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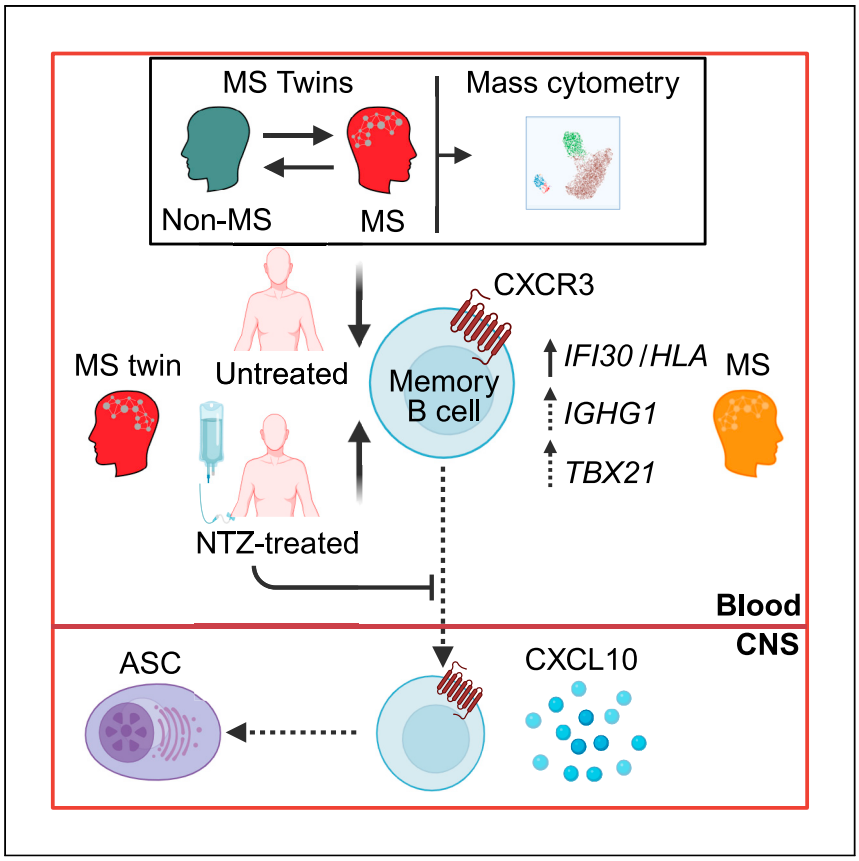
Twin study dissects CXCR3^{+} memory B cells as non-heritable feature in multiple sclerosis



Interrogating data of monozygotic twins discordant for multiple sclerosis (MS), Ingelfinger et al. identify decreased frequency of blood CXCR3^{+} B cells as a non-inheritable feature in MS. While the corresponding ligand, CXCL10, is increased in MS cerebrospinal fluid, therapeutically blocking cell migration reversed their reduction, indicating the tissue-infiltrating capacity of CXCR3^{+} B cells.

Florian Ingelfinger, Kirsten L. Kuiper, Can Ulutekin, ..., Joost Smolders, Marvin M. van Luijn, Burkhard Becher

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Highlights

Reduced frequencies of circulating CXCR3^{+} B cells is a non-heritable feature of MS

Reduction in circulating CXCR3^{+} B cells in MS twins is reversed by natalizumab

CXCR3 ligand CXCL10 levels are increased in MS cerebrospinal fluid compared to blood

CXCR3^{+} B cells have higher propensity to differentiate into antibody-secreting cells

Translation to Patients

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Report

Twin study dissects CXCR3$^+$ memory B cells as non-heritable feature in multiple sclerosis

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SUMMARY

Background: In multiple sclerosis (MS), B cells are considered main triggers of the disease, likely as the result of complex interaction between genetic and environmental risk factors. Studies on monozygotic twins discordant for MS offer a unique way to reduce this complexity and reveal discrepant subsets.

Methods: In this study, we analyzed B cell subsets in blood samples of monozygotic twins with and without MS using publicly available data. We verified functional characteristics by exploring the role of therapy and performed separate analyses in unrelated individuals.

Findings: The frequencies of CXCR3$^+$ memory B cells were reduced in the blood of genetically identical twins with MS compared to their unaffected twin siblings. Natalizumab (anti-VLA-4 antibody) was the only treatment regimen under which these frequencies were reversed. The CNS-homing features of CXCR3$^+$ memory B cells were supported by elevated CXCL10 levels in MS cerebrospinal fluid and their *in vitro* propensity to develop into antibody-secreting cells.

Conclusions: Circulating CXCR3$^+$ memory B cells are affected by non-heritable cues in people who develop MS. This underlines the requirement of environmental risk factors such as Epstein-Barr virus in triggering these B cells. We propose that after CXCL10-mediated entry into the CNS, CXCR3$^+$ memory B cells mature into antibody-secreting cells to drive MS.

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CONTEXT AND SIGNIFICANCE

Multiple sclerosis (MS) is the most common chronic demyelinating disorder of the central nervous system (CNS), affecting about 2.8 million people worldwide. The course of this disease is highly unpredictable, probably driven by the impact of genetic and environmental cues on the immune system. Although the success of anti-CD20 treatment and the role of Epstein-Barr virus (EBV) put forward B cells as key peripheral drivers of MS, we do not know which and how subsets are altered by such cues to enter and produce antibodies in the CNS. With the presence of CXCR3$^+$ B cells in the circulation as a non-inheritable MS feature, which is possibly the result of a prior EBV infection, we provide a new lens on the heterogeneity of the disease that may further support the development of more refined predictive tools and therapeutic strategies.

INTRODUCTION

B cells that express the T-box transcription factor T-bet are increasingly recognized to be involved in anti-viral responses and autoimmunity. The ultimate effector function of these B cells not only depends on their specificity but is also determined by their differentiation program and tissue-infiltrating capacity. In individuals with multiple sclerosis (MS), T-bet$^+$ B cells differentiate into CXCR3$^+$ subsets, which are prone to infiltrate and mature into antibody-secreting cells in the central nervous system (CNS).[1,2] This evidently differs from individuals affected by other autoimmune diseases, such as systemic lupus erythematosus, in which (auto)antibodies are produced by T-bet$^+$ B cells in the periphery.[3] In MS, peripheral B cells are directly influenced by genetic drivers and environmental risk factors (e.g., Epstein-Barr virus

[EBV]), which likely define their differentiation and effector function.[4] B cell-depleting agents have been highly efficient in the treatment of MS, although functional understanding of their contribution to disease remains largely enigmatic.[5] Thus, we discerned genetic predisposition from non-heritable, disease-related effects on the circulating B cell compartment in monozygotic twin pairs discordant for MS.

RESULTS

For this, we analyzed B cells of publicly available data obtained from peripheral blood mononuclear cells (PBMCs) of 57 monozygotic twin pairs discordant for MS using mass cytometry.[6] Dimensionality reduction using uniform manifold approximation and projection and unsupervised FlowSOM clustering identified dominant B cell populations in human blood: naive B cells, memory B cells, plasmablasts, marginal zone-like B cells, immature B cells, and transitional B cells (Figure 1A). In addition, FlowSOM clustering yielded a population of class-switched CXCR3$^+$ memory B cells, which have been reported to enter the CNS of individuals with MS (Figures 1A and 1B).[1]

To study whether CXCR3$^+$ memory B cells are controlled by genetic or non-heritable factors, we next assessed the composition of the B cell compartment in genetically identical twins with MS compared to their unaffected twin siblings. The frequencies of CXCR3$^+$ class-switched memory B cells were significantly reduced in blood samples of twins with MS compared to unaffected twins (Figure 1C). Of note, and in contrast to other chemokine receptors, such as CXCR5 and CCR6, CXCR3 was only expressed by a fraction of memory B cells and was the only chemokine receptor differentially expressed in memory B cells of twins discordant for MS (Figures S1A–S1C). This is in line with the results from a previous case-control study using genetically unrelated individuals with MS and suggests preferential recruitment into the CNS.[1] However, except for plasmablasts, each of the B cell subsets analyzed demonstrated significant alterations in their frequency (Figure 1C). Interestingly, when analyzing twin pairs in which the MS-affected twin did not receive immunomodulatory treatment, CXCR3$^+$ memory B cells stood out as the only subset affected by the disease (Figure 1D). These observations indicate that most immune perturbations in the remaining B cell clusters were elicited by disease-modifying therapy rather than by the disease per se (Figure S1D). To elucidate the transcriptional profile of CXCR3$^+$ memory B cells, we analyzed publicly available CITE-seq (cellular indexing of transcriptomes and epitopes) data of PBMCs from patients with MS.[7] We observed that CXCR3$^+$ memory B cells of patients with MS had increased expression of the interferon-γ-induced transcript *IFI30*, a concomitant increase in transcripts associated with antigen presentation, and a trend for increased expression levels of *IGHG1* and *TBX21* encoding for T-bet (Figures 1E and 1F), all features that link to the stimulation of CXCR3 expression.[1] In line with the concept that circulating CXCR3$^+$ memory B cells preferentially enter the CNS to mature into antibody-secreting cells in MS,[1,2,8] we additionally revealed that the concentration of CXCL10, the ligand of CXCR3, was increased in the cerebrospinal fluid (CSF) compared to paired serum from patients with MS (Figure 1G). Moreover, CXCR3$^+$ memory B cells were more prone to differentiate into antibody-secreting cells than CXCR3 counterparts under T cell-dependent *in vitro* conditions (Figure 1H).

Next, we assessed whether CXCR3$^+$ memory B cells were affected by disease-modifying therapy other than B cell-depleting agents. We analyzed the inter-twin-pair difference in frequency of CXCR3$^+$ memory B cells and stratified twin pairs by treatment regimen of the MS-affected twin. Of note, natalizumab

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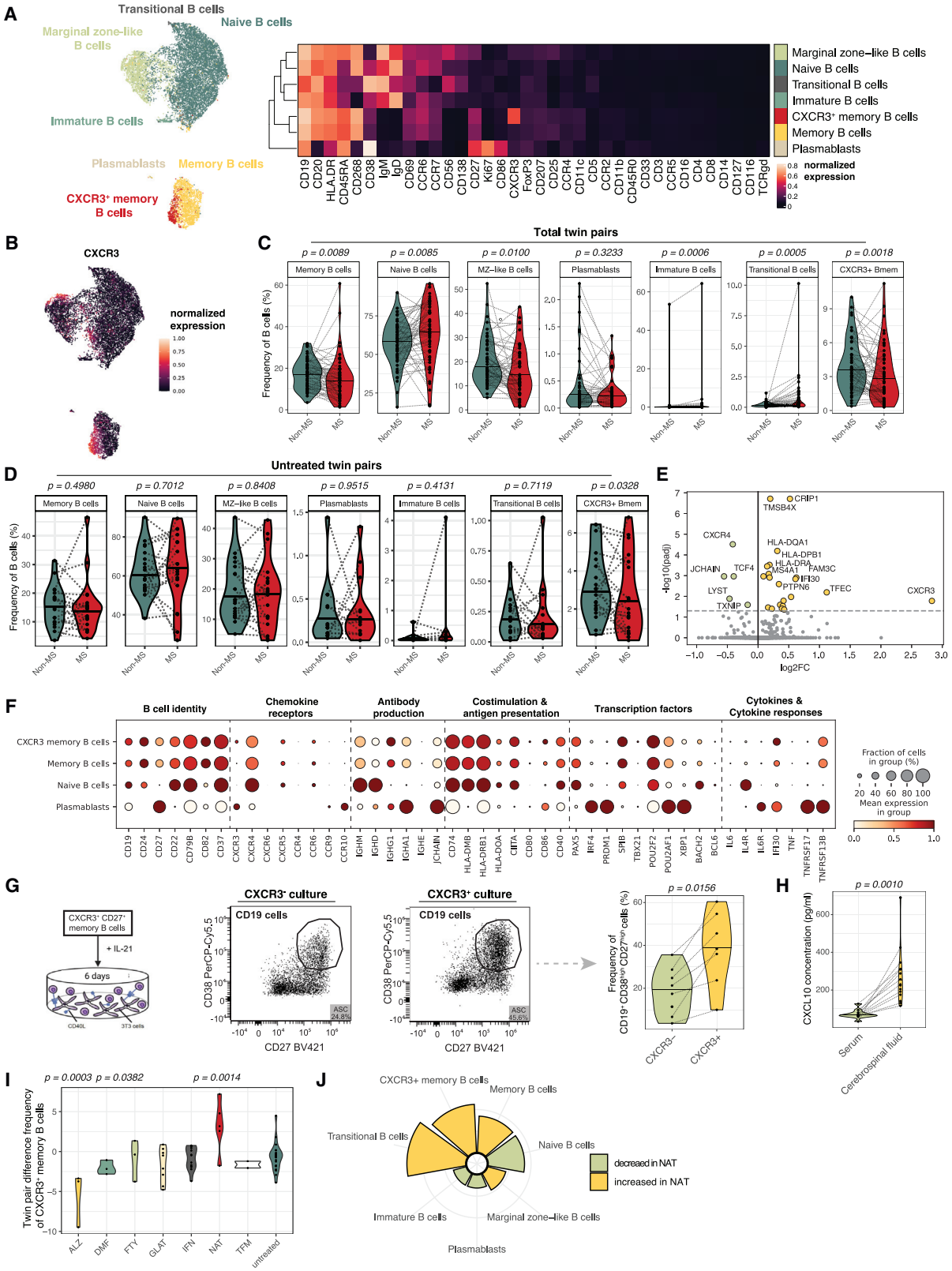
A
Transitional B cells
Naive B cells
Marginal zone-like B cells
Immature B cells
Plasmablasts
Memory B cells
CXCR3+ memory B cells
Marginal zone–like B cells
Naive B cells
Transitional B cells
Immature B cells
CXCR3+ memory B cells
Memory B cells
Plasmablasts
normalized expression
CD19 CD20 HLA.DR CD45RA CD268 CD38 IgM IgD CD69 CCR6 CCR7 CD56 CD138 CD27 Ki67 CD86 CXCR3 FoxP3 CD207 CD25 CCR4 CD11c CD5 CCR2 CD11b CD45R0 CD33 CD3 CCR5 CD16 CD4 CD8 CD14 CD127 CD116 TCRgd
B
CXCR3
normalized expression
C
Total twin pairs
p = 0.0089
p = 0.0085
p = 0.0100
p = 0.3233
p = 0.0006
p = 0.0005
p = 0.0018
Memory B cells
Naive B cells
MZ–like B cells
Plasmablasts
Immature B cells
Transitional B cells
CXCR3+ Bmem
Frequency of B cells (%)
Non-MS
MS
D
Untreated twin pairs
p = 0.4980
p = 0.7012
p = 0.8408
p = 0.9515
p = 0.4131
p = 0.7119
p = 0.0328
E
-log10(padj)
log2FC
CRIP1
TMSB4X
CXCR4
HLA-DQA1
HLA-DPB1
HLA-DRA
JCHAIN
TCF4
MS4A1
FAM3C
IFI30
PTPN6
TFEC
CXCR3
LYST
TXNIP
F
B cell identity
Chemokine receptors
Antibody production
Costimulation & antigen presentation
Transcription factors
Cytokines & Cytokine responses
CXCR3 memory B cells
Memory B cells
Naive B cells
Plasmablasts
Fraction of cells in group (%)
Mean expression in group
CD19 CD24 CD27 CD22 CD79B CD82 CD37 CXCR3 CXCR4 CXCR6 CXCR5 CCR4 CCR6 CCR9 CCR10 IGHM IGHD IGHG1 IGHA1 IGHE JCHAIN CD74 HLA-DMB HLA-DRB1 HLA-DOA CIITA CD80 CD86 CD40 PAX5 IRF4 PRDM1 SPIB TBX21 POU2F2 POU2AF1 XBP1 BACH2 BCL6 IL6 IL4R IL6R IFI30 TNF TNFRSF17 TNFRSF13B
G
CXCR3+ CD27+ memory B cells
+ IL-21
6 days
CD40L
3T3 cells
CXCR3- culture
CXCR3+ culture
CD19 cells
CD38 PerCP-Cy5.5
CD27 BV421
ASC 24.8%
ASC 45.6%
p = 0.0156
Frequency of CD19+ CD38high CD27high cells (%)
CXCR3–
CXCR3+
H
p = 0.0010
CXCL10 concentration (pg/ml)
Serum
Cerebrospinal fluid
I
p = 0.0003
p = 0.0382
p = 0.0014
Twin pair difference frequency of CXCR3+ memory B cells
ALZ
DMF
FTY
GLAT
IFN
NAT
TFM
untreated
J
CXCR3+ memory B cells
Memory B cells
Transitional B cells
Naive B cells
Immature B cells
Marginal zone–like B cells
Plasmablasts
decreaed in NAT
increased in NAT

Figure 1. Twin study identifies CXCR3$^+$ memory B cells as non-heritable component in multiple sclerosis

Mass cytometry analysis of the peripheral B cell compartment of 57 twin pairs discordant for multiple sclerosis (MS). Uniform manifold approximation and projection (UMAP) of 100 cells/sample randomly drawn from the combined dataset (A, left). Colors indicate FlowSOM clustering and concomitant manual annotation, and heatmap displays corresponding expression profiles (A, right). UMAP overlay showing the CXCR3 expression across B cells (B). Violin plots showing the frequency of B cell clusters among total B cells for twins with MS and non-MS twins across all twin pairs analyzed (n = 57; C) and twin pairs in which the twin with MS did not receive immunomodulatory treatment (n = 20; D). Dashed line indicates twinship. Volcano plot showing the differentially expressed genes between CXCR3$^+$ and CXCR3$^-$ memory B cells in CITE-seq data of patients with MS (n = 20; E). Dot plot showing gene expression of indicated B cell signature genes across B cell clusters in the CITE-seq dataset (n = 20; F). Schematic (left), representative biaxial flow cytometry plots (middle), and violin plot (right) showing CXCR3$^+$ and CXCR3$^-$ memory B cells derived from healthy blood donors differentiated into antibody-secreting cells in the presence of CD40L and interleukin-21 (IL-21) (n = 8; G). Violin plots showing the CXCL10 concentrations in the serum and cerebrospinal fluid of patients with newly diagnosed MS (n = 11 pairs; H). Violin plots showing the inter-twin-pair difference for the frequency of CXCR3$^+$ memory B cells separated by treatment of the MS-affected twin. ALZ, alemtuzumab (n = 3); DMF, dimethyl fumarate (n = 2); FTY, fingolimod (n = 4); GLAT, glatiramer acetate (n = 8); IFN, type 1 interferons (n = 14); NAT, natalizumab (n = 4); TFM, teriflunomide (n = 2; I). Effect size for the intra-twin-pair changes in B cell subset frequency of natalizumab-treated twin pairs (n = 4) compared to twin pairs in which the MS-affected twin did not receive immunomodulatory treatment (n = 20; J). Comparisons were performed using a two-sided paired non-parametric Wilcoxon signed-rank test (C, D, G, and H) or a two-sided unpaired non-parametric Mann-Whitney-Wilcoxon test (I and J). Differential gene expression was computed using a two-sided unpaired non-parametric Mann-Whitney-Wilcoxon test with Benjamini-Hochberg adjustment to control false discovery.

(anti-VLA-4 antibody) treatment of the MS-affected sibling reversed the relative decrease observed in untreated twins with MS (Figures 1D–1I and 1J). Notably, among the B cell subsets analyzed, natalizumab exerted its strongest effect on transitional B cells, followed by CXCR3$^+$ memory B cells and CXCR3$^-$ memory B cells (Figures 1J, S1D, and S1E). This observation implies that the migration of circulating CXCR3$^+$ memory B cells into the CNS could be blocked. In contrast, treatment using dimethyl fumarate and alemtuzumab further reduced their relative abundance in twins with MS, in the latter case possibly due to depletion of more differentiated leukocytes.

DISCUSSION

In conclusion, interrogating the B cells of 57 twin pairs discordant for MS revealed that CXCR3$^+$ memory B cells are hardwired by non-heritable cues in MS promoting migration into the CNS and potential differentiation into antibody-secreting cells. A major driver of disease could be infection with EBV, which is not only a prerequisite for developing MS in genetically susceptible individuals[4] but also positively corresponds to CXCR3 expression on class-switched memory B cells in individuals with MS.[8] As an underlying mechanism, EBV has been proposed as a transcriptional regulator of risk loci for MS and other autoimmune diseases,[2,4] which may shape the development and function of CXCR3$^+$ B cells even in a T-bet-independent manner.[9] With age being the major risk factor for MS disease progression, it could be expected that these types of B cells slowly accumulate and develop into antibody-secreting cells within the CNS to eventually contribute to MS pathology.[2,10]

Limitations of the study

Our study lacks CSF samples for comparison of CXCL10 levels between twin pairs discordant for MS. In addition, the observed reduction in frequencies of circulating CXCR3$^+$ memory B cells for twins with MS (and the reversal after natalizumab treatment) clearly supports but does not necessarily solidify the impact of non-heritable cues on the recruitment and maturation of this B cell subset within the CNS. Correlations between CSF CXCL10 and circulating CXCR3$^+$ memory B cells in twins with MS versus non-MS twins would have strengthened this notion. Our study would have been further enriched if blood samples of twins with MS were taken before and after disease-modifying treatment including natalizumab and if blood samples of healthy twins were included. Despite genetic similarities, the study group is representative of the total MS population (e.g., 3:1 female:male), for

which infection as well as treatment with antibiotics and steroids were used as exclusion criteria.[6]

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.medj.2024.02.013.

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AUTHOR CONTRIBUTIONS

F.I. and S.M. analyzed data. F.I., K.L.K., M.M.v.L., J.S., B.B., L.A.G., and S.M. interpreted the results. K.L.K., C.U., and L.R. performed experiments. L.A.G. provided clinical samples. F.I., M.M.v.L., J.S., and B.B. conceptualized the study. B.B. provided funding. F.I. and M.M.v.L. wrote the manuscript. B.B., S.M., J.S., and L.A.G. edited the manuscript. F.I., S.M., M.M.v.L., and B.B. had unrestricted access to all data. All authors read and approved the final article and take responsibility for its content.

DECLARATION OF INTERESTS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BV421 CD27	BD Biosciences	Cat# 562513; RRID: AB_11153497
BV785 CD19	Biolegend	Cat# 302240; RRID: AB_2563442
APC CD38	Biolegend	Cat# 303510; RRID: AB_314362
Alexa Fluor 700 CD3	Biolegend	Cat# 344822; RRID: AB_2563420
PE-CF594 IgD	BD Biosciences	Cat# 562540; RRID: AB_11153129
PE-Cy7 CXCR3	Biolegend	Cat# 353720; RRID: AB_11219383
Fixable Viability Dye eFluor™ 520	Thermo Fisher	Cat# 65-0867-18; RRID: -
PerCP-Cy5.5 CD38	Biolegend	Cat# 303522; RRID: AB_893314
Biological samples		
Human serum samples of MS patients	This paper	N/A
Human CSF samples of MS patients	This paper	N/A
Human PBMC samples of healthy individuals	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Human IL-21 Recombinant Protein	Thermo Fisher	PHC0215
Critical commercial assays		
Human CXCL10/IP-10 Quantikine ELISA Kit	R&D	DIP100
Deposited data		
Mass cytometry data of twin pairs discordant for MS	Ingelfinger et al.[6]	Mendeley Data: https://doi.org/10.17632/fzs5ph5p8s.1
CITE-seq data of MS patients undergoing vitamin D treatment	Galoppin et al.[7]	GSE239626
Experimental models: Cell lines		
Murine NIH3T3 fibroblasts expressing human CD40L (3T3-CD40L)	Acquired as described earlier (Rijvers, Van Langelaar et al.)[11]	https://doi.org/10.1172/jci.insight.160909
Software and algorithms		
R 4.1.2	R Foundation for Statistical Computing	https://www.r-project.org/
Python 3.9.7	Python Software Foundation	https://www.python.org
Analysis notebooks for mass cytometry and CITE-seq analysis	This paper	https://doi.org/10.5281/zenodo.10696551
Other		
Infinite M200 Pro Microplate Reader	Tecan Life Sciences	N/A
FACSAria™ Fusion cell sorter	BD Biosciences	N/A
RS320 X-ray	Beckhoff	N/A
Cytek Aurora flow cytometer	Cytek Biosciences	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Burkhard Becher (becher@immunology.uzh.ch).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This paper analyzes existing, publicly available data. Mass cytometry and CITE-seq data have been accessed from publicly available repositories and are listed in the key resource table. Raw ELISA data of CSF and serum of MS patients and

cytometry data of the long-term B cell cultures are available upon request by the lead author without any requirements, such as MTA.

- Notebooks to reproduce the mass cytometry and CITE-seq analyses are available at a public repository as listed in the key resource table. This study does not report original code. Any additional information required to reproduce the analysis is available from the lead author upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The study was approved by the local ethics committee of the Ludwig-Maximilian University Munich (project no. 163-16 and 18–419), and written informed consent was granted by all participants included in the study. Participants information on sex, age and study enrollment of the subjects is physician reported and is displayed in the Table S1.

METHOD DETAILS

Mass cytometry analysis of monozygotic twins

Mass cytometry data of the peripheral blood of monozygotic twin pairs discordant for MS has been accessed at Mendeley Data: https://doi.org/10.17632/fzs5ph5p8s.1 and analyzed in R. Based on provided cell type labels provided in the original manuscript[6] B cells have been selected and analyzed as described before.[12] In short, B cells have been clustered using *FlowSOM*[13] and manually merged. Dimensionality reduction was performed using UMAP. Visualizations have been drawn using *ggplot2* and *ComplexHeatmap*. Detailed analysis notebooks are listed in the key resource table.

CITE-seq analysis of the MS patients

CITE-seq data of the peripheral B cell compartment of MS patients have been accessed at GSE239626[7] and analyzed in python 3.9.7 using *scanpy*[14] and *scvi*.[15] Cells of both, MS patients receiving vitamin D treatment and placebo, were filtered for having more than 500 unique genes and less than 17 mitochondrial counts. RNA counts were normalized to each cell's library size and log1p transformed. The 5000 most highly variable genes were selected and cells from individual patients were integrated using *scvi* using default parameters. The scvi latent space was used to compute a neighbor graph, perform dimensionality reduction using umap and assign PBMCs into cell types using leiden clustering. B cells have been annotated based on marker genes and prior knowledge. Protein expression of the CITE-seq data has been normalized using a centered-log ratio transformation implemented in *muon*.[16] Memory B cells have been dissected into $CXCR3^{+}$ and $CXCR3^{-}$ memory B cells by manual gating on CXCR3 protein expression. Volcano plot was drawn in *seaborn*. Dot plots of selected genes were drawn in *scanpy*. Detailed analysis notebooks are listed in the key resource table.

Long-term B cell cultures

In vitro memory B-cell differentiation assays were performed as described earlier.[1] In summary, 1000 FACS-sorted $CXCR3^{+}$ or $CXCR3^{-}$ memory ($CD19^{+}CD38^{-/dim}CD27^{+}$) B cells were cultured on irradiated 3T3 fibroblasts expressing human CD40L in the presence of rhIL-21 (50 ng/mL; Thermo Fisher Scientific) to trigger ($CD19^{+}CD38^{high}CD27^{high}$) ASC outgrowth. Viable (live/$dead^{-}$) $CD19^{+}$ cells were analyzed using spectral flow cytometry upon 6 days of culturing.

Collection of serum and CSF of MS patients

CSF and serum samples were collected as a clinical diagnostic procedure to confirm the diagnosis of relapsing MS in treatment naive patients with clinical neurological

and MRI-findings highly suspicious of onset of MS. The study was approved by the local ethics committee of the Ludwig-Maximilian University Munich (project no. 163-16 and 18–419), and written informed consent was granted by all participants included in the study.

Analysis of CXCL10 in MS patients

The levels of CXCL10 in serum and liquor samples were determined through enzyme-linked immunosorbent assay (ELISA) using the human CXCL10/IP-10 Quantikine ELISA kit from R&D Systems, following the manufacturer's guidelines. All samples were processed and read in duplicates, and the averaged measurements were utilized for the subsequent analysis. Wavelength absorption was read using the Tecan infinite M200 Pro microplate reader.

QUANTIFICATION AND STATISTICAL ANALYSIS

Two-sided paired non-parametric Wilcoxon signed-rank test and two-sided unpaired nonparametric Mann-Whitney-Wilcoxon test have been performed in R using the *ggpubr* package to assess differences in B cell frequencies in the mass cytometry dataset. Differential gene expression has been performed using a two-sided unpaired nonparametric Mann-Whitney-Wilcoxon test with a false-discovery correction according to the Benjamini-Hochberg approach as implemented in *scanpy*. Details on the respective statistical tests, utilized sample sizes and definition of center, dispersion and precision measures are defined in the figure legends. P-values lower than 0.05 were considered statistically significant.