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Intrathecal Anti-*Akkermansia muciniphila* IgG Responses in Multiple Sclerosis Patients linked to CSF Immune cells and Disease Activity

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Abstract: (1) Background: Gut microbial dysbiosis, leaky gut and increased transepithelial translocation of commensal bacteria have been documented in multiple sclerosis (MS). Intrathecal IgGs specific for *Akkermansia muciniphila*, a gut bacterium, are increased in MS patients and associated with clinical disability. Our objective here was to explore the putative involvement of intrathecal anti-*A. muciniphila* IgG in MS pathogenesis by characterizing patients with different anti-*A. muciniphila* IgG indices. (2) Methods: Serum and intrathecal IgG specific for *A. muciniphila* and other gut bacteria as well as routine cerebrospinal fluid (CSF) parameters were measured in 61 MS patients. Examination of these patients included immunophenotyping of CSF-infiltrating and paired circulating lymphocytes, intrathecal markers of neurodegeneration and inflammation and a detailed characterization of demographic-, clinical-, and magnetic resonance imaging (MRI) features. (3) Results: MS patients with high anti-*A. muciniphila* IgG index also showed higher intrathecal IgG indices against other gut bacteria. Plasma blasts, B cells and Th2 cells that might be involved in antibody production were increased in the CSF of these patients as well as blood pro-inflammatory Th17 cells. Anti-*A. muciniphila* IgG indices were negatively associated with blood brain barrier (BBB) permeability and circulating monocytes and positively with brain lesion load. (4) Conclusions: The differences between patients with low and high anti-*A. muciniphila* IgG indexes regarding BBB permeability, CSF cell infiltrates, pro-inflammatory peripheral immune cells as well as imaging features, support a role of anti-*A. muciniphila* immune response in MS pathogenesis.

Keywords: multiple sclerosis; gut microbiota; dysbiosis; *Akkermansia muciniphila*; antibodies; cerebrospinal fluid

1. Introduction

MS is an immune-mediated demyelinating disease of the central nervous system (CNS) [1,2] that develops in genetically susceptible individuals and likely requires environmental triggers [3,4]. Recently, several studies have revealed a dysbiosis in the gut microbiota of MS patients that might play a role in disease pathogenesis [5]. Supporting this hypothesis, it has been demonstrated that the transfer of gut microbiota from MS patients but not healthy controls into mice can induce or exacerbate experimental autoimmune encephalomyelitis [6,7]. Since gut microbiota and their

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metabolites are important for maintaining the gut epithelial barrier [8] and influence systemic immunity [9,10], microbiota dysbiosis affect the intestinal barrier function and immune homeostasis. In addition, gut enrichment in mucin-degrader bacteria can reduce mucus thickness and facilitate mucosal damage [11]. A leaky gut can then enable the translocation of commensal bacteria across intestinal epithelium [12] enabling activation of the immune system. Bacterial lipopolysaccharides (LPS) and metabolites from translocated bacteria can access the blood and circulate to distant organs such as the brain and affect BBB permeability [13] as well as the maturation and function of microglia [14] and astrocytes [15]. Supporting a role of leaky gut in MS pathogenesis, altered biomarkers of gut barrier leakiness are common in MS patients and correlate with disease progression and with increased BBB permeability [16,17]. Furthermore, circulating LPS, as a measure of gut microbiota translocation, is increased in MS patients and correlates with disability [18].

Among the different bacteria reported to be enriched in MS patients [6,7,19], one of the most interesting is the mucin-degrader *Akkermansia muciniphila* [11]. We recently demonstrated higher intrathecal levels of anti-*A. muciniphila* immunoglobulin G (IgG) in MS patients compared with controls and a significant correlation with disease disability [20], which supports a putative role of this bacterium in MS. Furthermore, two studies demonstrated cross-recognition between recently identified MS autoantigens and *A. muciniphila* derived peptides by CD4+ T cells from MS patients [21,22].

In order to better understand the involvement of intrathecal anti-*A. muciniphila* IgG in MS, we identified MS patients with different anti-*A. muciniphila* IgG indices and characterized them in depth by analysis of CSF measures, ex vivo immunophenotyping of CSF and paired blood samples as well as a demographic-, clinical-, and MRI characteristics.

2. Materials and Methods

Patient material

Paired CSF and blood samples were collected from 61 untreated MS patients (Table 1). All CSF samples were obtained for diagnostic purposes. Patients were recruited from the Neuroimmunology and MS Research Section, Neurology Clinic, University Hospital Zurich (USZ). MS diagnosis was based on the revised McDonald criteria [23]. Fifty patients had never been treated.

Table 1. Demographic and clinical features.

	All	**Anti-*A. mucniniphila* IgG Index Low**	**Anti-*A. mucniniphila* IgG Index High**	**p[1]**
Number of patients	61	20	20	
Female/male ratio	2.05	1.2	3	0.32
Age at CSF puncture (years)	36.2 ± 10.1	36.9 ± 11.6	35.8 ± 9.3	0.89
Age at disease onset (years)	33.7 ± 9.2	35.0 ± 10.8	32.2 ± 9.4	0.54
Disease duration (Months)	31.9 ± 58.8	29.9 ± 60.1	42.4 ± 72.9	0.89
RIS /CIS (%)	19.6	30.0	10.0	0.23
RRMS (%)	73.7	60.0	85.0	0.15
PMS (%)	6.5	10.0	5.0	0.90
CSF OCB Type II (%)	81.9	70.0	100.0	0.02 *
HLA DR15 (%)	44.2	25.0	55.0	0.10
QAlb-QNorm>0 (%)	19.6	35.0	5.0	0.04 *

[1] Comparisons were performed using U-test (Mann-Whitney) and associations using Fisher's Exact Test.RIS: Radio Isolated syndrome. CIS=Clinically Isolated syndrome. RRMS= Relapsing-Remitting MS. PMS= Progressive MS.

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Eleven had previously been treated but were considered untreated at the time of lumbar puncture (7 patients received steroids, not during the last 4 weeks prior to enrolment and 4 patients received glatimer acetate not during the last 3 months prior to enrolment).

Quantification of anti-bacteria antibodies

ELISA tests were performed in paired serum and CSF samples as previously described to detect antibodies against *Akkermansia muciniphila* [20]. Bacterial proteins were coated at 1µg/ml in phosphate buffer saline (PBS) overnight at 4°C. Blocking was performed using Bovine serum albumin (Sigma Aldrich) at 1% in PBS during 1 hour at 37°C. Patient samples were incubated for 2 hours at 37°C in PBS, 1% bovine serum albumin (dilutions 1/100 for serum, 1/10 for CSF). Anti-human IgG antibodies coupled with horseradish peroxidase (Bethyl Laboratories) at 1/5 000, 1 hour at 37°C, were used for detection. The reaction with the substrate (3,3′,5,5′-Tetramethylbenzidine, BD Biosciences) was stopped with sulfuric acid (0.18M, Sigma Aldrich) after 10 min. Plates were read at 450 nm using a Spark 10M multimode microplate reader (Tecan).

Routine CSF and Serum/Blood Measures

CSF measures were determined as previously reported [24]. Intrathecal indices for anti-*A. muciniphila* IgG were calculated using the following formula (Ig Index= (Ig CSF/ Alb CSF)/(Ig Serum / Alb Serum)). Routine blood analyses including cell counts for neutrophils, eosinophils, basophils and monocytes were performed in the Hematology Department, USZ.

HLA Typing

Patients were typed for HLA-class II (DRB1*, DRB3*, DRB4*, DRB5*, DQA1* and DQB1*) as previously reported [24].

Immunophenotyping

Flow cytometric immunophenotyping of CSF-infiltrating and paired circulating lymphocytes was performed as previously reported [24,25]. Antibodies: anti-CD3 AF700, anti-CD4 PE TR, anti-CD8 BV510, anti-CD45RA BV711, anti-CCR7 BV421, anti-CD27 APC Cy7, anti-CD28 PE Cy7, anti-CCR4 APC, anti-CRTh2 PE, anti-CCR6 BV785, anti-CD19 PerCPCy5.5, anti-IgD BV605 and anti-CD138 FITC. SPHEROTM AccuCount Particles (Sperotech, Inc. Lake Forest, IL) were added to determine absolute counts following manufacturer's instructions. Sample acquisition was done in a LSR Fortessa cytometer (BD Biosciences, Franklin Lakes, NJ) and data was analyzed using FACSDiva (BD) and FlowJO (TreeStar Inc., Ashland, OR, USA) software. The gating strategy is summarized in Figure 1.

Magnetic Resonance Imaging

Patients were scanned with a 3T Philips Ingenia or 3T Siemens Skyra. The MRI protocol included a 3D fluid-attenuated inversion recovery (FLAIR) sequence. The number and the total volume in ml of all hyperintense lesions were determined from the FLAIR images by an automatic algorithm based on convolutional neural networks [26]. Whole brain volume in ml was determined on the pre-contrast MPRAGE image using the automatic processing pipeline Biometrica MS® analysis platform (version 2.1, jung diagnostics GmbH, Hamburg, Germany) [27].

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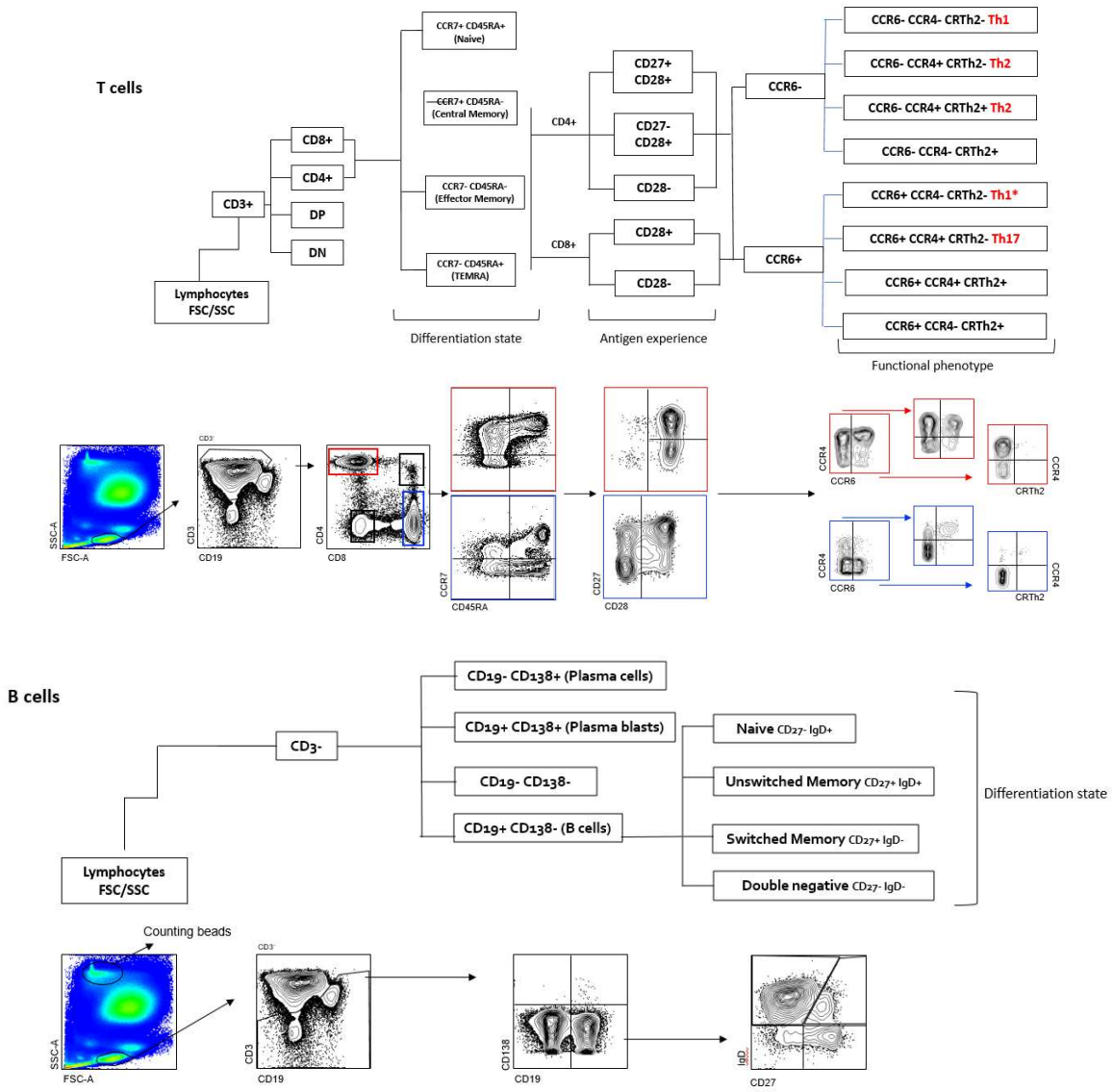

Figure 1. Gating strategy of T and B cells. Doublets were excluded, followed by identification of lymphocytes by size. Next, CD3- are identified and among them, plasma cells (CD19- CD138+), plasma blasts (CD19+ CD138+), B cells (CD19+ CD138-) and CD19- CD138- cells. Among B cells, naïve (IgD+ CD27-), unswitched memory (IgD+ CD27+), switched memory (IgD- CD27+) and doublé negative (IgD- CD27-) B cell subsets are also identified. In CD3+ T cells, CD3+ CD4+ and CD3+ CD8+ cells are first identified and then separated in CM (CCR7+ CD45RA-), EM (CCR7- CD45RA-), TEMRA (CCR7- CD45RA+) and naive (CCR7+ CD45RA+). CM, EM and TEMRA CD8+ T cells are then separated in CD28+ and CD28- while CM, EM and TEMRA CD4+ T cells in CD28+ CD27+, CD28+ CD27- and CD28-. Each one of these CD4+ and CD8+ T cells are separated first in CCR6- and CCR6+ and then in Th1 (CCR6- CCR4- CRTH2-), Th2-A (CCR6- CCR4+ CRTH2-), Th2-B (CCR6- CCR4+ CRTH2+), CCR6- CCR4- CRTH2+, Th1* (CCR6+ CCR4- CRTH2-), Th17 (CCR6- CCR4+ CRTH2-), CCR6+ CCR4+ CRTH2+ and CCR6+ CCR4- CRTH2+ cells.

Statistics

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, California, USA). For the comparison of two groups of patients we used U-test (Mann-Whitney) for not-normally distributed variables. For the comparison of more than two groups of patients we used Kruskal-Wallis test for not-normally distributed variables. Linear correlation between variables was tested using Spearman r for not-normally distributed variables. The significance level was set at $p < 0.05$. Associations were calculated using Fisher's Exact Test with 5% significance.

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3. Results

3.1. IgGs Specific for Gut Commensal Bacteria

Intrathecal and serum levels of anti-*A. muciniphila* IgG were measured in 61 MS patients (Figure 2a). In order to characterize patients differing in anti-*A. muciniphila* IgG index, we formed two patient groups, one with low and one with high index, by selecting the twenty patients with the lowest and highest anti-*A. muciniphila* IgG index. There were no significant differences between anti-*A. muciniphila* IgG indices in patients, who had never been treated and those with prior treatment (Figure 2b).

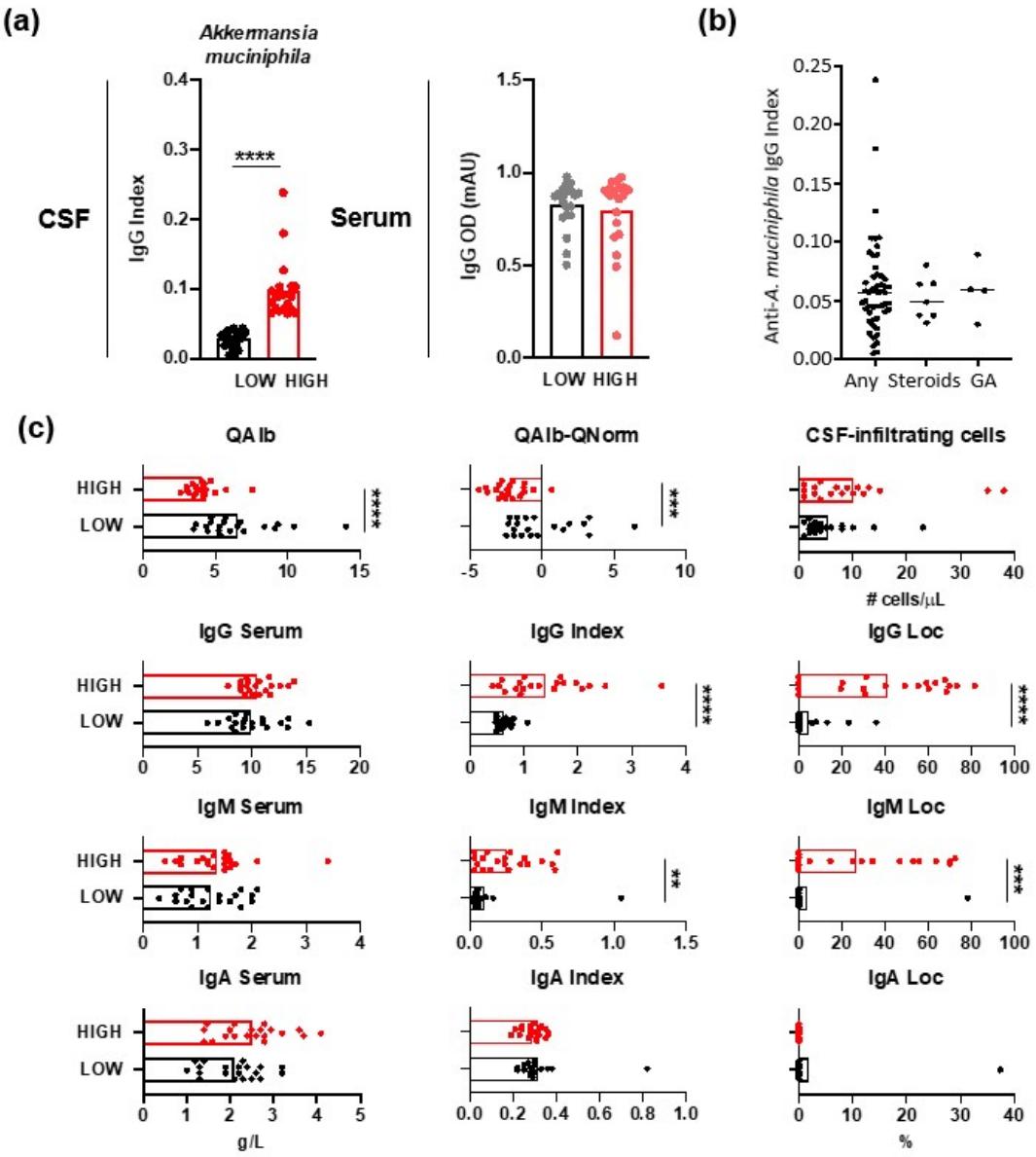

Figure 2. IgG specific for *A. muciniphila* bacteria in MS and their association with CSF measures. (**a**), IgG index and serum levels of IgG specific for *A. muciniphila* in MS patients. (**b**) Anti- *A. muciniphila* IgG indices in MS patients never treated, previously treated with steroids and previously treated with glatiramer acetate (GA). (**c**) CSF measures in patients with high (red) and low (black) anti-*A. muciniphila* IgG indices. Each dot in the graphs corresponds to a single patient and the bars show the mean. Kruskal-Wallis test was used to compare more than two groups of patients and Mann-Whitney test to compared two groups. Linear correlation between variables was tested using Spearman r correlation. Statistical significance (** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$) is shown.

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3.2. CSF Measures in MS Patients with Different Anti-A. muciniphila IgG Index

Patients with low anti-*A. muciniphila* IgG index showed significantly higher QAlb as well as QAlb-QNorm values, suggesting altered BBB permeability, but not higher numbers of CSF-infiltrating cells (Figure 2e). Intrathecal IgG and IgM indices and synthesis were increased in patients with high anti-*A. muciniphila* IgG index (Figure 2e).

3.2.1. B Cells in MS Patients with Different Anti-*A. muciniphila* IgG Index

Immunophenotyping of CSF samples demonstrated significantly elevated relative frequencies (Figure 3a) and absolute numbers (Figure 3b) of plasma blasts (CD3- CD138+ CD19+) and B cells (CD3- CD138- CD19+) in patients with high anti-*A. muciniphila* IgG index. Further, these frequencies (Figure 3a) and numbers (Figure 3b) correlated positively with the total- and anti-*A. muciniphila* IgG indices. The absolute numbers of circulating plasma blasts and B cells were also significantly higher in patients with high anti-*A. muciniphila* IgG index (Figure. 3c). While these counts did not correlate with the total IgG indices, they interestingly did with the anti-*A. muciniphila* IgG indices (Figure. 3c).

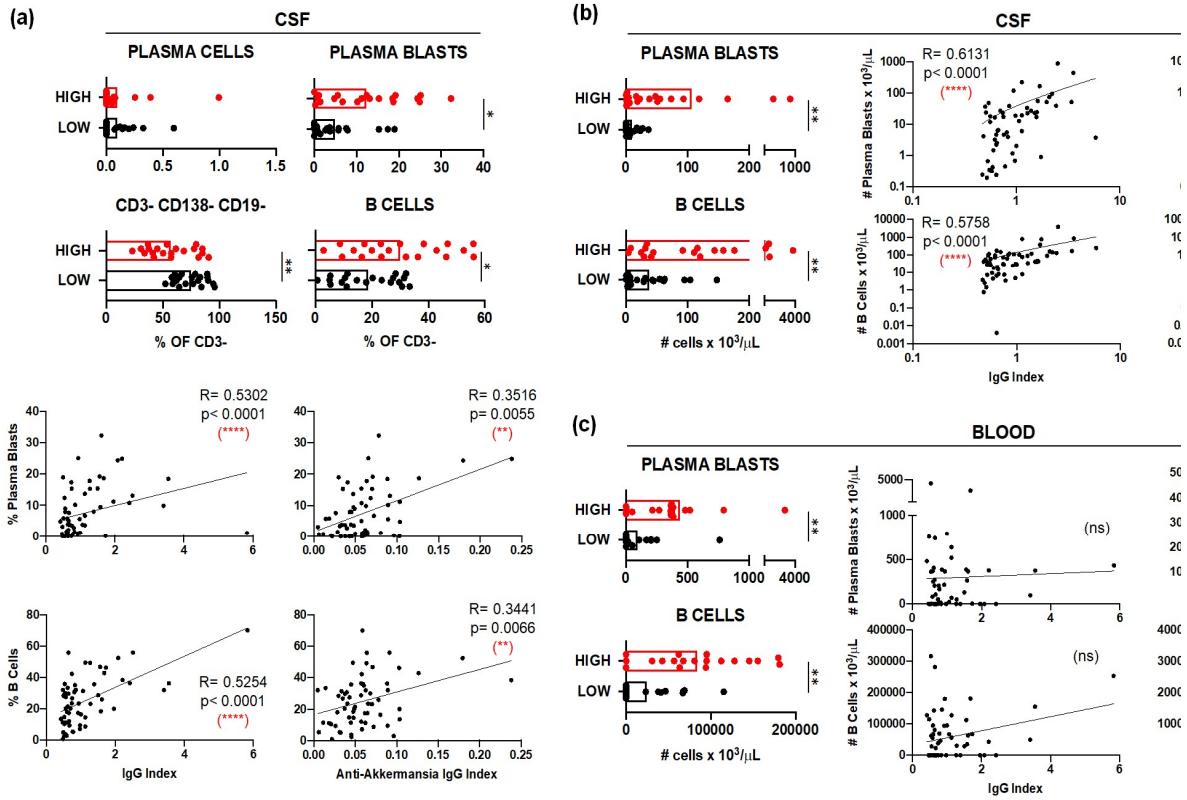

Figure 3. B cells and anti- *A. muciniphila* IgG indices. (**a**) Comparison between patients with high (red) and low (black) anti- *A. muciniphila* IgG indices of frequencies among CSF-infiltrating CD3- cells of plasma cells (CD138+ CD19-), plasma blasts (CD138+ CD19+), B cells (CD138- CD19+) and CD138- CD19- cells. Correlation between IgG indices (total- and anti-*A. muciniphila*) and frequencies of CSF-infiltrating plasmablasts and B cells. (**b** and **c**) Comparison between patients with high (red) and low (black) anti- *A. muciniphila* IgG indices of absolute numbers of CSF-infiltrating (**b**) and circulating (**c**) plasma blasts and B cells as well as correlations of these numbers with IgG indices (total- and anti- *A. muciniphila*) (**b** and **c**). Each dot in the graphs corresponds to a single patient and the bars show the mean. Mann-Whitney test was used to compare two groups of patients. Linear correlation between variables was tested using Spearman r correlation. Statistical significance (* p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001) is shown.

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3.2.2. Th2 Cells in MS Patients with Different Anti-*A. muciniphila* IgG Index

The relative frequencies and the absolute numbers of CD4+ central memory (CM, CCR7+ CD45RA-) T cells expressing the coreceptor CD28 but not CD27, and with a Th2-B (CCR6- CCR4+ CRTh2+) functional phenotype were significantly higher in the CSF of patients with high anti-*A. muciniphila* IgG index (Figure 4a). Furthermore, both the relative frequencies and absolute numbers correlated with the anti-*A. muciniphila* IgG indices (Figure 4b). In contrast, the relative frequencies and absolute numbers of these cells in peripheral blood did not show differences between patients nor did they correlate with anti-*A. muciniphila* IgG indices (Figure 4c,d).

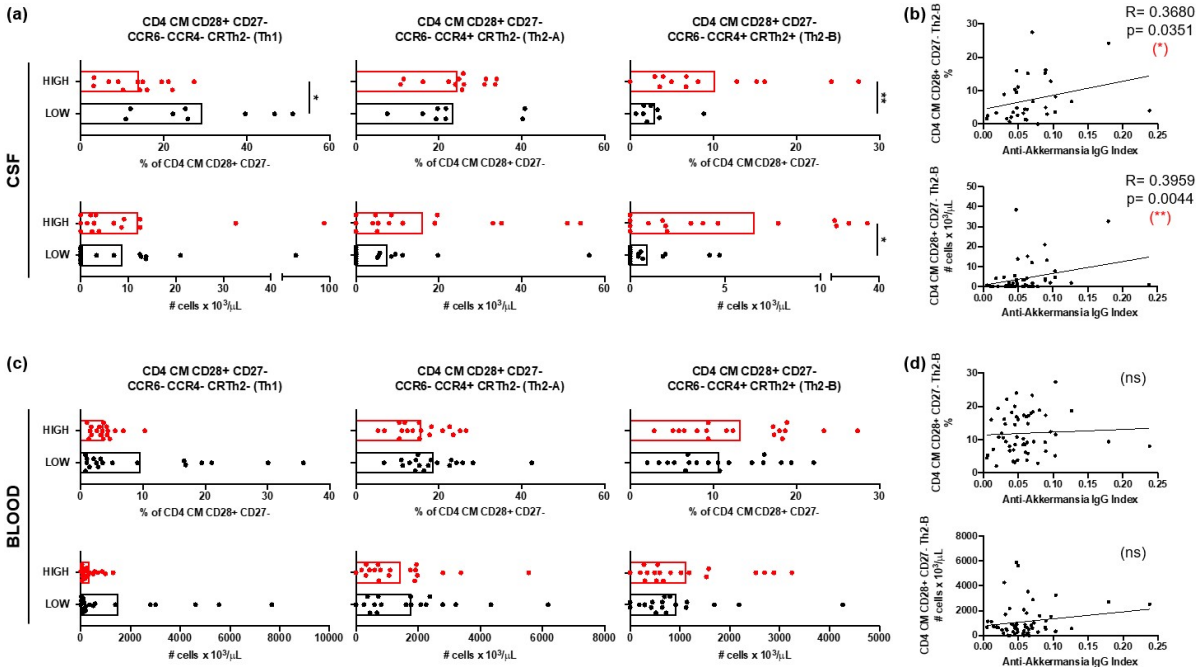

Figure 4. CSF-infiltrating T cells and anti- *A. muciniphila* IgG indices. (a and c), Relative frequencies and absolute numbers of CSF infiltrating **(a)** and circulating **(c)** CD4 CM CD28+ CD27- T cells with the following functional phenotypes Th1 (CCR6- CCR4- CRTh2-), Th2-A (CCR6- CCR4+ CRTh2-) and Th2-B (CCR6- CCR4+ CRTh2+) in patients with high (red) and low (black) anti- *A. muciniphila* IgG indices. (**b and d),** Correlation between IgG indices (total- and anti- *A. muciniphila*) and relative frequencies and absolute numbers of CSF-infiltrating **(b)** and circulating **(d)** CD4 CM CD28+ CD27- Th2- cells. Each dot in the graphs correspond to a single patient and the bars show the mean. Mann-Whitney test was used to compared two groups of patients. Linear correlation between variables was tested using Spearman r correlation. Statistical significance (* $p<0.05$, ** $p<0.01$) is shown.

Immunophenotyping of blood samples revealed that only T cells with a Th17 (CCR6+ CCR4+ CRTh2-) functional phenotype were increased in patients with high anti-*A. muciniphila* IgG indices (Figure 5). Circulating CM and TEMRA CD4+ T expressing the co-stimulatory molecules CD28 and CD27 as well as EM and TEMRA CD8+ T cells expressing the co-stimulatory molecule CD28 and all with a Th17 functional phenotype were more frequent and/or more abundant in blood from patients with high anti-*A. muciniphila* IgG indices (Figure 5).

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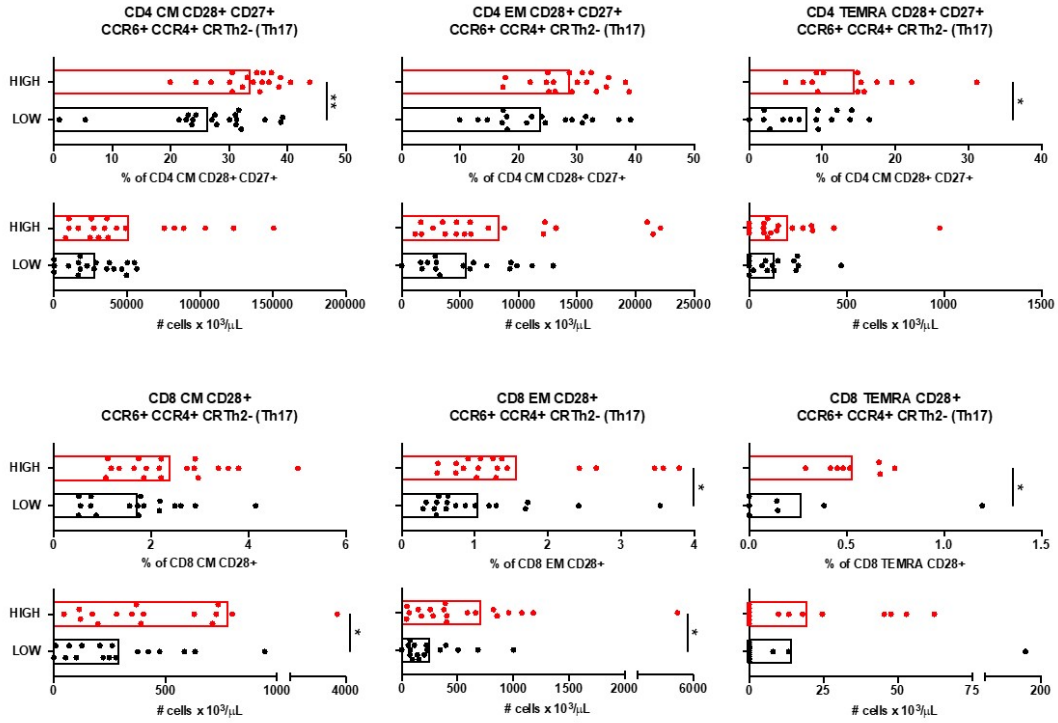

Figure 5. Circulating T cells and anti- ***A. muciniphila*** **IgG indices.** Relative frequencies and absolute numbers of circulating CD4 CD28+ CD27- and CD8 CD28+ T cells that have CM, EM and TEMRA differentiation state and a Th17 (CCR6+ CCR4+ CRTh2-) functional phenotype in patients with high (red) and low (black) anti- *A. muciniphila* IgG indices. Each dot in the graphs corresponds to a single patient and the bars show the mean. Mann-Whitney test was used to compare two groups of patients. Statistical significance (* $p<0.05$, ** $p<0.01$) is shown.

3.3. Characterization of Patients with Different Anti-A. muciniphila IgG Index

Demographic and clinical features did not differ between patients with low and high anti-*A. muciniphila* IgG index (Table 1).

Although brain MRI scans that have been obtained at the time of lumbar puncture were only available from twelve patients, these showed a statistically significant correlation between anti-*A. muciniphila* IgG indices and the total number and -volume of FLAIR T2 lesions, but not with the total brain volume (Figure 6a). We also addressed CNS damage and inflammation by using neurofilament light chain (NF-L) [28] and chitinase 3-like 1 (CHI3L1) [29] as biomarkers. Neither intrathecal NF-L nor CHI3L1 correlated with anti-*A. muciniphila* IgG indices (Figure 6b).

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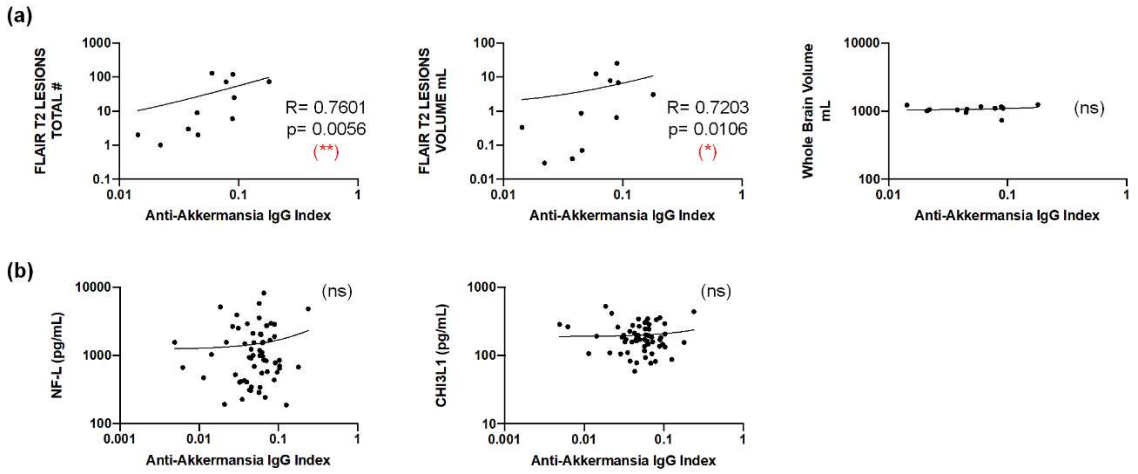

Figure 6. Association of anti-*A. muciniphila* IgG indices with MRI features. (**a**) Correlation between anti-*A. muciniphila* IgG indices and total number and volume of FLAIR T2 lesions and total brain volume. (**b**), Correlation between anti-*A. muciniphila* IgG indices and intrathecal NF-L, CHI3L1 and number of monocytes in whole blood. Each dot in the graphs correspond to a single patient and the bars show the mean. Linear correlation between variables was tested using Spearman r correlation. Statistical significance (* p<0.05, ** p<0.01) is shown.

Finally, we compared the number of circulating neutrophils, eosinophils, basophils and monocytes and found a negative correlation between the number of circulating monocytes and the anti-*A. muciniphila* IgG indices (Figure 7).

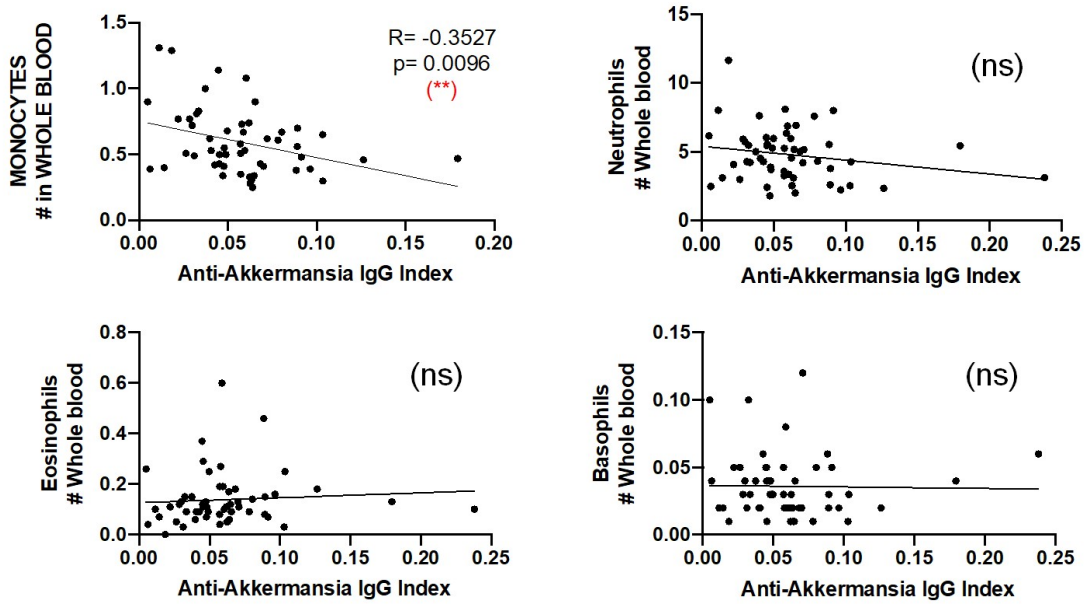

Figure 7. Correlation between anti-*A. muciniphila* IgG indices and number of neutrophils, eosinophils and basophils in whole blood. Each dot in the graphs corresponds to a single patient and the bars show the mean. Kruskal-Wallis test was used to compare more than two groups of patients and Mann-Whitney test to compared two groups. Linear correlation between variables was tested using Spearman r correlation.

4. Discussion

In this study, we aimed to find new evidence supporting a role of intrathecal anti- *A. muciniphila* IgG in MS pathogenesis and thoroughly characterized patients with different anti-*A. muciniphila* IgG

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index. The comparison of patients with low and high intrathecal IgG production against this gut bacterium identified significant differences regarding BBB permeability, CSF infiltrates, pro-inflammatory circulating immune cells as well as imaging features that indicate a role of these antibodies in MS pathogenesis.

MS patients with high anti-*A. muciniphila* IgG index also produced higher intrathecal IgG against other gut bacteria such as *P. melaninogenica*, *E. Coli* and *B. fragilis*, suggesting a leaky gut and a general translocation of gut commensal bacteria in these patients. However, intrathecal production of anti-*A. muciniphila* IgG did not correlate with serum levels of IgG specific for gut bacteria, which may indicate a selective recruitment into the CNS compartment of B cells producing these antibodies. The intrathecal synthesis of anti-*A. muciniphila* IgG is supported by higher intrathecal IgG and IgM synthesis in patients with high anti-*A. muciniphila* IgG index and also by higher amounts of CSF-infiltrating cells, which may be involved in antibody production such as plasma blasts, B cells and CD4+ CM CD28+ CD27- Th2-B cells. Furthermore, both the relative frequencies and absolute numbers of these cells correlated with anti-*A. muciniphila* IgG indices. CD4+ CM CD28+ CD27- Th2-B cells are probably relevant for providing B cell help since the downregulation of CD27 indicates repetitive stimulation with antigen [30], and the expression of CCR4 [31] and CRTh2 [32] a Th2 phenotype. Unexpectedly, MS patients with high anti-*A. muciniphila* IgG index had lower QAlb, suggesting that trafficking of albumin and cells through the BBB use different mechanisms.

Patients with high anti-*A. muciniphila* IgG index also showed slightly higher pro-inflammatory Th17 cells. This is of interest since Th17 cells have been associated with many autoimmune diseases and are crucial in immune responses against bacterial infections [33] and also against bacteria translocation [34].

Finally, despite the low number of patients from whom brain MRI scans were available at the time of lumbar puncture, anti-*A. muciniphila* IgG indices nicely correlated with the number and volume of FLAIR T2 lesions in the brain, suggesting a possible involvement of these antibodies in demyelination. The total brain volume and markers of CNS damage/inflammation such as NF-L [28] and CHI3L1 [29] did not correlate with anti-*A. muciniphila* IgG indices, which renders an involvement of these antibodies in neurodegeneration unlikely.

5. Conclusions

Our results demonstrate an association between intrathecal anti-*A. muciniphila* IgG and CSF-infiltrating cells that are known to be involved in antibody production consistent with an intrathecal synthesis of anti-gut microbiota antibodies and a selective recruitment of specific immune cells into the CNS. The significant differences between patients with low and high anti-*A. muciniphila* IgG index regarding BBB permeability, MRI lesion load or peripheral inflammation, while preliminary, suggest an involvement of these antibodies in MS pathogenesis.

Author Contributions: Conceptualization, M.S. and L.B.; methodology, C.M., C.C.; validation, C.C., and C.M.; formal analysis, M.S., L.B.; investigation, X.X.; resources, X.X.; data curation, X.X.; writing—original draft preparation, M.S.; writing—review and editing, A.B.N., D.A.L., R.M., M.S. and L.B.; visualization, X.X.; supervision, X.X. D.A.L., R.M., M.S. and L.B.; project administration, X.X.; funding acquisition, R.M., D.A.L., M.S. and L.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The Cantonal Ethics Committee of Zurich approved the study procedures (EC-No. 2013-0001).

Informed Consent Statement: Informed consent was obtained from all subjects and their relatives involved in the study.

Data Availability Statement: Data are available upon reasonable requests.

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