

Natalizumab Treatment Induces Proinflammatory CD4 T Cells Preferentially in the Integrin β 7+ Compartment

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Abstract

Background and Objectives

Natalizumab, a monoclonal humanized antibody targeting integrin α 4, inhibits the transmigration of lymphocytes into the CNS by preventing the interaction of integrin α 4 β 1 with V-CAM expressed on brain vascular endothelial cells. Although natalizumab treatment reduces the clinical relapse rate in patients with relapsing-remitting MS, its discontinuation after reactivation of the JC virus is associated with a rebound of the disease in 20% of patients. The mechanisms of this rebound are not elucidated, but natalizumab increases the frequencies of circulating CD4 T cells expressing proinflammatory cytokines as well as the proportion of circulating Th17/Th1 cells (Th1-like Th17 cells). Gut-derived memory CD4 T cells are a population of growing interest in the pathogenesis of MS, but whether and how their properties are affected by natalizumab is not known. Here, we studied the phenotype and cytokine expression profile of circulating gut-derived memory CD4 T cells in patients with relapsing-remitting MS under natalizumab.

Methods

We identified gut-derived memory CD4 T cells by their expression of integrin β 7 and compared their properties and those of integrin β 7- memory CD4 T cells across healthy donors and patients with relapsing-remitting MS treated or not with natalizumab. We also compared the capacity of integrin β 7- and integrin β 7+ CD4 T-cell subsets to transmigrate in vitro across a model of blood-brain barrier.

Results

The proportions of proinflammatory Th17/Th1 cells as well as of IL-17A+IFN γ + and IL-17A+GM-CSF+ cells were higher in memory CD4 T cells expressing integrin β 7 in patients receiving natalizumab compared with healthy donors and patients with relapsing-remitting MS not receiving natalizumab. By contrast, integrin β 7 negative memory CD4 T cells only presented a modest increased in their proportion of Th17/Th1 cells under natalizumab. We further observed that integrin β 7+ Th17/Th1 cells migrated as efficiently as integrin β 7- Th17/Th1 across a monolayer of brain microvascular endothelial cells.

Discussion

Our study shows that circulating integrin β 7+ memory CD4 T cells of patients with relapsing-remitting MS under natalizumab are enriched in proinflammatory cells supporting the hypothesis that integrin β 7+ memory CD4 T cells could play a pathogenic role in the disease rebound observed at natalizumab discontinuation.

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Glossary

EAE = experimental autoimmune encephalomyelitis; MS = multiple sclerosis; NTZ = natalizumab; PBMCs = peripheral blood mononuclear cells; RRMS = relapsing-remitting phase.

Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, and neurodegenerative disease resulting from the autoimmune destruction of myelin and associated collateral tissue within the CNS.¹ Studies in experimental autoimmune encephalomyelitis (EAE) animal models and on patient samples have established the CD4 T-cell subsets Th17, Th1, and more recently Th17/Th1 (also known as Th1-like Th17²) as a central component in the pathogenesis.³ These subsets, notably Th17/Th1 cells, infiltrate the CNS and are believed to be one of the main drivers of CNS inflammation and lesion formation during the inflammatory relapsing-remitting phase (RRMS) of the disease.³⁻⁵

Natalizumab (NTZ), a humanized monoclonal antibody targeting integrin (int.) α 4 (CD49d), inhibits the transmigration of inflammatory lymphocytes across the BBB reducing clinical relapse rate in RRMS. Mechanistically, NTZ decreases the expression on lymphocytes of the subunits int. α 4 and int. β 1⁶ of VLA-4 and int. α L^{e1} (CD11a) of LFA-1. VLA-4 and LFA-1, respectively, bind to V-CAM and ICAM expressed on brain endothelial cells mediating the transmigration of lymphocytes across the BBB. Despite its high efficacy, NTZ treatment is associated with the risk of developing progressive multifocal leukoencephalopathy because of the reactivation in the CNS of the JC virus,⁷ and the treatment is therefore frequently interrupted. In 20% of patients, NTZ discontinuation is followed by a rebound of the disease, defined as a higher relapse rate after cessation of natalizumab than before natalizumab.^{8,9} The mechanisms involved in this rebound are not currently elucidated. However, NTZ treatment increases the frequencies of CD4 T cells expressing proinflammatory cytokines and the proportion of Th1/Th17 cells in the blood of patients with RRMS, suggesting that these cells might be involved in the disease rebound.^{2,10,11} Beside int. β 1, int. α 4 also associates on lymphocytes with int. β 7 to form the gut homing receptor int. α 4 β 7, and accordingly, NTZ is efficient in treating patients with Crohn disease.¹² In addition, int. β 7+ memory CD4 T cells also express int. β 1 albeit at lower levels than int. β 7- cells.^{13,14} Studies in mouse models of autoimmune diseases, including MS, have shown that intestinal lymphocytes migrate to target organs of autoimmune disease and participate to the pathogenesis.¹⁵⁻¹⁸ In humans, CD4 T cells expressing the gut homing receptors int. β 7 and/or CCR9 are detected in the CSF of patients with MS and noninflammatory neurologic diseases.^{19,20} Moreover, more than half IgA+ B cells localized in the brain of patients with MS express the gene encoding int. β 7.²¹ Determining whether and how the phenotype and properties of memory CD4 T cells expressing int. β 7 are altered under NTZ might therefore participate in a better understanding of the mechanisms responsible for the rebound of the disease but also of the pathogenesis.

In this study, we compared the phenotype and cytokine expression profile of int. β 7+ and int. β 7- memory CD4 T cells in healthy donors and patients with RRMS treated or not with natalizumab. We found that int. β 7 positive memory CD4 T cells contain higher proportions of Th17/Th1 as well as IL-17A/IFN γ and IL-17A/GM-CSF coexpressing cells in NTZ-treated patients compared with non-natalizumab-treated patients with RRMS and healthy donors. This increased proinflammatory profile of circulating memory CD4 T cells was only marginally observed in int. β 7 negative cells, suggesting that despite their lower representation in the bloodstream, gut-derived memory CD4 T cells might play an important role in the pathogenesis of MS notably at the withdrawal of natalizumab treatment.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by the Institutional Review Board of the local Ethical Committee (Comité de Protection des Personnes Sud-Ouest et Outre Mer III), and informed consent was obtained from all the participants or their legal guardian according to the Declaration of Helsinki.

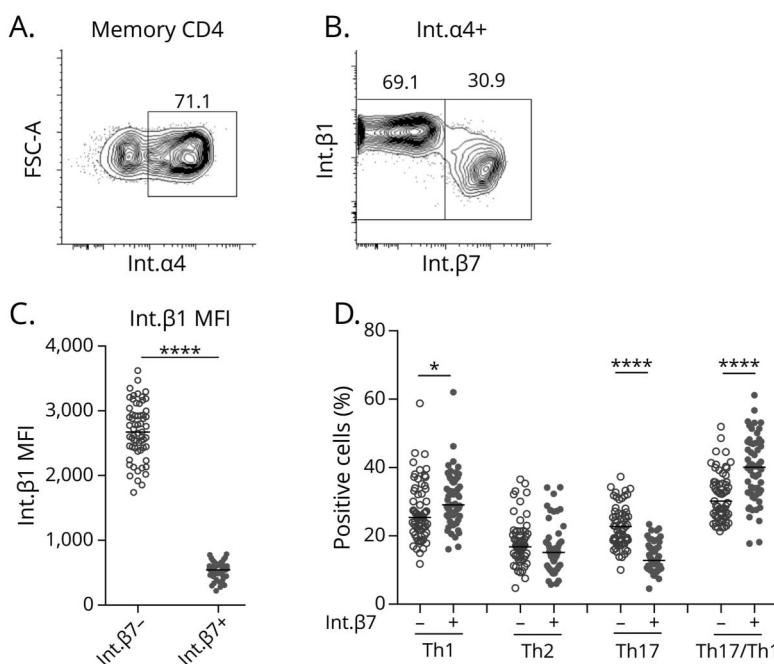
Patient and Healthy Donor Samples

Blood samples from healthy donors (EDTA tubes or buffy coats) and patients with RRMS (EDTA tubes) in remission (eTables 1 and 2, links.lww.com/NXI/A903) were obtained, respectively, from the établissement français du sang and the Neurology Department of Bordeaux hospital. Patients with RRMS were diagnosed according to the McDonald 2017 criteria²². Whole blood (100 μ L) was stained with tetraCHROME CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5 antibody cocktail (Beckman Coulter), red blood cells were lysed with Versalyse (Beckman Coulter), and absolute counts were measured using Flow-Count Fluorospheres (Beckman Coulter), NAVIOS or FC500 cytometers (Beckman Coulter), and Kaluza software (Beckman Coulter). Peripheral blood mononuclear cells (PBMCs) isolated by density gradient centrifugation (Ficoll-Paque, Cytivia) were either used directly or stored in liquid nitrogen.

Phenotyping of PBMCs by Flow Cytometry

Freshly isolated PBMCs were incubated with Zombie Aqua fixable viability marker (Biolegend) and antibodies against CD4 (OKT4), int. β 7 (FIB504), int. β 1 (TS2/16), int. α 4 (9F10), CXCR5 (J252D4), CXCR3 (G025H7), CCR6 (G034E3), CD226 (11A8), ICOS (C398.4A), PD-1 (EH12 2H7), CD146 (P1H12) (Biolegend), CD3 (UCHT1), CXCR5 (RF8B2), CD20 (2H7) (BD), and CD45RA (2H4)

Figure 1 Integrin β 7+ Memory CD4 T Cells Comprised a Higher Proportion of Th17/Th1 Cells Than Integrin β 7- Memory CD4 T Cells



(A) Representative flow cytometry plots of the expression of int. α 4 in memory CD4 T cells. (B) Representative flow cytometry plot showing the expression of int. β 1 and int. β 7 on int. α 4+ memory CD4 T cells. (C) MFI of int. β 1 on int. α 4+ int. β 7- and int. α 4+ int. β 7+ memory CD4 T cells in healthy individuals ($n = 61$). (D) Percentages of Th1 (CXCR3+CCR6-), Th2 (CXCR3-CCR6-), Th17 (CXCR3-CCR6+), and Th17/Th1 (CXCR3+CCR6+) cells in int. β 7- and int. β 7+ CXCR5-memory CD4 T cells from healthy donors ($n = 61$). The paired Student *t* test.

(Beckman Coulter) for 15 minutes at room temperature. The staining for CD146 expression was performed on frozen/thawed PBMCs. Cells were acquired on a BD LSRII Fortessa and analyzed with FlowJo software (BD).

Intracellular Cytokine Staining

Frozen/thawed PBMCs were stimulated with phorbol 12-myristate 13-acetate (PMA, 25 ng/mL) and ionomycin (1 μ g/mL) for 5 hours in RPMI supplemented with 10% FCS, L-glutamine, penicillin-streptomycin, 1 mM sodium pyruvate, nonessential amino acids, 25 mM HEPES (Life Technologies), and 50 μ M 2-mercaptoethanol (Sigma) in the presence of GolgiStop (BD) and Brefeldin (eBioscience) for the last 3.5 hours. PBMCs were then stained with Zombie Aqua fixable viability marker and antibodies against CD3 (UCHT1), CD8 (SFCI21-Thy2D3, Beckman Coulter), and int. β 7 (FIB504, BD). After fixation and permeabilization, cells were incubated with IFN γ (4SB3), TNF α (Ma β 11), IL-22 (2G12A41), IL-17A (BL168), IL-10 (JES3-9D7), GM-CSF (BVD2-21C11), CD45RA (HI100) (Biolegend), MIP-1 β (D21-1351), IL-13 (JES10-5A2), and IL-17F (O33-782) (BD) mAbs. Cells were acquired on a LSRII Fortessa and analyzed using FlowJo software.

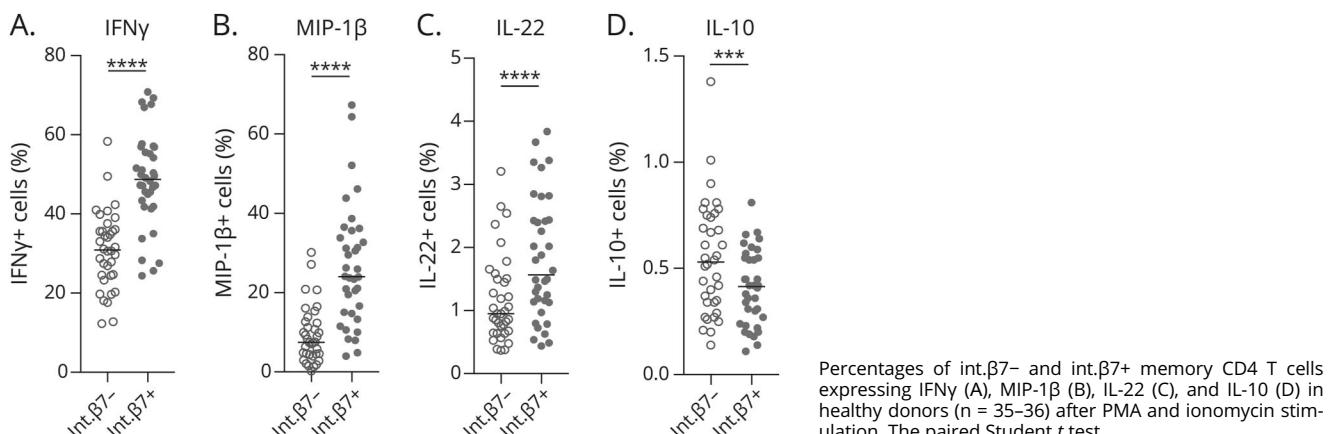
Transmigration Assay

hCMEC/D3 cell line^{e3} was obtained from Cedarlane and cultured in Endothelial Basal Medium (EBM-2, Lonza) supplemented with 5% FCS, ascorbic acid (5 μ g/mL, Sigma), 1% chemically defined lipid concentrate (Life Technologies), human basic fibroblast growth factor (1 ng/mL, Sigma), hydrocortisone (1.4 μ M, Sigma), HEPES (10 mM), penicillin, and streptomycin (100U/mL each)

in culture flasks coated with Cultrex Rat Collagen I at 150 μ g/mL (R&D systems). For transmigration assay, hCMEC/D3 were seeded at 4.5×10^4 cells/cm² on 12-well plate transwell inserts, pore size 3 μ m (Falcon) coated with Cultrex Rat Collagen I, and then cultured for 7 days with the addition of TNF α (100U/mL, Peprotech) for the past 24 hours.

Because the expression of the chemokine receptors CXCR3 et CCR6 is altered following cell activation, we first enriched PBMCs from healthy donors in memory CD4 T cells using memory CD4 $^+$ T-cell isolation kit (Miltenyi Biotec) and labeled them with antibodies against CD4 (RPA-T4), CD45RA, CD56 (HCD56, Biolegend), CD8 (RPA-T8, Biolegend), CXCR3, and CCR6. CXCR3+CCR6-, CXCR3-CCR6-, CXCR3-CCR6+, and CXCR3+CCR6+ cells among CD4 $^+$ CD56 $^-$ CD8 $^-$ CD45RA-lymphocytes were then purified with a FACSaria (BD - cell purity >98%). Each subset was labeled with CellTrace Violet (Life Technologies) and mixed back with autologous unlabeled PBMCs (ratio 1:4). To determine the transmigration capacity of int. α 4+ int. β 7+ and int. α 4+ int. β 7+, we used total PBMCs because the expression of these markers is stable on short-term culture. In both assays, the PBMCs were stimulated overnight with dynabeads coated with CD3 and CD28 mAbs (Dynal) to achieve optimal activation of integrins.²² After removal of the dynabeads, 1×10^6 PBMCs resuspended in 500 μ L of RPMI supplemented with 5% FCS, L-glutamine (2 mM), ascorbic acid, chemically defined lipid concentrate, human basic fibroblast growth factor, hepes, penicillin, and streptomycin were added to the insert containing the hCMEC/D3 previously washed. Cells were then allowed to migrate for 8 hours at 37°C, and top

Figure 2 Integrin $\beta 7^+$ Memory CD4 T Cells Display a Higher Proinflammatory Profile Compared With Integrin $\beta 7^-$ Memory CD4 T Cells



and bottom chambers were separately harvested and rinsed with phosphate buffer saline containing EDTA (0.1 mM). Cells were then labeled with Zombie Aqua viability dye and mAbs against CD4, CD45RA, int. $\beta 7$, int. $\beta 1$, and CXCR5. CountBright Absolute Counting Beads (Life Technologies) were added to each fraction. The percentages of migration were determined by calculating the ratio between the numbers of int. $\beta 7^-$ int. $\beta 1^+$ or int. $\beta 7^+$ int. $\beta 1^{low}$ CXCR5- CellTrace Violet positive cells contained in the top chamber and the total number of these cells (top and bottom chambers).

Statistical Analysis

The significance of the difference between groups in the experiments was evaluated using the unpaired Student t test, paired Student t test, or one-way ANOVA followed by the Tukey multiple comparison test. A value of $p < 0.05$ was considered significant (* <0.5 , ** <0.1 , *** <0.001 , **** <0.0001).

Data Availability

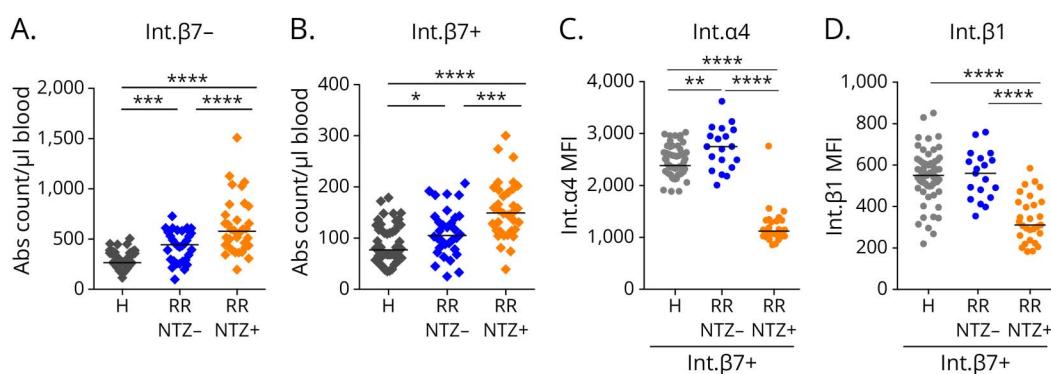
Anonymized data published within this article are available on reasonable request to qualified investigators for the purposes of replicating procedures and results.

Results

Integrin $\beta 7^+$ Memory CD4 T Cells Display a Higher Proinflammatory Profile Compared With Integrin $\beta 7^-$ Memory CD4 T Cells at Steady State

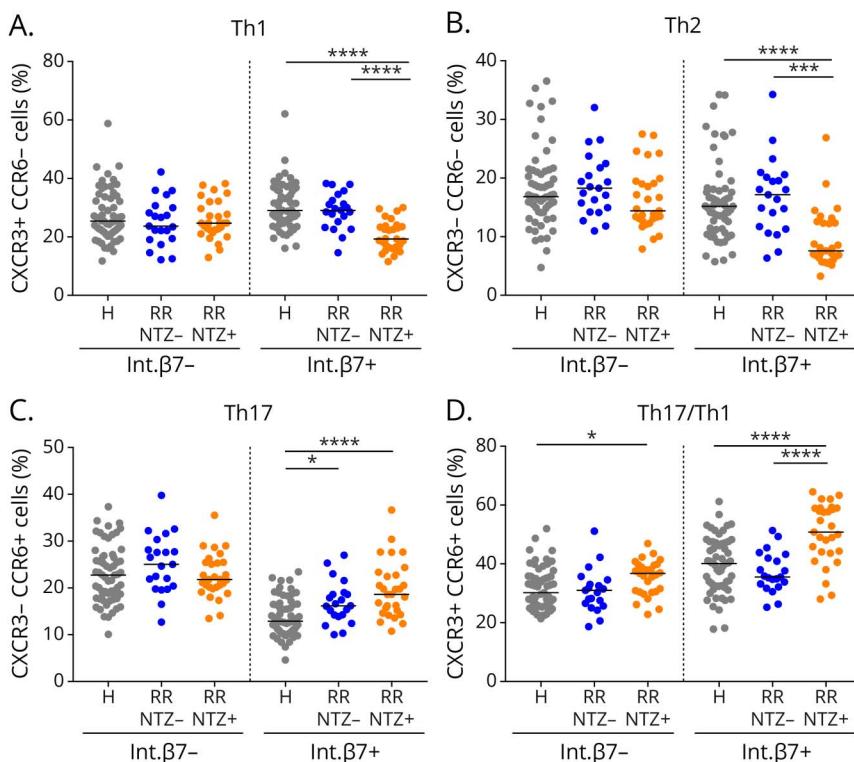
The target of natalizumab, int. $\alpha 4$, was expressed in healthy donors by most ($62.3 \pm 8.1\%$, median \pm SD) circulating memory CD4 T cells with $30.2 \pm 6.4\%$ (median \pm SD) of int. $\alpha 4^+$ cells coexpressing int. $\beta 7$ (Figure 1, A and B, eFigure 1A, links.lww.com/NXI/A901). In agreement with previous studies,^{13,14,23} both int. $\beta 7^-$ and int. $\beta 7^+$ memory CD4 T cells expressed int. $\beta 1$ with a lower level of expression on int. $\beta 7^+$ cells

Figure 3 Natalizumab Treatment Decreases the Expression of Brain Homing Molecules at the Surface of Integrin $\beta 7^+$ Memory CD4 T Cells and Induces Their Retention in Periphery



(A and B) Absolute numbers per μ l of blood of int. $\beta 7^-$ (A) and int. $\beta 7^+$ (B) memory CD4 T cells in healthy individuals ($n = 57$) and patients with RRMS not treated (NTZ-, $n = 32$) or treated (NTZ+, $n = 34$) with natalizumab. (C and D) Median of fluorescence intensity of int. $\alpha 4$ (C) and int. $\beta 1$ (D) expressed by int. $\beta 7^+$ memory CD4 T cells in healthy donors ($n = 61$), RRMS NTZ- ($n = 19$) and RRMS NTZ+ ($n = 29$) groups. One-way ANOVA followed by the Tukey multiple comparison test.

Figure 4 Integrin β 7+ Memory CD4 T Cells From Patients With RRMS Treated With Natalizumab Contain an Increased Proportion of Th17/Th1



Percentages of Th1 (A), Th2 (B), Th17 (C), and Th17/Th1 (D) in int. β 7- CXCR5- and int. β 7+ CXCR5+ memory CD4 T cells in healthy donors ($n = 61$), RRMS NTZ- ($n = 21$), and RRMS NTZ+ ($n = 29$) groups. One-way ANOVA followed by the Tukey multiple comparison test.

(Figure 1, B and C, eFigure 1B). The differential expression of the chemokine receptors CXCR3 and CCR6 allowed us to identify Th1 (CXCR3+CCR6-), Th2 (CXCR3-CCR6-), Th17 (CXCR3-CCR6+), and Th17/Th1 also known as Th1-like Th17² (CXCR3+CCR6+) subsets in both int. β 7- and int. β 7+ CXCR5- CD45RA- CD4⁺ T cells (eFigure 1C). We observed that int. β 7+ memory CD4 T cells contained higher percentages of Th17/Th1 and Th1 cells and a lower percentage of Th17 cells compared with int. β 7- memory CD4 T cells (Figure 1D). The study of the cytokine expression profile of int. β 7+ memory CD4 T cells revealed higher proportions of cells expressing the proinflammatory cytokines IFN γ , MIP-1 β , TNF α , and IL-22 compared with int. β 7- memory CD4 T cells (Figure 2 and eFigure 2). By contrast, int. β 7+ memory CD4 T cells were less potent at expressing IL-10 and IL-13 compared with int. β 7- memory CD4 T cells. These results indicate that, at steady state, int. β 7+ memory CD4 T cells display a higher inflammatory profile compared with int. β 7- memory CD4 T cells.

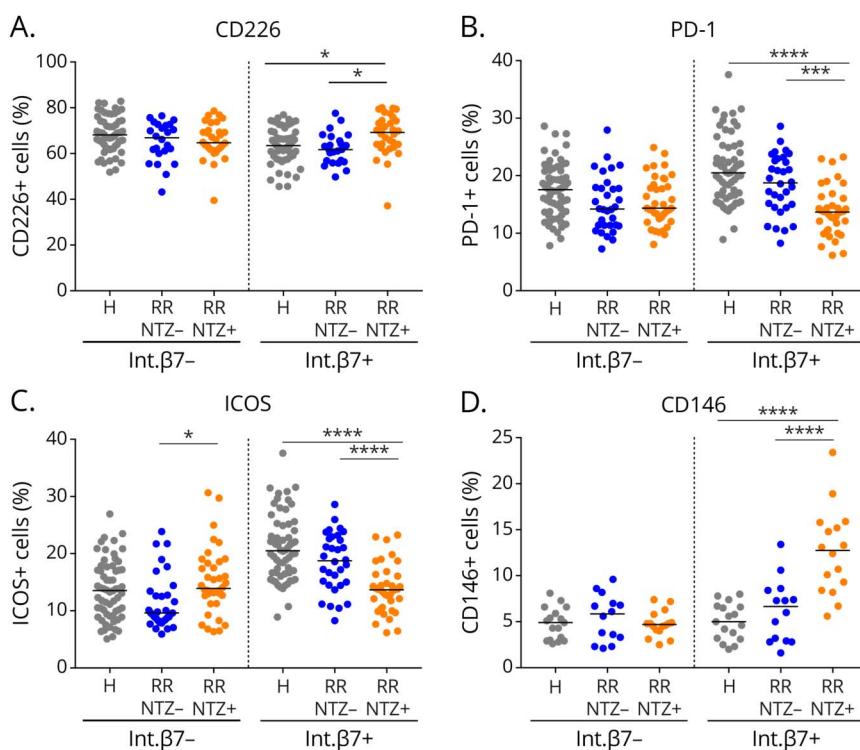
Natalizumab Treatment Decreases the Expression of Brain Homing Molecules at the Surface of Integrin β 7+ Memory CD4 T Cells

We next compared the phenotype of int. β 7- and int. β 7+ memory CD4 T cells from healthy donors, patients with RRMS untreated or receiving disease-modifying therapies excluding natalizumab (RRMS NTZ-), and patients with

RRMS under natalizumab (RRMS NTZ+) (eTables 1 and 2, links.lww.com/NXI/A903). As expected, the level of expression of int. α 4 was decreased on memory CD4 T cells by NTZ treatment (eFigure 3, A and B, links.lww.com/NXI/A901). This decrease of expression was accompanied by a decrease of the intensity of int. β 7 expression on int. β 7+ memory CD4 T cells (eFigure 3A and C). However, int. β 7 expressing and nonexpressing memory CD4 T cells still clearly segregated by flow cytometry in NTZ+ patients regarding their expression of int. β 7 (eFigure 3D). Accordingly, the percentages of int. β 7+ memory CD4 T cells did not differ between patients with RRMS treated or not with NTZ. This conserved bimodal expression of int. β 7 under NTZ allowed us to study the impact of the treatment on int. β 7+ and int. β 7- memory CD4 T cells.

NTZ is known to induce increased levels of peripheral immune cells, including CD4 T cells.²⁴ Here, we observed that the absolute blood counts of total CD4 T cells, memory CD4 T cells, int. β 7-, and int. β 7+ memory CD4 T cells were increased in NTZ+ patients compared with NTZ- patients (median fold change of 1.30 and 1.41 for int. β 7- and int. β 7+ memory CD4 T cells, respectively) and healthy donors (median fold change of 2.17 and 1.93 for int. β 7- and int. β 7+ memory CD4 T cells, respectively) (Figure 3, A and B and eFigure 3E, links.lww.com/NXI/A901). We next asked how NTZ alters the expression of the brain homing molecules

Figure 5 Integrin β 7+ Memory CD4 T Cells From Patients With RRMS Treated With Natalizumab Display Higher Expression of Markers Associated With Pathogenicity



(A–C) Percentages of CD226 (A), PD-1 (B), and ICOS (C) expressing cells in int.β7- and int.β7+ memory CD4 T cells of healthy donors ($n = 61$ – 62), RRMS NTZ- ($n = 24$ – 33), and RRMS NTZ+ ($n = 32$ – 38) groups. (D) Percentages of CD146 expressing cells in int.β7- and int.β7+ memory CD4 T cells of healthy donors ($n = 17$), RRMS NTZ- ($n = 14$), and RRMS NTZ+ ($n = 16$) groups. One-way ANOVA followed by the Tukey multiple comparison test.

int.α4 and int.β1 (the 2 subunits of VLA-4) and observed that the expression of these 2 molecules was strongly reduced by NTZ treatment in both int.β7- and int.β7+ memory CD4 T cells (Figure 3, C and D, and eFigure 4).

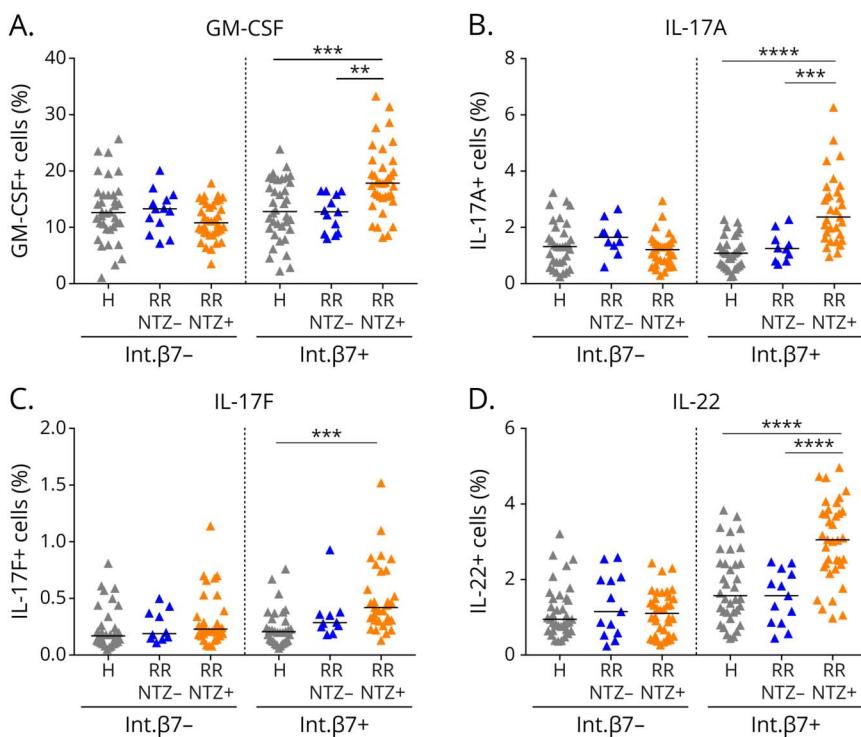
Integrin β 7+ Memory CD4 T Cells From Patients With RRMS Treated With Natalizumab Contain an Increased Proportion of Th17/Th1 Cells

As NTZ treatment increases the proportion of circulating Th17/Th1 cells in patients with RRMS,² we next compared the impact of NTZ treatment on the repartition of Th subsets in int.β7- and int.β7+ memory CD4 T cells. In the int.β7+ compartment, we observed that the proportion of Th1 and Th2 was decreased in favor of Th17/Th1 in NTZ+ patients compared with NTZ- patients and healthy donors (Figure 4). Indeed $50.8\% \pm 10.2\%$ (median \pm SD) of int.β7+ memory CD4 T cells from NTZ+ patients display a Th17/Th1 phenotype against $35.5\% \pm 6.9\%$ (median \pm SD) in NTZ- patients and $40.1\% \pm 9.4\%$ (median \pm SD) in healthy donors. Of note, the proportion of Th17 cells in int.β7+ cells was higher in NTZ- and NTZ+ patients than in healthy donors but did not differ between the 2 groups of patients with RRMS. Concerning int.β7- cells, the alterations in the repartition of Th subsets were less marked than in the int.β7+ compartment with only a modest increase of the percentage of Th17/Th1 in NTZ+ patients ($36.8\% \pm 5.9\%$, median \pm SD) compared with healthy donors ($30.2\% \pm 7.1\%$, median \pm SD) but not with NTZ- patients ($31.0\% \pm 7.3\%$, median \pm SD).

Because the shift toward Th17/Th1 observed in the int.β7+ compartment could be due to the higher past activity of the disease in NTZ+ patients (eTable 1, links.lww.com/NXI/A903 and eFigure 5, links.lww.com/NXI/A901) rather than to natalizumab treatment by itself, we next determine whether the proportion of Th17/Th1 in int.β7+ CD4 T cells correlates with clinical parameters in patients with RRMS. We did not observe any significant correlation between the percentages of Th17/Th1 in int.β7+ memory CD4 T cells and the clinical score (EDSS), the duration of the disease, or the number of past relapses (eFigure 6). This suggests that the higher proportion of Th17/Th1 cells in int.β7+ memory CD4 T cells is induced by natalizumab treatment.

We next assessed the impact of NTZ on the expression of CD226, PD-1, ICOS, and CD146 by int.β7- and int.β7+ memory CD4 T cells. CD226 promotes the differentiation and proliferation of proinflammatory CD4 T-cell subsets including their secretion of IFNγ and IL-17.²⁵ In addition, genome-wide association studies have defined CD226 allelic variants as a risk factor for MS²⁶ and blocking CD226 in EAE reduced the disease onset.²⁷ Concerning PD-1, its polymorphism is associated with disease progression,^{28,e4} and its level of expression is reduced on CD4 T cells in acute MS.^{29,e5} Accordingly, mice deficient for PD-1 develop more severe EAE.³⁰ ICOS promotes the expansion of Th17/Th1 in humans,³¹ but mice deficient for ICOS present an enhanced susceptibility to EAE³² suggesting a different role of ICOS

Figure 6 Integrin β 7+ Memory CD4 T Cells From Patients With RRMS Under Natalizumab Display an Increased Capacity to Express GM-CSF, IL-17A, IL-17F, and IL22



Percentages of int.β7- and int.β7+ memory CD4 T cells expressing GM-CSF (A), IL-17A (B), IL-17F (C), and IL-22 (D) after PMA and ionomycin stimulation in healthy controls ($n = 32\text{--}36$), RRMS NTZ- ($n = 10\text{--}13$), and RRMS NTZ+ ($n = 31\text{--}36$) groups. One-way ANOVA followed by the Tukey multiple comparison test.

during the disease. Here, we observed higher percentages of cells expressing CD226 and lower percentages of cells expressing PD-1 and ICOS in int.β7+ memory CD4 T cells of NTZ+ patients compared with those of NTZ- patients and healthy donors (Figure 5, A-C). By contrast, no alteration in the expression of CD226, PD-1, and ICOS could be detected in int.β7- cells in NTZ+ patients. CD146+ memory CD4 T cells are enriched in IL-17+ as well as in IL-17+ IL-22+ and IL-17+ IFN γ + cells,³³ and CD146 has been proposed as an alternative route to mediate the trafficking of CD4 T cells into the CNS under VLA-4 blockade.³⁴ Studies on MS samples showed that CD146+ memory CD4 T cells were increased in the CSF and PBMCs of long-term NTZ+ RRMS patients.^{34,35} In agreement with the preferential increased of Th17 and Th17/Th1 subsets in the integrin β 7+ compartment, we observed that the upregulation of CD146+ cells under NTZ was only detectable in the integrin β 7+ compartment (Figure 5D).

These results suggest that int.β7+ memory CD4 T cells possess an increased inflammatory and pathogenic phenotype in patients with RRMS under NTZ.

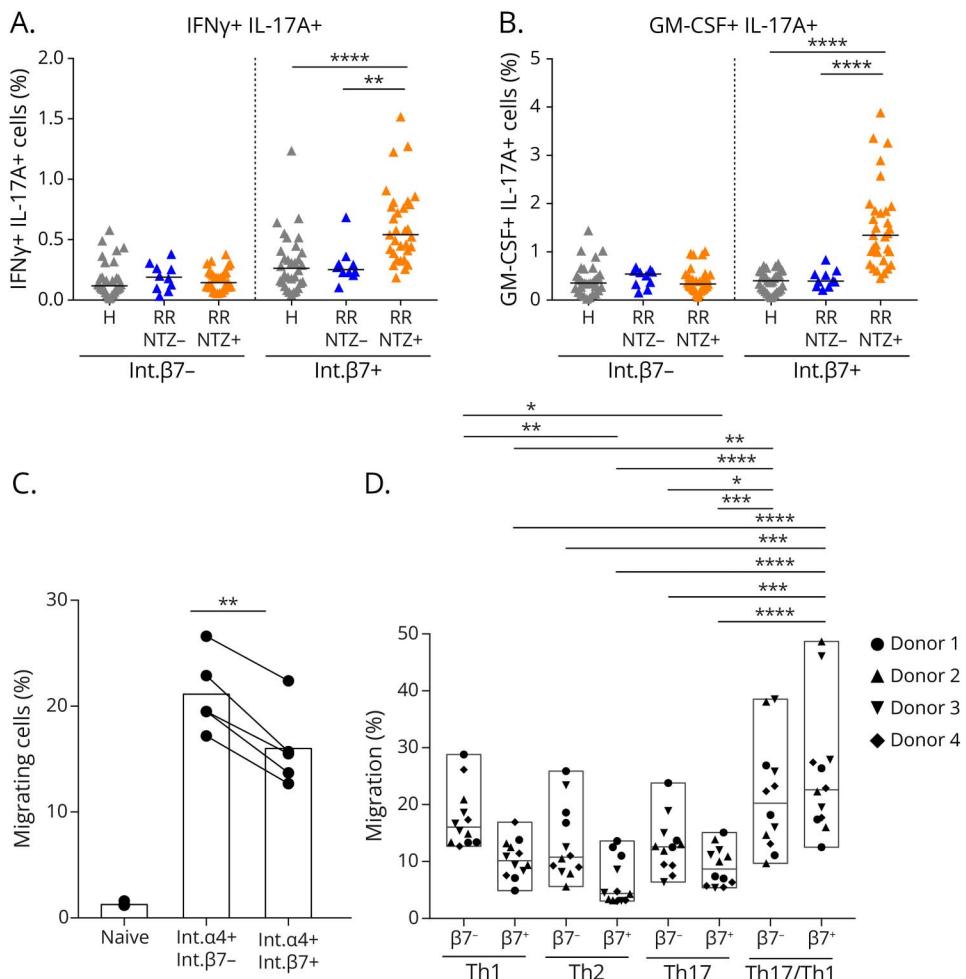
Natalizumab Treatment Induces an Increased Coexpression of Th1 and Th17 Cytokines by Integrin β 7+ Memory CD4 T Cells

Previous studies have shown an increased expression of GM-CSF, IFN γ , and IL-17A by CD4 T cells under NTZ.^{2,11} Here, we assessed the cytokine expression profile of int.β7- and

int.β7+ in patients with RRMS under NTZ (Figure 6 and eFigure 7A, links.lww.com/NXI/A901). We observed that the proportions of int.β7+ memory CD4 T cells expressing GM-CSF, IL-17A, IL-17F, and IL-22 were higher in NTZ+ patients compared with NTZ- patients and healthy donors. Concerning IFN γ and MIP-1 β , their expressions by int.β7+ memory CD4 T cells in NTZ+ patients were lower than in healthy donors but similar to those observed in NTZ- patients. The proportion of IL-10 expressing int.β7+ memory CD4 T cells in NTZ+ patients was lower than in NTZ- patients but did not differ compared with healthy donors. By contrast, in int.β7- memory CD4 T cells, the expression of GM-CSF, IL-17A, IL-17F, IL-22, IFN γ , or IL-10 did not differ between NTZ+ and NTZ- patients or healthy donors.

Because Th17/Th1 cells are known to coexpress Th1 and Th17 cytokines,³⁶ we next asked whether the higher proportion of Th17/Th1 observed at the phenotypical level in NTZ+ patients was associated with increased proportions of IFN γ +IL-17A+ and/or GM-CSF+IL-17A+ cells. We determined that despite their global reduced proportion of IFN γ positive cells (eFigure 7A, links.lww.com/NXI/A901), int.β7+ memory CD4 T cells from NTZ+ patients contained higher proportions of IFN γ +IL-17A+ as well as GM-CSF+IL-17A+ cells compared with healthy donors and NTZ- patients (Figure 6, A and B and eFigure 7B). By contrast, no modification in the proportion of IFN γ +IL-17A+ or GM-CSF+IL-17A+ cells could be detected in the int.β7- compartment.

Figure 7 Integrin β 7+ Memory CD4 T Cells Are Enriched in IFN γ +IL-17A+ and GM-CSF+IL-17A+ Cells



(A and B) Percentages of IFN γ +IL-17A+ (A) and of GM-CSF+IL-17A+ (B) cells in int. β 7- and int. β 7+ memory CD4 T cells in healthy donors ($n = 32$), RRMS NTZ- ($n = 10$), and RRMS NTZ+ ($n = 31$) groups. One-way ANOVA followed by the Tukey multiple comparison test. (C) Percentages of naive CD4 T cells, integrin α 4+ integrin β 7-, and integrin α 4+ integrin β 7+ memory CD4 T cells migrating across a monolayer of hCMEC/D3 prestimulated during 24 h with TNFa. Data from one experiment performed in quadruplicate representative of 5 experiments performed with different healthy donor. Data points are paired per transwell. Paired Student *t* test. (D) Percentages of migration of the indicated Th subsets expressing or not int. β 7 across a monolayer of hCMEC/D3 stimulated (C). Data from 4 experiments each performed in triplicate with different healthy donor are shown in scattered dot plot. Floating bars (min and max) with line at median are indicated. One-way ANOVA followed by the Tukey multiple comparison test.

As for Th17/Th1, we asked whether the percentages of IFN γ +IL-17A+ and GM-CSF+IL-17A+ memory CD4 T cells in patients with RRMS correlate with clinical parameters. In contrast to what we observed with Th17/Th1 cells, the percentages of IFN γ +IL-17A+ and in a lesser extend of IL-17A+GM-CSF+ memory CD4 T cells contained in the int. β 7+ compartment positively correlated with the disease severity and duration (eFigure 8A-B, links.lww.com/NXI/A901). Although the correlation coefficients were moderate (r^2 values < 0.3), this indicate that the capacity of int. β 7+ memory CD4 T cells to coexpress IL-17A and IFN γ or GM-CSF increases with the disease severity. However, at equivalent clinical parameters (EDSS and disease duration), NTZ+ patients consistently presented higher percentages of IFN γ +IL-17A+ and GM-CSF+IL-17A+ cells in int. β 7+ memory CD4 T cells compared with NTZ- patients (eFigure 8, C and D). Altogether, our data indicate that the proportion of Th17/Th1 not only identified by their phenotype but also by their coexpression of IL-17A and IFN γ or GM-CSF is increased by NTZ treatment, particularly in int. β 7+ memory CD4 T cells.

Th17/Th1 CD4 T Cells Expressing Integrin β 7+ Efficiently Transmigrate Through an in Vitro Model of Blood-Brain Barrier

Th17/Th1 cells coexpressing IFN γ and IL-17 infiltrate the CNS of patients with MS and preferentially cross the blood-brain barrier both in vitro and in EAE model.⁴ Because the proportion of these cells is increased in the int. β 7+ compartment under NTZ, we next assessed the capacity of the different int. β 7+ memory CD4 T-cell subsets to transmigrate through the BBB. We used a previously described^{e3} artificial in vitro model of BBB hinged on a monolayer of hCMEC/D3, a human endothelial cell line derived from brain microvascular endothelial cells, grown on culture insert. We first assessed the transmigration capacity of total int. α 4+int. β 7- and int. α 4+int. β 7+ memory CD4 T cells. We observed that int. α 4+int. β 7+ memory CD4 T cells efficiently migrated through the monolayer of hCMEC/D3, although approximately 20% less efficiently than int. α 4+int. β 7- memory CD4 T cells (Figure 7C). These results are in agreement with the proportions of gut-derived CD4 T cells (expressing integrin β 7 or CCR9) detected in the CSF from patients with noninflammatory neurologic

diseases and MS.^{19,20} Next, we determined that among int. β 7+ subsets, Th17/Th1 cells transmigrated most efficiently through the hCMEC/D3 layer (Figure 7D). Furthermore, int. β 7+ Th17/Th1 cells transmigrate as efficiently as int. β 7- Th17/Th1 and Th1 cells and more efficiently than int. β 7- Th2 and Th17.

These data indicate that int. β 7+ Th17/Th1 possess a capacity to transmigrate across the BBB similar to those of int. β 7- Th17/Th1 and Th1 cells known to be pathogenic in MS and EAE³⁶ and are therefore consistent with a pathogenic role of int. β 7+ Th17/Th1 in MS.

Discussion

In this study, we compared the phenotype and cytokine expression profile of int. β 7- and int. β 7+ memory CD4 T cells in healthy donors and patients with RRMS receiving natalizumab or not. We also investigate the capacity of int. β 7+ memory CD4 T-cell subsets to transmigrate through an artificial model of BBB.

Kebir et al.⁴ showed that Th17/Th1 cells identified by their coexpression of not only IFN γ and IL-17 but also ROR γ t and T-bet are present in the CNS of patients with MS and that IL-17+ IFN γ + CD4 T cells are preferentially recruited in the CNS in EAE model. Comparative analysis of CSF, brain tissues, and blood from patients with MS confirmed that Th17/Th1 cells are abundant in their CNS.² Confirming the importance of Th17/Th1 cells in MS pathogenesis, circulating CCR6+ myelin-reactive CD4 T cells from patients with MS show an enhanced production of GM-CSF, IL-17A, and IFN γ compared with healthy controls.³⁷ In agreement with their in vivo abundance into the CNS, in vitro transmigration assay on PBMCs from healthy donors and patients with MS have demonstrated that, among CD4 T cell subset, Th17/Th1 cells possess the highest ability to cross the BBB.^{4,38} Here, we showed that int. β 7+ Th17/Th1 cells transmigrate as efficiently as int. β 7- Th17/Th1 and Th1 cells across a monolayer of brain microvascular endothelial cells, which is consistent with a pathogenic role of int. β 7+ Th17/Th1 cells in MS. Although lymphocytes expressing gut homing molecules are observed in significant proportion in human CSF in pathologic conditions, including in patients with MS,^{20,21} the molecular interactions mediating their transmigration through the BBB are currently unknown. In the absence of NTZ treatment, int. β 7+ memory CD4 T cells express int. α 1 at low levels compared with int. β 7- memory CD4 T cells. Although these levels are inferior to those observed on int. β 7- memory CD4 T cells during NTZ treatment, further studies are required to determine whether they permit or facilitate the transmigration of memory CD4 T cells across the BBB in the presence of physiologic levels of int. α 4. In EAE, studies in mice deficient for int. α 4 or treated with an anti-int. α 4 antibody demonstrated that int. α 4 is critical for the trafficking of Th1 but not Th17 into the CNS.^{39,40} In these studies, the entry of Th17 cells inside the CNS in EAE mice was abolished

by the blockade of LFA-1 (int. α L β 2), indicating that Th17 cells can migrate into the CNS in an int. α 4 β 1-independent, LFA-1-dependent manner. Concerning int. α 4 β 7, studies on its involvement in the migration of memory CD4 T cells into the CNS in EAE have produced inconsistent results probably because of the differences in the models and experimental procedures used.⁴¹⁻⁴⁴ Notably, whether int. α 4 β 7 can switch its ligand specificity from MadCAM-1 to VCAM-1 under inflammatory conditions in vivo, as described in vitro,⁴⁶ remains to be determined.

In healthy donors, the greater proinflammatory phenotype and cytokine profile observed in int. β 7+ compared with int. β 7- memory CD4 T cells is consistent with the high inflammatory profile of the gut environment caused by the continuous exposure of gut immune cells to microbiota components. Under natalizumab, previous studies have shown that circulating memory CD4 T cells display increased proinflammatory properties with an elevated expression of CCR6 (expressed by Th17 and Th17/Th1 cells) and a higher expression of IL-17A, IFN γ , and TNF α .¹¹ Longitudinal study further showed that the proportion of a subpopulation of Th17/Th1 cells (CXCR3+CCR6+CCR4- cells) as well as the proportion of Th17/Th1 cells coexpressing IFN γ and GM-CSF are increased in the blood of patients with RRMS under natalizumab.² In this study, we found that patients with RRMS under natalizumab display a marked increase of the proportion of Th17/Th1 cells in the int. β 7+ compartment. The higher proportion of IFN γ +IL-17A+ and GM-CSF+IL-17A+ cells observed in the int. β 7+ compartment in these patients strengthened this observation. Int. β 7+ memory CD4 T cells further expressed lower levels of ICOS and PD-1 and higher levels of CD226 and CD146 which might potentiate the pathogenic properties of these cells in the context of MS.^{27,30,32,34} The modifications of phenotype and cytokine expression profile were mainly restricted to the int. β 7+ compartment as we only observed a modest increase of the proportion of Th17/Th1 cells and no modification of the cytokine expression profile in int. β 7- memory CD4 T cells under NTZ.

Previous studies on blood samples have found that GM-CSF, IL-17, and IL-22 are expressed by a higher proportion of circulating CD4 T cells in NTZ- patients than in healthy controls.^{45,47} In our study, we did not observe such modifications neither in int. β 7- nor in int. β 7+ memory CD4 T cells most likely because the patients enrolled in the RRMS NTZ- group (eTable 1, links.lww.com/NXI/A903) were in remitting phase and either treated with immunomodulatory/immunosuppressive drugs or at an early stage of the disease.⁴⁸ Of note, we did not observe differences in the phenotype or cytokine expression profile between male and female in healthy donors or in the RRMS NTZ- group between the untreated patients and the patients under interferon β 1, glatiramer acetate, or teriflunomide. Patients with RRMS under dimethyl fumarate, rituximab, or fingolimod were excluded from this study as their treatment affected the studied

parameters. Therefore, whether and how the properties of int. β 7+ memory CD4 T cells are altered in active/untreated MS remain to be determined.

Altogether, our results show that circulating int. β 7+ memory CD4 T cells acquire pathogenic features under natalizumab and are therefore a subset of interest to better understand the rebound of the disease observed in 20% of patients with RRMS at natalizumab discontinuation.^{8,9}

In humans, the pathogenic features of int. β 7+ memory CD4 T cells in MS remain to be determined. Studies in mice have shown that alterations in the composition of the gut microbiota largely modify the pathogenic properties of CD4 T cells found in the gut and in the CNS as well as the susceptibility to EAE.^{49,50} In agreement with these observations, gut-derived lymphocytes have been shown to migrate to target organs and participate to disease pathogenesis in several mouse models of autoimmune diseases, including EAE.¹⁵⁻¹⁸ Indeed, in the opticospine EAE mouse model, Smad7 overexpression in intestinal CD4 T cells favor their expansion and migration into the CNS promoting autoimmunity.¹⁸ CNS IgA-producing plasma cells were also shown to partly originate from the gut and suppress neuroinflammation in EAE.¹⁷ In humans, memory CD4 T cells expressing the gut homing receptors CCR9 are detected in the CSF of patients with MS,¹⁹ while gut microbiota-specific IgA+ B cells were found to traffic to the CNS in active patients with MS.²¹

In conclusion, although the pathogenicity of int. β 7+ CD4 T cells is not currently defined in MS, studies in mouse models and in humans support a relevance of these cells in the development of the disease. Our study, by evidencing a preferential dysregulation of the int. β 7+ compartment in patients with RRMS under natalizumab points out toward a potential role of gut-derived CD4 T cells in the disease rebound observed at the interruption of natalizumab treatment.

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