-2), and mixed active/inactive and demyelinating (MS-3).⁴⁸ (C) Coexpression of CD4 (red) and MDR1 (green) in 4',6-diamidino-2-phenylindole (blue)-positive cells in these brain tissues, as determined by immunofluorescence staining. (D) Representative gating of ex vivo CCR6⁺ Th subsets and MDR1⁺ fractions of Th17.1 (CCR6⁺CXCR3⁺CCR4^{-dim}) in the blood, meninges, and brain tissue from the same MS donor. (E) Frequencies of MDR1⁺ Th17, Th17 DP, and Th17.1 cells in freshly isolated single-cell suspensions from postmortem blood, meninges, and brain tissues from 8 late-stage MS donors, as determined by FACS. A total of 14 brain tissues from 8 donors were analyzed. Data were compared using a nonrepeated measurement 2-way ANOVA with a post hoc Bonferroni multiple comparison test. * $p < 0.05$ and **** $p < 0.0001$. MDR1 = multidrug resistance protein 1; Th = T helper.

regulation of MDR1 such as altered intracellular trafficking and localization may be different, especially after induction by cyclosporin A.³⁹ For GR, expression levels were downregulated in all CCR6⁺ Th subsets from the blood of natalizumab responders and were further reduced in nonresponders. A possible explanation for this is that HPA axis hyperactivity is more pronounced in patients with progressive MS such as patients

not responding to natalizumab, thus decreasing GR levels.⁴⁰ Another limitation of this study is that the frequencies and phenotype of Th17.1 were not analyzed before natalizumab treatment, which possibly differs between responders and nonresponders. GR can also be alternatively controlled post-transcriptionally, but remains a challenge to detect at the protein level, let alone in distinct Th subsets.

Apart from the role of MDR1 and GR in GC resistance, both molecules also play a critical role in the induction of neuroinflammation. Increased GR signaling in pathogenic T cells protects from CNS autoimmunity, whereas deletion of GR abrogates this phenomenon in EAE.^{8,41} Deletion of *ABCB1* resulted in a similar protective phenotype.⁴² These studies at least suggest that the low GR and high MDR1 expression in brain-homing Th17.1 drives neuroinflammation. Quantification of in situ localization of MDR1⁺ Th cells also for patients with other neurologic diseases should be performed to better interpret their role in MS brain pathology. Because of their involvement in GC resistance and local inflammation, MDR1 and GR are promising targets to improve GC efficacy in early MS. The need for improving this efficacy is underlined by the increased MDR1-mediated efflux ratio of methylprednisolone,⁴³ the standard treatment of acute MS relapses.

A recent study showed that low vitamin D levels are associated with GC-resistant relapses in patients with MS.²⁰ Although controversial results were obtained from clinical trials with respect to disease activity, increasing vitamin D levels improved GC efficacy and suppressed EAE induction in mice via Th-cell intrinsic upregulation of *NR3C1*.²⁰ Because vitamin D also downregulates CCR6 on Th cells,¹⁷ this steroid hormone may be exploited to enhance GC efficacy in MS by sensitizing Th17.1 cells. MDR1 activity can also be blocked by other types of steroids⁴⁴ and even by anti-CD20 antibodies.⁴⁵ The selective targeting of MDR1 is further supported by its additional role in proinflammatory cytokine excretion⁴⁶ and trafficking⁴⁷ of T cells across the blood-brain barrier. However, the implementation of such strategies should also be taken with care because MDR1 is expressed by many other cell types. In-depth analysis of Th17.1 cells by currently available single-cell platforms would reveal additional targets that can be used for the design of small-molecule therapeutics. The predominance of Th17.1 in the CSF of patients with early MS could set the stage for its use as a marker to predict disease activity.

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Jamie van Langelaar	Erasmus MC and MS Center ErasMS, University Medical Center, Rotterdam, the Netherlands	Designed, performed, and analyzed the data and contributed to the study concept
Katelijnn M. Blok, MD	Erasmus MC and MS Center ErasMS, University Medical Center, Rotterdam, the Netherlands	Collected clinical samples, patient information, and contributed to the study concept
Thierry P.P. van den Bosch, PhD	Erasmus MC, University Medical Center, Rotterdam, the Netherlands	Performed experiments and analyzed data
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Joost Smolders MD, PhD	Erasmus MC and MS Center ErasMS, University Medical Center, Rotterdam, the Netherlands; Netherlands Institute for Neuroscience, Amsterdam, the Netherlands	Contributed to the study concept
Erik Lubberts, PhD	Erasmus MC, University Medical Center, Rotterdam, the Netherlands	Contributed to the study concept
Helga E. de Vries, PhD	Amsterdam University Medical Center and MS Center Amsterdam, Amsterdam, the Netherlands	Selected and provided postmortem brain tissues and contributed to the study concept
Marvin M. van Luijn, PhD	Erasmus MC and MS Center ErasMS, University Medical Center, Rotterdam, the Netherlands	Designed the experiment, wrote the paper, supervised the project, and acquired funding

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