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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-heritable 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.

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

Florian Ingelfinger, Kirsten L. Kuiper, Can Ulutekin, ..., Joost Smolders, Marvin M. van Luijn, Burkhard Becher
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Twin study dissects CXCR3⁺ memory B cells as non-heritable feature in multiple sclerosis

Florian Ingelfinger,1,8 Kirsten L. Kuiper,5 Can Ulutekin,1 Lukas Rindlisbacher,1 Sarah Mundt,1 Lisa Ann Gerdes,2,3,4 Joost Smolders,5,6,7 Marvin M. van Luijn,5,9 and Burkhard Becher1,9,10,*

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.
Funding: This work was supported by Nationaal MS Fonds (OZ2021-016), Stichting MS Research (19-1057 MS, 20-490f MS, and 21-1142 MS), the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation program grant agreement no. 882424, and the Swiss National Science Foundation (733 310030_170320, 310030_188450, and CRSII5_183478).

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. B cell-depleting agents have been highly efficient in the treatment of MS, although functional understanding of their contribution to disease remains largely enigmatic. 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. 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).

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). 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. 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. 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. In line with the concept that circulating CXCR3+ memory B cells preferentially enter the CNS to mature into antibody-secreting cells (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 intertwin-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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(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−/C0 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 but also positively corresponds to CXCR3 expression on class-switched memory B cells in individuals with MS. As an underlying mechanism, EBV has been proposed as a transcriptional regulator of risk loci for MS and other autoimmune diseases, which may shape the development and function of CXCR3+ B cells even in a T-bet-independent manner. 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.

**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.

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.

ACKNOWLEDGMENTS
We thank the study participants that contributed to this study. This work was funded by the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation program grant agreement no. 882424 (to B.B.), the Switzerland National Science Foundation (733 310030_170320, 310030_188450, and CRSII5_183478 to B.B.), Nationaal MS Fonds (OZ2021-016), Stichting MS Research (19-1057 MS, 20-490f MS, and 21-1142 MS), Deutsche Forschungsgemeinschaft (DFG; German Research Foundation) under Germany’s Excellence Strategy within the framework of the Munich Cluster for Systems Neurology (EXC 2145 SyNergy: ID 390857198), the Gemeinnützige Hertie Stiftung, Bavarian Association, and National Association of the German MS Society (DMSG), Dr. Leopold and Carmen Ellinger Foundation, and the association “Verein zur Therapieforschung für MS Kranke e.V.” F.I. is recipient of an EMBO postdoctoral fellowship (ALTF 723-2022).

AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
J.S. received lecture and/or consultancy fees from Biogen, Merck, Novartis, and Sanofi Genzyme. M.M.v.L. received research support from EMD Serono, Merck, GSK, Novartis, and Idorsia Pharmaceuticals, Ltd. L.A.G. has received speaker honoraria,
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REFERENCES


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## STAR★METHODS

### KEY RESOURCES TABLE

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## 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 avail-
able 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 Uni-
versity 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 manuscript6 B cells have been selected and analyzed as described
before.12 In short, B cells have been clustered using FlowSOM13 and manually
merged. Dimensionality reduction was performed using UMAP. Visualizations
have been drawn using ggplot2 and ComplexHeatmap. Detailed analysis note-
books 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 ac-
cessed at GSE2396267 and analyzed in python 3.9.7 using scanpy14 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 anno-
tated based on marker genes and prior knowledge. Protein expression of the
CITE-seq data has been normalized using a centered-log ratio transformation imple-
mented 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 anal-
ysis 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−/dimCD27+)
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+CD38high
CD27high) 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.