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Article

Keywords:

Posted Date: March 23rd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8544428/v1>

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Additional Declarations: **Yes** there is potential Competing Interest. DG is an employee of Merck AB, Solna, Sweden, an affiliate of Merck KGaA. CS is currently an employee at AstraZeneca, Mölndal, Sweden, but had no disclosures at the time of the contribution to the present work. OT has received compensation for lectures and an advisory board from Merck and Pfizer respectively, which have no relation to the content of this study. TO has received lecture/advisory board honoraria from Merck, Novartis, Biogen and Sanofi, and unrestricted MS research grants from the same companies, none of which have contributed to this study. NR has received a research grant from Pfizer. FP has received research grants from Denka, Janssen, Merck KGaA, Novartis, Pfizer and UCB, and fees for serving on DMC in clinical trials with Lundbeck and Roche, and preparation of expert witness statement for Novartis. All other authors declare no competing interests.

Immune reprogramming following B-cell depletion in MS involves NF-κB activation and durable suppression of EBV host-pathogen interaction

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Abstract: 142 words (max 150)

Main text (excluding methods, references and legends): 3677 words (max 4000)

Figures and tables: 5 figures, 1 table (max 6 in total)

Supplementary material: 9 figures, 5 tables (no maximum)

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ABSTRACT (142/150)

Emerging data supports a durable immune reprogramming effect of B-cell-depleting therapies (BCDT) in relapsing-remitting multiple sclerosis (MS), although the underlying mechanisms remain unclear. We performed immunologic and transcriptomic profiling of repopulating B cells, including single-cell RNA sequencing of paired naïve and memory B cells before and after rituximab (RTX), at a time when peripheral B-cell counts had normalized. Differential expression analysis revealed upregulation of NF-κB-associated genes across naïve and memory B-cell clusters, correlating with increased circulating B-cell activating factor (BAFF). In contrast, repopulating switched memory B cells showed marked downregulation of an Epstein-Barr virus (EBV) lytic-infection gene signature, accompanied by reduced T-cell activation to EBV antigens and to anoctamin-2, a candidate autoantigen. Our findings support a mechanistic link between active EBV replication in memory B cells and autoreactive T-cell responses, a dysregulated immune axis that is attenuated in the reconstituted B-cell compartment.

INTRODUCTION

Epstein-Barr virus (EBV) infection appears to be a central event preceding the loss of tolerance to central nervous system (CNS) antigens in relapsing-remitting multiple sclerosis (RRMS).¹ EBV establishes a long-lasting infection within memory B cells, enabling expression of viral peptides that exhibit molecular mimicry with an expanding set of MS-associated candidate autoantigens, including glial cell adhesion molecule (GlialCAM), alpha-crystallin B (CRYAB), and anoctamin-2 (ANO2).²⁻⁵ Growing evidence further indicates that B cells promote inflammation in MS through dysregulated cytokine production and aberrant antigen presentation.^{6,7} Consistent with this, bidirectional communication between B and T cells is perturbed in MS,⁷⁻⁹ and ex vivo-isolated memory B cells from people with MS (pwMS) can induce proliferation and activation of memory effector T cells with encephalitogenic properties even in the absence of stimulation.¹⁰

B-cell-depleting therapies (BCDT), including the anti-CD20 monoclonal antibodies ocrelizumab, ofatumumab, ublituximab and rituximab (RTX), are strikingly effective in suppressing inflammatory disease activity in RRMS and are increasingly used in clinical practice.^{11,12} However, chronic exposure to BCDT is accompanied by increased risk of severe infections, reduced vaccine responses and hypogammaglobulinemia.¹³⁻¹⁵ During the COVID-19 pandemic, several centers implemented moderately extended or individualized dosing intervals based on the reappearance of circulating B cells,¹⁶⁻²⁰ whereas others adopted substantial interval extensions regardless of repopulation kinetics.^{21,22} Notably, relapse rates and magnetic resonance imaging (MRI) activity remained stable even after full numerical B-cell repopulation, which on average typically occurs 12 months after the last dose.²¹ Arguably, proportional imbalances favoring naïve over memory B-cell subtypes at repopulation do not fully explain the lack of disease activity, given that also memory B cells recover after BCDT, albeit more slowly.²² Together, these observations suggest a durable effect of BCDT driven by altered functional properties of repopulating cells rather than a constitutional feature of B cells, which would require constant depletion to maintain efficacy. Although increased expression of activation markers on repopulating B cells has been reported,²³ comprehensive phenotyping of B-cell populations following BCDT in MS remains limited and challenging due to their limited abundance.

The objective of this study was to define phenotypic alterations associated with sustained suppression of inflammatory disease activity after RTX treatment by characterizing the transcriptome of peripheral blood naïve and memory B cells from pwMS before and after RTX exposure using single-cell RNA sequencing (scRNA-seq). We further validated major findings at the RNA and protein levels and performed functional analyses in an expanded cohort of pwMS at different stages after RTX exposure.

RESULTS

Study design

Eighty-nine pwMS treated with RTX were included in the study (**Figure 1a**), of whom 67 had at least one paired baseline sample prior to RTX initiation, either in absence of any disease-modifying therapy (“Untreated”, n=57, contributing 59 samples) or during natalizumab treatment (“NTZ”, n=11, contributing 12 samples). One pwMS contributed to both groups. RTX-treated samples were categorized in relation to time point of collection: i) within 3 months of RTX initiation (“RTX 1-2M”, n=9), ii) between 3 and 6 months since RTX initiation, before the second infusion (“RTX 3-6M”, n=25), iii) during a regular biannual RTX dosing schedule (“RTX depl”, n=23), and iv) during an extended-interval dosing schedule with >6 months (median 14.7 months, interquartile range 12.2-19.1) since the most recent RTX dose and detectable B-cell levels on routine flow cytometry (“RTX repop”, n=45 individuals, contributing 53 distinct samples). Individual sample contributions to each group are shown in **Supplementary Table 1** and **Supplementary Figure 1**. For reference, we also included 39 pwMS on continuous NTZ treatment (for a total of 51 NTZ samples) and 41 healthy controls (HC).

Demographic characteristics are provided in **Table 1**. Untreated patients had a mean annualized relapse ratio (ARR) in the year preceding the sampling (ARR1y) of 0.71, while patients under long term NTZ or RTX treatment had an ARR1y <0.1. Specifically, during RTX treatment, ARR during the whole follow-up was 0 in all groups except RTX depl, where two relapses were recorded (mean ARR 0.06, SD ± 0.19). All RTX groups, except for RTX 1-2M, also displayed minimal rates (<0.05) of inflammatory disease activity on neuroimaging, calculated as number of MRI scans with new/enlarging T2 lesion or contrast-enhancing lesion per year of treatment.

From the overall cohort, samples were selected according to biomaterial availability (live frozen peripheral blood mononuclear cells – PBMC –, fresh frozen PBMC pellet, plasma or whole blood) for multilayer characterization, with sample contribution to each experiment as reported in **Figure 1b**.

B cells repopulating after RTX show a generalized activation related to the NF-κB pathway

B-cell repopulation dynamics, analyzed with spectral flow cytometry, varied markedly between individuals and showed no correlation with time since the last RTX infusion in the RTX repop group (**Figure 2a**). At the group level, however, RTX 3-6M samples were characterized by profound B-cell depletion, whereas RTX repop samples displayed nearly normalized total B-cell counts compared with baseline (**Figure 2b**). Analysis of B-cell subsets by flow cytometry revealed increased proportions of memory B cells in NTZ-treated pwMS relative to HC, while B cells repopulating after RTX predominantly exhibited transitional or naïve phenotypes (**Figure 2c**, **Supplementary Figure 2a**).

To explore features of repopulating B cells in greater detail, we selected paired samples from a subset of individuals (n = 10) with samples available at baseline (5 untreated and 5 NTZ) and at B-cell repopulation for scRNA-seq of sorted naïve and memory B and T cells. Follow-up samples were collected at the time of complete numerical B-cell reconstitution (mean B-cell count $0.16 \times 10^9/L$, range $0.03-0.56 \times 10^9/L$) (**Supplementary Figure 2b-c**). Memory B cells were enriched at a 1:2 naïve:memory ratio, given the proposed importance for MS pathogenesis of the memory subsets.^{6,10} Clustering of the resulting transcriptomes identified 12 clusters (**Figure 2d**), which were manually annotated based on immunoglobulin heavy chain expression (**Supplementary**

Figure 2d), characteristic immune genes (**Figure 2e**), and Monaco reference annotation scores (**Supplementary Figure 2e**).

As expected from quantitative changes between baseline and follow-up, non-memory clusters demonstrated a relative increase in transitional B cells (cluster Naïve1) compared with naïve cells (cluster Naïve2; **Figure 2f**). Within the memory compartment, cluster Switched2 and Unswitched1 were proportionally less abundant, whereas clusters Switched1, Switched3 and Mixed2 were increased at repopulation (**Figure 2f**).

We next examined qualitative transcriptomic changes in repopulating B cells using paired pseudobulk differential expression analyses on pooled naïve (clusters Naïve1-3) and memory (Unswitched1-3, Switched1-3, Mixed1-2 and Exhausted) clusters. Numerous immune genes were differentially regulated after RTX, and both naïve and memory B cells at repopulation exhibited upregulation of genes associated with immune activation, including *CD69*, *CD80*, *CD83*, *NFKBIA*, and the kinases *JUN* and *FOS* (**Figure 2g**). We then repeated differential expression analysis within each cluster and assessed enrichment of Gene Ontology Biological Processes (GO_BP) and Hallmark gene sets (**Figure 2h**). Multiple immune-related GO terms were upregulated in repopulating naïve and memory B cells, and Hallmark gene set enrichment analysis revealed an even more pronounced activation profile after RTX, including “IL6-JAK-STAT3 signaling,” “IL2-STAT5 signaling,” “interferon gamma response,” “complement,” and “TGFβ signaling,” along with a striking upregulation of “TNFα signaling via NF-κB” and “hypoxia” in 10 and 9 of the 12 clusters, respectively (**Figure 2h**).

Applying the same analysis to scRNA-seq data from total T cells sorted from the same individuals (**Supplementary Figure 3a-c**) revealed highly similar activation signatures after RTX, with significant enrichment of Hallmark’s “TNFα signaling via NF-κB,” “hypoxia,” and “mTORC signaling” in 9 of 10 T-cell clusters identified (**Supplementary Figure 3d**). These transcriptomic observations were validated at the protein level by flow cytometry on B cells, which showed increased expression of CD69 and CD86 after RTX treatment (**Figure 2i**).

Thus, repopulating B cells after RTX display robust, cluster-wide immune activation gene signatures (dominated by NF-κB-related pathways), shared by T cells, and confirmed at the protein level by upregulation of cell surface activation markers.

Persistent BAFF/APRIL elevation is associated with generalized B-cell activation after RTX

To assess whether the widespread activation observed across nearly all B-cell clusters, and also affecting T-cell clusters, was reflected in circulating proteins, we performed a multiplex bead assay on plasma from 32 HC, 42 NTZ-treated pwMS, 38 untreated pwMS before RTX, and 72 pwMS after RTX initiation (9 RTX 1-2M, 16 RTX 3-6M, 18 RTX depl, and 29 RTX repop). RTX exposure was associated with significantly elevated levels of BAFF, APRIL (**Figure 3a**), and CCL3, CXCL11, CXCL12 and CXCL13 chemokines (**Supplementary Figure 4**) compared with HC, untreated and NTZ groups. BAFF and APRIL increased shortly after treatment initiation, showed a partial reduction by 6 months, and reached their highest levels with prolonged B-cell depletion; notably, their concentrations did not fully normalize despite complete numerical B-cell repopulation. Activation markers CD27, CD40 and CD40L were also increased in the RTX 1-2M groups, while no significant difference was evident in the longer treatment duration group (**Supplementary Figure 4**). TNFα concentrations displayed a similar pattern to activation markers (**Figure 3a**), but levels were generally below the assay’s standard curve (6.9 pg/μl) and therefore could not be reliably quantified.

In samples profiled with scRNA-seq, BAFF levels showed a strong correlation ($r=0.82$, $p<0.0001$) with the average “TNF α signaling via NF- κ B” gene signature score in B cells, followed by APRIL, CCL3 and TNFR2, while levels of activation markers such as soluble CD40 and CD40L, and TNF α correlated poorly with this molecular signature (**Figure 3b** and **Supplementary Figure 5a**). This may suggest that persistently increased B-cell growth factors contribute to the observed activation. Interestingly, BAFF, APRIL, IL6R α and TNFR2 also correlated with the same signature in T cells (**Supplementary Figure 5b**).

Given the observed state of activation across virtually all B- and T-cell clusters, we hypothesized that this signature would also be detectable in PBMCs analyzed by bulk RNA sequencing (RNA-seq), performed on 16 HC, 25 untreated and 43 RTX treated pwMS (5 RTX 1-2M, 8 RTX 3-6M, 21 RTX depl, and 9 RTX repop). Indeed, averaging z-scores of genes belonging to the “TNF α signaling via NF- κ B” Hallmark signature within treatment groups revealed increased expression in the RTX 1-2M and RTX depl groups, i.e. during profound B-cell depletion, with no effect in the RTX 3-6M and RTX repop groups (**Figure 3c**). GSEA of Hallmark terms confirmed a significant enrichment of “TNF α signaling via NF- κ B” in the RTX depl compared with the untreated group (**Figure 3d**). Conversely, the RTX repop group showed a significant downregulation of this pathway, potentially reflecting longer intervals since the last RTX dose in this bulk RNA-seq cohort compared with the scRNA-seq cohort (27 vs. 18 months, respectively), or a diluted signal in the bulk PBMC setting.

Overall, plasma soluble molecules and bulk transcriptomics confirmed the broad NF- κ B-driven activation signature during RTX treatment detected in single-cell analyses, with BAFF/APRIL elevation strongly correlating with immune activation during and after RTX-induced B-cell depletion.

B-cell depletion alters EBV-host interaction dynamics

While B-cell repopulation was associated with cytokine-driven, generalized immune activation affecting both B and T cells, notably this activation did not translate into detectable signs of clinical or neuroradiological disease activity (**Table 1**). To define immune responses linked to disease biology, we next focused on downregulated genes in repopulating B cells. This analysis revealed a significant enrichment of GO terms related to protein translation, particularly in switched memory and exhausted memory B-cell clusters (**Figure 4a**). Many of these transcripts included ribosomal protein genes, which showed marked overlap with a molecular signature previously described in B cells undergoing lytic EBV infection and reported as enriched in B cells from deep cervical lymph nodes of pwMS.^{24,25}

Due to these findings, we next quantified expression of the EBV lytic gene set (**Supplementary Table 2**) across our dataset. At the B-subtype level (**Figure 4b**), switched memory and exhausted B cells displayed lower EBV lytic scores after RTX compared with their pretreatment counterparts and with HC (**Figure 4c**). Enrichment analyses of ranked differentially expressed genes further confirmed the significant downregulation of the EBV lytic signature in all switched memory clusters, followed by Exhausted and Unswitched1 clusters, after RTX (**Figure 4d**). Conversely, NTZ-treated baseline samples displayed upregulation of this signature in the Exhausted and Switched1 clusters compared with HC (**Supplementary Figure 6**).

To understand the functional implications of reduced EBV lytic transcription in switched memory B cells after RTX, we assessed whether EBV status was modified by RTX exposure. Quantitative PCR (qPCR) performed on whole blood revealed a numerical, though non-significant, reduction in the proportion of EBV-positive samples, which may reflect the limited

sensitivity of the assay due to the rarity of infected cells in peripheral blood (**Figure 4e**). We therefore examined indirect markers of EBV infectious burden; detection of anti-EBV antibodies by ELISA showed a significant reduction in anti-EBNA1 IgG levels at repopulation, while anti-BZLF1 IgG exhibited a downward trend (**Figure 4f** and **Supplementary Figure 7a-b**). This reduction was not accompanied by a significant reduction of total IgG concentrations as determined by clinical routine analyses (**Supplementary Figure 7c-d**).

To unravel the possible implications of a reduced EBV load on the functional properties of T cells, we evaluated T-cell responses to EBV proteins and MS-associated candidate autoantigens. A FluoroSpot assay measuring Th1- and Th17-associated cytokines was performed in 15 individuals with paired samples at baseline (untreated) and at B-cell repopulation, assessing responses to bead-coupled recombinant EBNA1, BZLF1 (n = 4), myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), ANO2 and a viral control (cytomegalovirus antigen pp65). Anti-CD3 stimulation and albumin binding domain (ABD)-coupled beads were used as positive and negative controls, respectively. Numbers of IFN γ -secreting cells in response to EBNA1 were significantly reduced after RTX compared with baseline, whereas responses to control stimulations (α CD3, ABD, pp65) remained unchanged (**Figure 4g**). Responses to BZLF1 also tended to be lower, although sample size limited statistical power. Interestingly, IFN γ responses to the autoantigen ANO2 were also significantly decreased at B-cell repopulation, while responses to MBP and MOG remained unaltered (**Figure 4g**). In contrast, IL-17A and IL-22 responses to all tested antigens showed no significant differences between baseline and post-RTX samples (**Supplementary Figure 8**).

In summary, these findings indicate a reduction of EBV burden during B-cell repopulation, accompanied by diminished T-cell reactivity to EBV antigens and a downregulated EBV lytic molecular footprint in repopulating B cells. These results support a model in which RTX may have immune reprogramming effects in RRMS.

Reduced Th17 and Th1/17 proportions and functions during B-cell depletion are restored at B-cell repopulation

To understand whether a long-lasting RTX effect was evident also on putatively pathogenic T-cell phenotypes, we performed flow cytometric characterization of T cells in samples used for B-cell immunophenotyping. No striking differences were observed between subpopulation of total CD4 $^{+}$ cells, except for a higher proportion of naïve T cells in untreated pwMS and RTX repop, and an increased presence of Th1/17 and CXCR5 $^{+}$ PD1 $^{-}$ cells, paralleled by a significant decrease of regulatory T cells (CD25 $^{+}$ CD127 $^{-}$) in NTZ-treated pwMS (**Figure 5a-b** and **Supplementary Figure 9**). Focusing on memory CD4 $^{+}$ cells, significantly decreased circulating T follicular helper (Tfh) compared to untreated, and T helper 17 (Th17) and Th1/17 compared to NTZ were observed shortly after RTX start (RTX 3-6M), but with full normalization under B-cell repopulation (**Figure 5c-e**). To confirm this result at the transcriptomic level and during long-term RTX depletion, we modeled our bulk RNA-seq data on PBMC to correct for the impact of B-cell depletion and highlight T-cell transcriptomic changes (see online methods). Expression pattern analysis identified 9 clusters of genes displaying similar behavior across treatment groups, as depicted in **Figure 5f**. Ingenuity pathway analysis (IPA) on these gene clusters showed an enrichment for T-cell-related functions, including “Th17 activation pathway” and several costimulatory molecule signaling in Th cells (OX40, ICOS-ICOSL, CD28 and CTLA4) in genes that were downregulated after the first RTX dose (RTX 1-6M compared to untreated, cluster “-100”) and in genes that were persistently downregulated during B-cell depletion (RTX 1-6M and RTX depl compared to untreated) but recovered at B-cell repopulation (cluster “-1-10”) (**Figure 5g**). No T-cell-relevant

261 pathway was enriched in gene clusters that were persistently downregulated in the RTX repop
262 group compared to untreated (“00-1” and “-1-1-1”).

263 These findings indicate a phenotype shift in Th populations, especially among memory CD4+
264 cells, with decreased Th17 and Th1/17 functions during B-cell depletion, which is not sustained
265 when B-cell repopulation occurs. Therefore, the sustained suppression of inflammatory disease
266 activity observed during RTX treatment in RRMS is not associated with noticeable changes in T-
267 cell phenotypes, in turn pointing towards the relevance of altered T-cell-specificities detected by
268 FluoroSpot.

DISCUSSION

The advent of BCDT has transformed management of RRMS, providing a treatment modality yielding long-lasting suppression of inflammatory disease activity in the vast majority of treated pwMS. Interestingly, this effect outlasts not only the presence of antiCD20 monoclonal antibody in the circulation, but also the suppression of B-cell concentrations.^{21,22,26} Yet, the biological basis for an induction effect, i.e. the continued absence of disease activity even after numerical B-cell repopulation, remains poorly explored. In this study, we leveraged a comprehensive, multi-layered dataset with paired sampling before treatment and at various stages of B-cell repopulation to dissect how BCDT, such as RTX, reshape the immune landscape. Beyond clarifying BCDT treatment effects, our results also provide new insights into the immunopathogenesis of MS, demonstrating that repopulating B cells lose transcriptional features linked to lytic EBV infection and that T-cell reactivity to EBV antigens, CNS and autoantigens diminishes at B-cell repopulation. Thereby our findings support the notion of an EBV-driven and cross-reactive immune response representing a key pathological axis in MS. This notion is also supported by our recent study demonstrating that EBNA1- and ANO2-specific CD4⁺ T cells are cross-reactive, cytotoxic in animal models of MS, and enriched in pwMS.⁴ Together, these observations provide evidence of a coupling of reactivity to EBV with adaptive autoimmune encephalitogenic responses in RRMS.⁴

A strength of this work is the availability of paired single-cell transcriptomes of naïve and memory B cells, together with parallel analyses of T cells, plasma biomarkers, and bulk RNA sequencing. This integrated approach revealed a consistent and unexpected finding, namely that repopulating B cells display a broad activation of inflammatory pathways, including NF-κB, IL-6-JAK-STAT3, IL-2-STAT5 and interferon-related gene signatures. Notably, a similar activation profile was observed across T-cell clusters, indicating that immune remodeling after BCDT is not restricted to the B-cell compartment. Interestingly, widespread upregulation of the NF-κB pathway across different leukocyte populations has also been recently described by Wei et al.²⁷ during B-cell depletion, and our data extends the persistence of this molecular signature well into a stage of full numerical B-cell repopulation. Importantly, plasma cytokine and chemokine profiling corroborated these observations, showing elevated levels of BAFF, APRIL, and multiple lymphoid cytokines after RTX. Nevertheless, despite this widespread molecular activation, clinical and radiological disease activity remained virtually absent, similar to recent clinical observations in larger cohorts.^{26,28}

The label of BCDT approved for RRMS is based on a fixed dosing schedule associated with prolonged and persistent depletion of B cells. However, emerging observational data shows that disease activity remains controlled even with extended dosing intervals that allow for B-cell repopulation. Further, a recent randomized clinical trial showed that yearly dosing of RTX was not inferior to a bi-annual dosing schedule.²⁹ Our findings now provide an important biological basis for these observations by showing that transcriptomic and functional properties differ markedly from the pre-treatment state, in turn suggesting that the induction effect is explained by phenotypic characteristics of repopulating B cells rather than sheer numbers.

A key novel finding is the marked suppression of EBV lytic molecular signature in repopulating switched memory B cells. This signature, dominated by ribosomal and other translation-related transcripts linked to lytic EBV infection^{24,25}, was consistently downregulated after RTX across clusters of switched memory and “exhausted” memory B cells. This latter cluster expresses markers of “atypical” or “age-associated” B cells, such as CXCR3, CD11c, T-bet and ZEB2,³⁰ which have recently been described as expanded by EBV primary infection and capable of CNS

homing in a humanized mouse model of MS.³¹ The notion of a reduced EBV burden in the repopulated memory B-cell pool is supported by declining anti-EBNA1 IgG levels at repopulation and by diminished T-cell responses to EBV antigens (as well as to ANO2, a CNS autoantigen with pathogenic potential⁴), while responses to control antigens remained unchanged. Collectively, these findings suggest that repopulating B cells are depleted of EBV-infected and/or EBV-reactive clones, consistent with the concept that EBV-CNS cross-reactive B- and T-cell responses contribute to MS pathogenesis.³²⁻³⁴

T-cell phenotypic makeup, on the other hand, showed reduced Th17 and Th1/17 subpopulations, which were previously associated with MS pathogenesis.³⁵⁻³⁷ Importantly, however, this feature was present only during B-cell depletion and was normalized at repopulation, thus unlikely explaining an induction effect of BCDT. Instead, these observations point toward the relevance of altered T-cell specificities rather than sustained gross T-cell immunophenotype changes.

Collectively, these findings are compliant with a mechanistic model in which BCDT eliminates EBV-infected B cells, while simultaneously reducing EBV-reactive and CNS-cross-reactive T cells, potentially involving initial elimination of CD20^{dim} T cells with encephalitogenic potential.^{38,39} Repopulation then proceeds from a more naïve, less pathogenic precursor pool, in an environment marked by elevated BAFF/APRIL and homeostatic immune activation. Such activation, however, appears to reflect physiological immune reconstitution rather than a re-emergence of MS-associated inflammation, given the lack of re-emergence of clinical or neuroradiological activity.

The study has limitations. The single-cell cohort, although enriched in memory B cells and deeply profiled, was modest in size, and sequencing depth may have limited detection of rare subpopulations. Bulk RNA-seq provided additional temporal granularity but lacked paired samples, reducing sensitivity. Functional T-cell assays required large cell numbers and were conditioned on sample availability. Moreover, most participants initiated RTX as a first- or second-line therapy, and therefore translatability to later disease stages remains uncertain.

In summary, we find that exposure to RTX reshapes B- and T-cell immunity in MS through two complementary processes: an early reduction of B-cell-related pathways and selected T-cell functions immediately following depletion, and the subsequent emergence of an activated B-cell compartment with a reduced EBV footprint. Together, these findings provide a biological foundation for understanding the durable effect of BCDT, and offer a framework for developing biomarkers and individualized dosing strategies that maintain therapeutic efficacy while minimizing long-term risks, ultimately improving the benefit-risk balance of this highly effective RRMS treatment modality.

ONLINE METHODS

Clinical materials and samples

Samples were obtained from pwMS enrolled in the STOPMS II observational study and treated with rituximab (RTX) in clinical routine at the Academic Specialist Centrum, Stockholm, Sweden. From this existing biobank, we identified samples collected during RTX at one of the following time points: i) <3 months from RTX initiation (“RTX 1-2M”); ii) 3 to 6 months after initiation (“RTX 3-6M”); iii) during a regular biannual infusion schedule with continued B-cell depletion (“RTX depl”); or iv) after repeated RTX doses with >6 months since the most recent infusion and at least partial B-cell repopulation (“RTX repop”). These categories reflect adaptations introduced in clinical routine during the COVID-19 pandemic, when RTX dosing intervals were extended to 18-24 months, starting after the first two doses.²¹ Flow cytometry for quantifying B cells was performed on fresh samples 1-2 weeks before each scheduled RTX infusion as part of clinical routine (Department of Clinical Immunology, Karolinska University Hospital). For most of the individuals a baseline sample was available in the biobank before RTX start, collected either in absence of disease-modifying treatment (“untreated”) or during natalizumab (NTZ) treatment, switching to RTX due to JC virus seropositivity. For comparative purposes, we also included additional samples from pwMS treated with NTZ within STOPMS II, as well as a healthy control (HC) group. Experimental setups using different sub-cohorts are shown in **Figure 1**, **Supplementary Figure 1** and **Supplementary Table 1**. Written informed consent was obtained from all participants (Stockholm Regional Ethics Board, 2009/2107-31/2, last amendment 2024-07377-02).

Clinical data were extracted from the Swedish MS Registry (SMSReg). Annualized relapse rate (ARR) was calculated by dividing the number of relapses occurring after RTX initiation by the number of patient-years until sample collection. “Active” scans were defined as the presence of contrast-enhancing lesions or new/enlarging T2 lesions compared to a prior MRI performed after RTX start. Annualized MRI activity was calculated analogously to ARR, substituting the count of relapses with the number of active scans. IgG total levels were also extracted from the SMSreg, and considered relevant to research samples when assessment was performed +/-90 days since research sampling date.

PBMC were collected and stored as previously described.⁴⁰ Briefly, blood was drawn into Vacutainer® CPT™ tubes (BD Biosciences) and centrifuged for 15 minutes at 1700 g. PBMC were recovered from the Ficoll™ (Cytiva)-plasma interface, washed with phosphate-buffered saline (PBS), resuspended in fetal bovine serum (FBS; Sigma-Aldrich) supplemented with 10% dimethyl sulfoxide (Sigma-Aldrich), and stored in liquid nitrogen. Plasma was obtained from blood collected in EDTA tubes, centrifuged at 1700 g for 15 minutes at room temperature, and stored at -80°C until use.

Flow cytometry

PBMC were thawed in complete RPMI (cRPMI) medium (RPMI 1640 (R8758, Sigma Aldrich) supplemented with 10% heat inactivated fetal bovine serum (F7524, Sigma Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (P4458, Sigma Aldrich), washed and stained with Fixable Viability Dye eFluor™ 455UV Live Dead (eBioscience) before proceeding with surface markers staining using the fluorochrome-conjugated antibodies in serum-free RPMI for 30 min at room temperature (**Supplementary Table 3**). Measurements were performed on a 5-laser Aurora spectral cytometer (Cytek), and data were analyzed with FlowJo v.10 (Tree Star).

Sorting of B and T cells and library preparation for single cell RNA sequencing

PBMC were quickly thawed at 37°C in complete RPMI medium (10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, Sigma Aldrich), rinsed twice in cold PBS and then incubated for 30 minutes in the dark at 4°C with an antibody mix including fluorochrome-conjugated FACS antibodies (Supplementary Table 3), a viability stain (LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, Invitrogen), and oligonucleotide-conjugated antibodies for surface marker detection in single cell sequencing data (TotalSeq™-C, BioLegend, **Supplementary Table 4**). TotalSeq anti-human hashtag antibodies (BioLegend), targeting β 2-microglobulin (β 2M) were added to baseline (Hashtag oligonucleotide 1 - HTO1) or follow-up (HTO2) samples in order to multiplex both samples from the same individual in one single-cell reaction. Cells were then washed in cold MACS buffer (Miltenyi Biotec) and filtered through a 40 µm cell strainer (Corning) into a polypropylene tube for sorting on a BD Influx™ cell sorter.

Sorted naïve (CD20+CD27-IgD+) and memory (CD20+CD27-IgD- and CD20+CD27+) B cells, and T cells (CD3+) were pooled in a 1:2:3 ratio to a single-cell suspension of 32 000 cells for each sample. Paired samples were included in the same reaction to minimize batch effect. Cells immediately underwent Chromium Next GEM Single Cell 5' v2 (10x Genomics) kit protocol in order to be individually indexed and generate transcriptomic, VDJ and surface marker sequencing libraries according to the manufacturer's instructions. Briefly, 36,3 µl of Master Mix for retrotranscription was added to 38,7 µl the single-cell suspension, mixed and loaded on a Chromium Next GEM Chip K for Gel Bead Emulsion (GEM) generation and single cell encapsulation. GEMs were then incubated overnight, followed by cDNA amplification and cleanup with SPRIselect beads (Beckman Coulter). Subsequently, longer fragments (cleanup pellet) proceeded to Gene Expression (GEX) library construction (Library Construction Kit, 10x Genomics), while shorter fragments (cleanup supernatant) underwent Cell Surface Protein library generation (5' Feature Barcode Kit, 10x Genomics). The resulting libraries were finally pooled and sequenced on Novaseq S2 2x100bp v1.5 platform.

PBMC RNA extraction and library preparation for bulk sequencing

Frozen PBMC pellets were thawed on ice and RNA extraction was performed with RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA concentration and quality were assessed with Qubit® RNA HS Assay Kit (Invitrogen) and Bioanalyzer/RNA 6000 Pico Kit (Agilent), respectively. Libraries were prepared using the Illumina TrueSeq Stranded mRNA (polyA) method and subsequently sequenced on NovaSeq 6000 in paired-end 101 bp mode.

Single cell RNA sequencing analysis

10x Genomics single-cell data, including gene expression, cell surface protein quantification using antibody-derived tags (ADT), and V(D)J libraries, were processed and aligned to the human GRCh38 reference genome using the Cell Ranger multi pipeline with default parameters (Cell Ranger v7.0.0). Assays were imported into Seurat objects for each sample and analyzed using Seurat (v4.3.0).⁴¹ Gene expression data were normalized using log-normalization, whereas centered log-ratio transformation was applied to normalize ADT and HTO protein expression assays.

To demultiplex cells by sample of origin and identify cross-sample doublets, the Seurat function HTODemux was adapted to the experimental design using two HTOs. Cells were designated as singlets if exclusively positive for either HTO1 or HTO2, or as doublets if positive for both. HTO-negative cells were excluded from downstream analyses.

A second round of doublet detection was performed using DoubletFinder (version 03/31/2020),⁴² and the union of doublets identified by both methods was removed from all samples.

Next, we followed the standard pre-processing workflow for scRNA-seq data in Seurat. Cells with at least 200 detected genes were retained, and genes detected in fewer than three cells were removed. For each sample, a mitochondrial read threshold was defined to exclude cells with high mitochondrial content, and mitochondrial genes as well as MALAT1 were removed from downstream analyses.

Individual Seurat objects were log-normalized (LogNormalize), and the 2,000 most variable genes were identified and used for scaling and PCA-based dimensionality reduction. The nearest neighbor graph was computed using FindNeighbours, followed by graph-based clustering with FindClusters.

Individual samples were merged into a single Seurat object. The merged data was normalized, and a new set of 2,000 variable genes was identified across all samples. Scaling, PCA-based dimensionality reduction (npcs = 20), and UMAP embedding were then performed. The merged object was subsequently split by sample using SplitObjects, and integration features were selected using SelectIntegrationFeatures. Data was scaled and reduced to 50 dimensions with RunPCA, and integration was performed using Harmony (RunHarmony).⁴³ Integrated clusters were inspected and removed based on the following criteria: (i) overall low quality (e.g., lack of V(D)J information or mixed B/T-cell composition), (ii) cluster size < 50 cells, or (iii) domination by a single sample.

Differentially expressed genes per cluster were identified using FindAllMarkers with the MAST test and gene set enrichment analysis (GSEA) for Gene Ontology, biological processes (GO_BP) was performed (**Supplementary Figure 2f**).⁴⁴ Non-protein-coding genes, IGHV, TCR, ribosomal genes and sex-chromosome-encoded genes were excluded. Cluster-level annotation was performed manually based on characteristic gene expression patterns and Monaco annotation scores⁴⁵ (**Figure 2e** and **Supplementary Figure 2d-e**).

Cluster proportion differences across sample groups were analyzed using the propeller method in the speckle R package (v0.0.3).⁴⁶ Pseudobulk differential expression analysis was performed with edgeR (v3.36.0), with scaling to equalize cell numbers across clusters to avoid bias due to differing cell proportions. GSEA was performed using clusterProfiler (v4.3.1),⁴⁷ based on differentially expressed genes ranked by decreasing log fold change, using GO_BP and Hallmark gene sets. Gene signature scores (Hallmark NF- κ B pathway, EBV lytic²⁵) were calculated using AddModuleScore either per cell (**Figure 4c**) or per sample (**Figure 3b**), with the latter obtained by averaging scores across all cells originating from the same sample.

Bulk PBMC RNA sequencing analysis

Sequencing data were converted to FASTQ format using bcl2fastq (v2.20). The nf-core/rnaseq pipeline (v3.0) was used for processing, including quality control with FastQC (v0.11.9), adapter trimming with TrimGalore (v0.6.6), alignment to the GRCh38 reference genome using STAR (v2.6.1d), and gene-level quantification with Salmon (v1.4.0). Additional quality metrics were assessed using RSeQC (v3.0.1), Picard (v2.23.9), and Qualimap (v2.2.2-dev). Cell-type deconvolution was performed using CIBERSORTx.⁴⁸

Downstream analyses were performed in R (v4.4.1). Low-abundance genes were filtered using edgeR (v4.2.1), retaining genes with ≥ 10 counts expressed in at least 90% of samples in each

group. Initial analyses (**Figure 3c-d**) were conducted on voom-transformed counts, fitting linear models using limma (v3.60.6). Resulting logarithmic fold change (logFC) values from pairwise comparisons between RTX -treated and HC compared to baseline untreated MS samples were used to rank genes for GSEA using Hallmark gene sets via clusterProfiler.

To isolate transcriptomic changes not attributable to B-cell abundance, baseline samples were used to model the effect of B-cell frequency (predicted by CIBERSORTx) on gene expression and incorporate this adjustment into the final regression framework (**Figure 5f-g**). Filtered baseline count data were voom-transformed, and a linear model was fitted with limma, adjusting for age, sex, and frequencies of the major cell types (B memory, B naïve, NK resting, CD8, CD4 naïve, CD4 memory resting, CD4 memory activated). Remaining cell types were represented by the first two principal components (PC1 and PC2). Gene-level expression values were then adjusted by subtracting the estimated B memory- and B naïve-associated expression components derived from the baseline model, and the resulting B-cell-adjusted expression values were used for downstream differential expression analyses.

A second linear model was fitted using limma to identify inter-group differences, adjusting for age, sex, and non-B-cell frequencies. Differential expression analysis with limma applied t-tests relative to a threshold ($TREAT^{49}$), testing whether the absolute log2 fold change exceeded 0.5. RTX 1-2M and RTX 3-6M samples were grouped together for this analysis (RTX 1-6M) given the limited number of samples and the absence of significant differences in gene expression. The pattern of expression of significant DEGs across different time points of RTX treatment was then visualized by heatmap (ComplexHeatmap v2.16) and clustered by direction in a 3-digit label, where each digit indicated the result of the comparison against untreated samples of RTX 1-6M, RTX depl and RTX repop samples, respectively. In this label, “1” indicates significantly higher expression in RTX treated groups compared to baseline, “-1” significantly lower expression in RTX samples, and “0” no significant differential expression. Functional annotation of such clusters of genes was performed with Ingenuity Pathway Analysis (IPA) following manual curation of immune-relevant pathways. To further ensure independence from B-cell abundance, genes correlated with MS4A1 ($|Pearson\ r| \geq 0.5$) were removed.

Multiplex bead assay (Luminex)

Levels of 27 proteins (see complete list in **Supplementary Table 5**) in plasma were determined with a Luminex Discovery Assay (R&D Systems, cat. no. LXSAM), with addition of TGF-beta1 measured with Luminex Performance (R&D Systems, cat. no. FCSTM17). In the first instance, 60 ul of plasma were diluted 1:2 with the provided diluent, and 50 ul were used in each of the two premixed multiplex assays. For TGF-beta1 detection, 25 ul of plasma were incubated with 5 ul HCl for 10 minutes at room temperature, followed by neutralization with 5 ul NaOH/HEPES and dilution to a final 1:15 ratio with the provided diluent, and 50 ul were used for the assay. Diluted samples and appropriate standard curves and blanks were then processed according to the manufacturer’s instructions. Plates were then read using a Bio-Plex 200 system (Bio-Rad).

FluoroSpot

Antigen reactivity to viral and self-proteins before RTX and at B-cell repopulation was assessed on paired samples using FluoroSpot Plus assay for IFN γ /IL-22/IL-17A (Mabtech, cat.no. FSP-011803). PBMC were quickly thawed at 37°C in complete RPMI medium (10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin, Sigma Aldrich), rinsed twice in cold PBS and then counted with LUNA-II automated cell counter (Logos Biosystems) using trypan blue to detect dead cells. A total of 250,000 live PBMC was seeded on precoated FluoroSpot plates together

with protein-coupled beads which were produced as previously described,⁵ in a 10 beads:1 cell ratio. The recombinant proteins included albumin binding domain (ABD, an irrelevant bacterial protein used in bead production), as negative control, EBV latent antigen EBNA1₁₋₁₂₀ and EBNA1₃₈₀₋₆₄₁ in 1:1 ratio, EBV lytic antigen BZLF1, CMV protein pp65, human proteins MOG, MBP and ANO2₁₋₂₇₅. A total of 125,000 live PBMC was seeded and stimulated with anti-CD3 as a positive control. Paired samples were analyzed on the same plate to minimize inter-plate variability. The plates were then incubated 44 hours at 37°C with 5% CO₂ and then developed as per the manufacturer's instructions, and fluorescence was read by an IRIS FluoroSpot reader (Mabtech).

EBV qPCR on whole blood

Total DNA was extracted from 10 ml of whole blood collected in EDTA tubes using QIAamp DNA Blood Maxi Kit (Qiagen) and quantified by Qubit® DNA HS Assay Kit (Invitrogen). EBV viral content was assessed by quantitative PCR using TaqMan Fast Advanced Master Mix and Gene Expression Assays for EBV Pa03453399_s1 FAM (Applied Biosystems). Two experiments were run, one using 135 ng of total DNA per sample/per well and a second using 540 ng/per well to maximize detection, both in triplicate. Serial dilution of commercially available viral genomes (Advanced Biotechnologies Inc.) spanning from 18,000 to 1.15 viral copies was used as a standard curve in each experiment, and a no-template control with nuclease free water was also included. Amplification and signal detection was performed on a CFX Opus 384 Real-Time PCR system (Bio-Rad) with the following program: 50°C for 2 min, 95°C for 20 s, 95°C for 3 s, 60°C for 30s, for 50 cycles. Positivity was evaluated after removing wells that had Ct values above the lower point of the standard curve on the same plate, and considering as positive samples that had amplification in at least 2 out of 3 wells in the 540 ng run, or, in case of single-well amplification in the 540 ng run, at least 2 wells with amplification in the 135 ng run.

ELISA

Enzyme-linked Immunosorbent Assay for IgG against EBNA1 and BZLF1 was performed on plasma of patients before and after RTX treatment, and samples obtained from the same individual were run on the same plate to minimize batch effect and allow for paired analysis. Ninety-six well plates (Nunc 96-Well Flat Bottom MaxiSorp Immuno Plates) were coated overnight at 4°C with 50 µl of recombinant EBNA (2.5 µg/ml in PBS), recombinant BZLF1 (1 µg/ml in PBS), and BSA (1% in PBS) as negative control. After washing 5 times with washing buffer (PBS with 0.05% Tween-20, PBS-T), plates were then blocked with BSA 1% in PBS-T for 2 hours at room temperature on a plate shaker at 350 rpm. After another 5x washing step, plasma samples were dispensed in duplicates, 50 µl/well, after dilution to 1:15000 for anti-EBNA IgG and to 1:500 for anti-BZLF1 IgG in PBS-T with 0.2% BSA. The plate was then incubated for another 2 hours at room temperature and on a plate shaker at 350 rpm, followed by another 5x washing step. Fifty microliters of HRP-conjugated, goat anti-human IgG (Thermofisher), diluted to 1:2000 in PBS-T with 0.2% BSA were then added and incubated for 1 hour in the dark at room temperature and on a plate shaker at 350 rpm, followed by 5x washing step. For detection of IgG levels, 50 µl of tetramethyl benzidine (TMB Substrate Reagent Set, BioLegend) was added to the wells and incubated for 3 minutes, after which the reaction was stopped with 50 µl H₂SO₄ 1M. Absorbance was then measured at 450 nm. Data is presented as adjusted optical density (OD) obtained by subtracting the duplicate average of BSA absorbance to the duplicate average of the absorbance for the EBV-specific IgG for the same sample.

Statistical analysis

571 Experimental setups including partially paired sample structures (FACS, Luminex) were analyzed
572 using a mixed effect linear model including a random intercept for each individual and
573 considering individual ID as covariate in the model to account for paired data. Pure paired
574 analyses (ELISA) were analyzed using Wilcoxon rank signed test. Differential abundance of
575 clusters (DA) and pseudobulk differential expression (DE) analysis in scRNA-seq were both
576 analyzed with a linear model that included individual ID as a covariate to account for paired
577 data, using the propeller package for DA and the limma package for DE. Correlations were
578 calculated with Pearson correlation test, except when using percentages (e.g., Figure 2a) where
579 Spearman correlation test was used. Comparison between percentages in qPCR data were
580 analyzed by Fisher exact test given the low number of positive samples. FluoroSpot data were
581 analyzed with ratio t test for logarithmic-transformed paired data. GraphPad (v9) and Rstudio
582 (v4.5.2) were used to perform statistical analyses and graphical representations.

Funding

This study received funding from Neuro Sweden, Neuro Stockholm, MS Forskningsfonden, the Swedish Brain Foundation, Swedish Research Council and the Horizon Europe Framework Programme (project no.101136991). OT was supported by Gunvor och Josef Anérs Stiftelse, Neuro Sweden, Neuro Stockholm, MS Forskningsfonden and Jeansson's Stiftelse and the Horizon Europe Framework Programme (project no. 101136991). NR was supported by Neuro Sweden, Neuro Stockholm, MS Forskningsfonden, Svenska Läkaresällskapet, Bertil & Ebon Norlins foundation for medical Research and the Horizon Europe Framework Programme (project no. 101136991). MJ's team was supported by the European Research Council (Epi4MS, grant agreement no. 818170), Knut and Alice Wallenberg Foundation, Swedish Research Council, Swedish Brain Foundation, Neurofonden, MS Forskningsfonden, StratNeuro, Stockholm County Council - ALF project and the Horizon Europe Framework Programme (project no. 101136991). FP was supported by the Swedish Research Council (grant no. 2023-00533), the Region Stockholm (grant no. FoUI-987565), the Swedish Brain Fund (grant no. FO2023-0336), Erling Persson's Foundation and the Horizon Europe Framework Programme (project no. 101136991).

Disclosures

DG is an employee of Merck AB, Solna, Sweden, an affiliate of Merck KGaA.
CS is currently an employee at AstraZeneca, Mölndal, Sweden, but had no disclosures at the time of the contribution to the present work.
OT has received compensation for lectures and an advisory board from Merck and Pfizer respectively, which have no relation to the content of this study.
TO has received lecture/advisory board honoraria from Merck, Novartis, Biogen and Sanofi, and unrestricted MS research grants from the same companies, none of which have contributed to this study.
NR has received a research grant from Pfizer.
FP has received research grants from Denka, Janssen, Merck KGaA, Novartis, Pfizer and UCB, and fees for serving on DMC in clinical trials with Lundbeck and Roche, and preparation of expert witness statement for Novartis.
All other authors declare no competing interests.

Acknowledgements

We deeply appreciate and express our gratitude to people with MS and healthy donors that contribute with time and biological material to research, as well as the healthcare personnel that make the biomaterial collection possible.
We thank Dr. Annika van Vollenhoven for the help with FACS sorting and the Center for Molecular Medicine Flow Cytometry Core for providing research facilities. We thank Dr. Mingmei Shang for her help with Luminex setup.
The computations were enabled by resources provided by the National Academic Infrastructure for Supercomputing in Sweden (NAISS), partially funded by the Swedish Research Council through grant agreement no. 2022-06725. The authors acknowledge support for Illumina Sequencing from the National Genomics Infrastructure (NGI) in Stockholm funded by Science for Life Laboratory, the Knut and Alice Wallenberg Foundation, and the Swedish Research Council (through grant agreement no. 2022-06725).

Author contributions

629 CSC, MJ, NR and FP designed the study. CSC performed most of the experiments with help from
630 GL for Luminex, NR for lymphocyte sorting, CS for scRNA-seq library preparation, KB and SDO
631 for FluoroSpot. GL and NR performed FACS immunophenotyping and analysis, with the help of
632 ML. JBB performed qPCR. OD performed PBMC bulk RNA-seq analysis with the help of EE and
633 MN, and ÅB and SER provided the core pipeline for scRNA-seq analysis, which CSC used for
634 analysis. CSC performed enrichment analysis on transcriptomic data. OGT, UR and AG provided
635 material and guidance for FluoroSpot assays, as well as recombinant proteins used in ELISA
636 assays. MJP provided material and guidance for qPCR assays. MK managed sample biobanking.
637 TO contributed with sample collection and provided substantial intellectual input throughout
638 the study. DG and MN provided guidance with the transcriptomic analysis. CSC, MJ, NR and FP
639 wrote the manuscript with contributions from all authors. All authors read and approved the
640 final manuscript.

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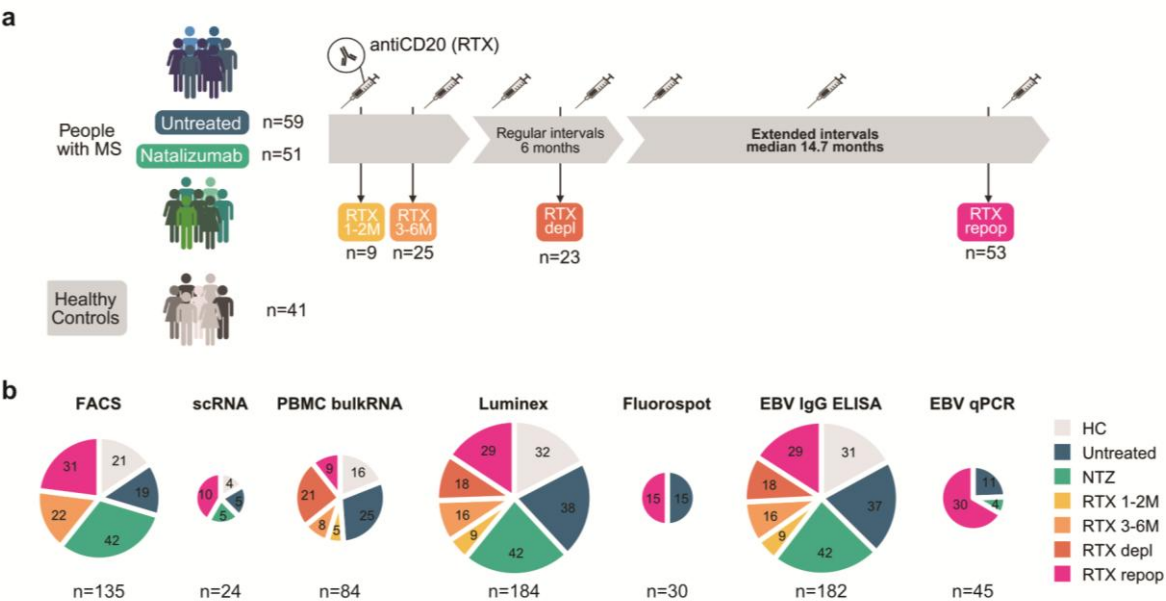
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775 **Table 1. Demographic characteristics of the study cohort.**

Characteristic	HC N = 41	Untreated N = 59	NTZ N = 51	RTX			
				1-2M N = 9	3-6M N = 25	depl N = 23	repop N = 53
Age ¹	33.1 (28.3, 38.4)	35.3 (26.9, 41.8)	43.8 (36.7, 50.4)	39.3 (34.4, 53.3)	40.6 (32.8, 46.8)	38.3 (32.6, 47.6)	40.1 (32.4, 46.5)
Female sex ²	28 (68%)	38 (64%)	37 (73%)	6 (67%)	18 (72%)	10 (43%)	44 (83%)
Disease duration (y) ¹	-	0.7 (0.2, 2.5)	16.2 (9.4, 21.9)	1.8 (0.5, 21.0)	8.0 (6.5, 13.2)	5.1 (2.7, 6.7)	10.1 (5.3, 15.5)
Months since RTX start ¹	-	-	-	1 (1, 1)	6 (6, 6)	25 (21, 39)	49 (41, 77)
No. of RTX doses ¹	-	-	-	1 (1, 1)	1 (1, 1)	4 (3, 7)	5 (3, 8)
Months since last RTX ¹	-	-	-	0.6 (0.5, 0.7)	5.8 (3.6, 6.0)	5.7 (3.9, 6.0)	14.7 (12.2, 19.1)
EDSS ¹	-	2.00 (1.00, 2.50)	2.00 (1.00, 2.50)	3.00 (2.50, 3.50)	2.50 (1.00, 3.50)	1.50 (1.00, 2.50)	1.50 (1.00, 3.00)
ARR in the previous year ³	-	0.71 (0.70)	0.06 (0.42)	0.44 (0.53)	0.32 (0.56)	0.04 (0.21)	0.00 (0.00)
ARR on RTX treatment ³	-	-	-	0.00 (0.00)	0.00 (0.00)	0.06 (0.19)	0.00 (0.00)
Annualized MRI activity on RTX treatment ³	-	-	-	0.00 (0.00)	0.08 (0.41)	0.04 (0.15)	0.03 (0.08)

¹ Median (Q1, Q3); ²n (%); ³Mean (SD)



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779 **Figure 1. Cohort overview.** **a**, Biological samples were collected from untreated pwMS before

780 undergoing RTX treatment, and during rituximab (RTX) treatment at different time point, i.e. <3

781 months since RTX start (RTX 1-2M), between 3 and 6 months since RTX start (RTX 3-6M), during

782 regular biyearly dosing schedule corresponding to persistent B-cell depletion (RTX depl) and

783 during extended dosing interval schedule with >6 months since previous RTX dose and at least

784 partial B-cell repopulation (RTX repop). A group of healthy control (HC) and natalizumab-treated

785 pwMS (NTZ) were also included for comparison. Image created with biorender.com. **b**, Group-

786 wide sample contribution to performed analyses.



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since last RTX infusion in samples characterized by FACS. Within-group Spearman correlation coefficients are reported. **b**, Boxplot of percentage of total B cells across sample groups. Significant pairwise comparisons are reported according to a mixed-effects model including individual ID to account for paired data. **c**, Stacked bar plot of B-cell subtypes percentages out of total B cells across sample groups. **d**, Uniform Manifold Approximation and Projection (UMAP) plot of single-cell transcriptomes obtained by droplet-based single cell performed on sorted naïve (IgD+CD27-) and memory (IgD+/-CD27+ and IgD-CD27-) B cells, pooled in a 1:2 ratio. **e**, Heatmap of B-cell-relevant gene expression across single-cell clusters. **f**, Volcano plot of cluster differential abundance analysis performed with propeller. Given the artificial pooling ratio between naïve and memory B cells, proportion were calculated separately for naïve clusters (Naïve1-3) and the remaining clusters (Unswitched1-3, Switched1-3, Mixed1-2 and Exhausted). **g**, Volcano plot of pseudobulk gene expression analysis within naïve and memory B-cell clusters, comparing RTX repop samples to baseline with a paired design. **h**, Gene-set enrichment analysis (GSEA) for Gene Ontology and Hallmark terms for differentially regulated genes before vs after RTX within single-cell clusters. Adj.p: false discovery rate (FDR)-adjusted p-value (FDR threshold 0.1); geneRatio: proportion of core enrichment genes on the total of pathway genes. **i**, FACS analysis of activation marker expression on total B cells, with representative mean fluorescence intensity (MFI) traces on the left and boxplot of percentages of positive B cells across groups on the right. Data analyzed with mixed-effects models, including individual ID to account for paired data. * p<0.05, **p<0.01, ***p<0.001.

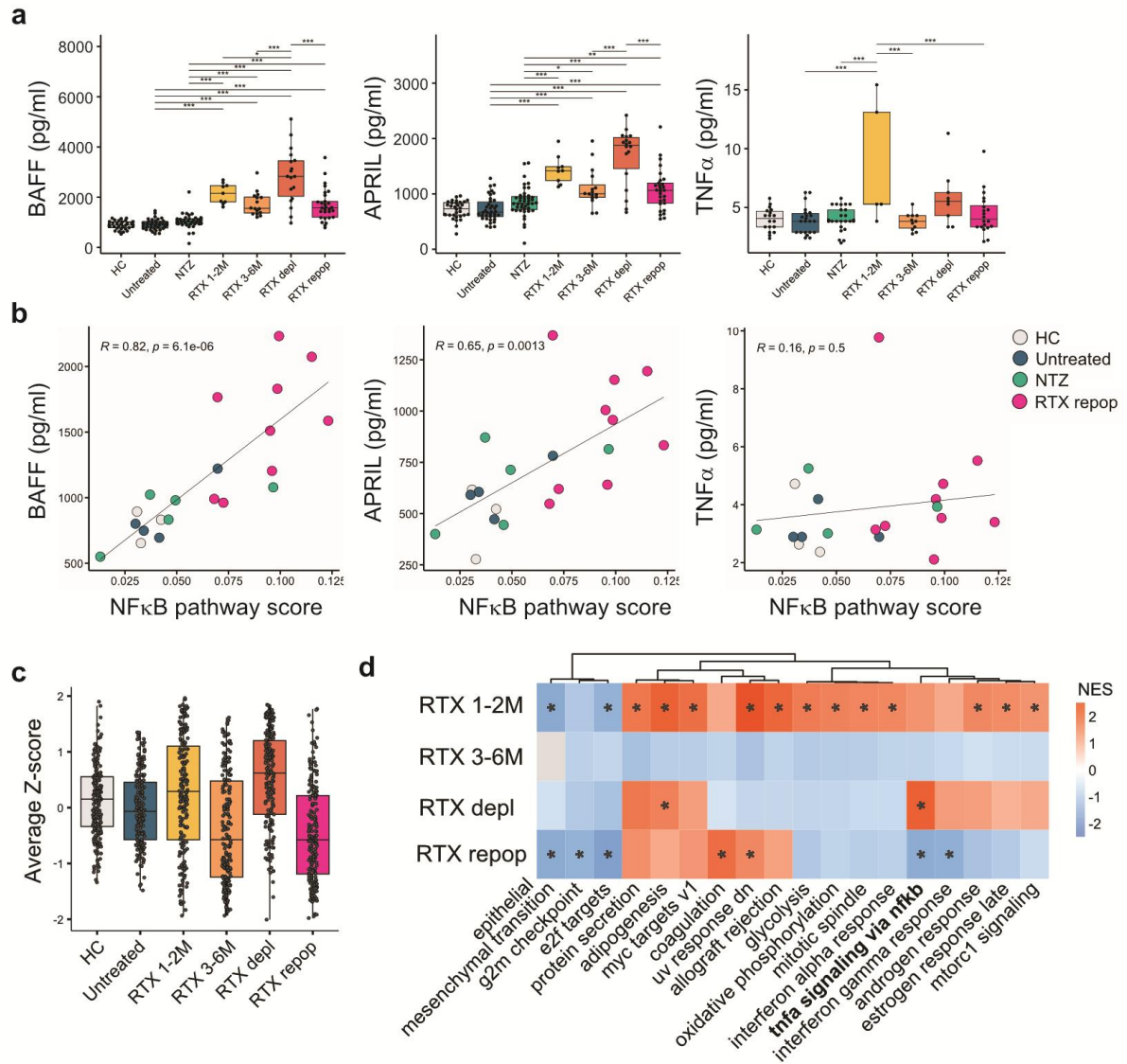


Figure 3. Persistent elevation of BAFF and APRIL at B-cell repopulation contributes to NF-κB activation. **a**, Plasma levels of BAFF, APRIL and TNFα as measured by Luminex assay across sample groups. Only significant pairwise comparison between RTX treated samples and untreated pwMS are reported on the graph for clarity reasons. P values are calculated using a mixed-effects model, including individual ID to account for paired data. **b**, Pearson correlation between BAFF, APRIL, TNFα and average B-cell "TNFα signaling via NF-κB" signature score in single-cell samples. **c**, Boxplot of average Z-score from PBMC bulk RNA sequencing for genes included in the "TNFα signaling via NF-κB" across sample groups. **d**, Heatmap of normalized enrichment score (NES) from GSEA of Hallmark terms in differentially expressed genes in RTX treated samples compared to untreated. Positive NES indicates upregulation of the term in RTX samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

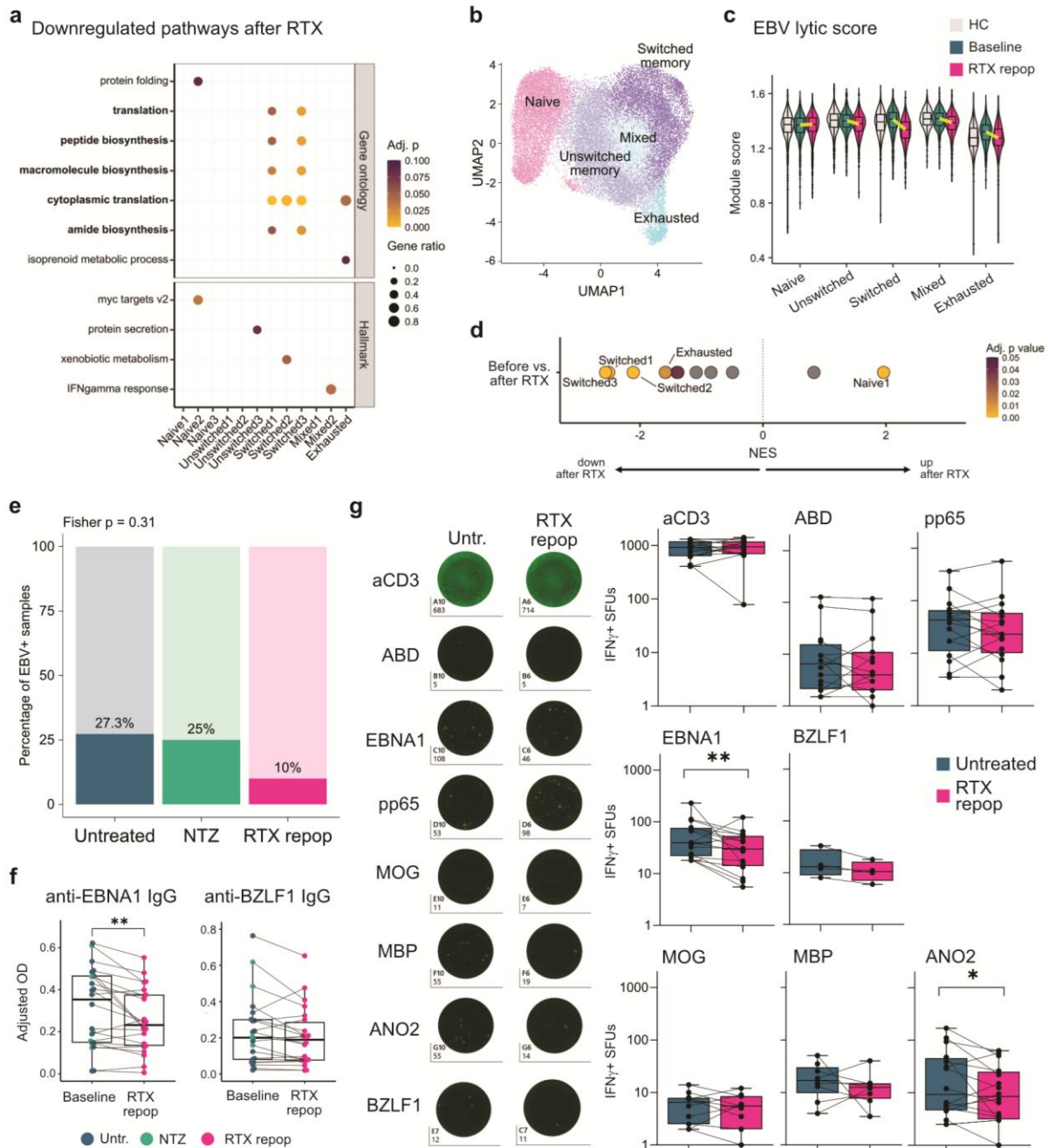


Figure 4. Molecular signature linked to EBV lytic infection of B cells is reduced in memory B cells at repopulation, accompanied by decreased B- and T-cell responses to EBV. a, Downregulated Gene Ontology and Hallmark terms according to GSEA in single-cell B-cell clusters after RTX treatment. Adj.p: false discovery rate (FDR)-adjusted p-value (FDR threshold 0.1); geneRatio: proportion of core enrichment genes on the total of pathway genes. **b,** UMAP of B-cell subtypes classified by original cluster annotation. **c,** Violin-boxplot for EBV lytic scores in single-cell transcriptome of B-cell subtypes across HC and paired baseline/RTX repop groups. Yellow lines join median levels of the transcriptomic score in the paired pwMS groups. **d,** GSEA of EBV lytic signature in differentially expressed genes per B-cell cluster after RTX vs baseline. Positive NES indicates upregulation of the term in RTX repop samples. Adj.p: false discovery rate (FDR)-adjusted p-value (FDR threshold 0.1). **e,** Percentage of whole-blood samples yielding positive qPCR across untreated, NTZ-treated and RTX repop samples. **f,** Boxplot of adjusted

835 optical density (OD) of anti-EBNA1 (left) and anti-BZLF1 (right) IgG in paired baseline/RTX repop
836 samples, analyzed by Wilcoxon ranked signed test. **g**, Fluorospot analysis of EBV- and self-
837 antigen T-cell reactivity in individuals with paired untreated and RTX repop samples. Left panel:
838 representative image of IFN γ + spot forming units (SFUs) in response to stimulation of the same
839 individual before and after RTX treatment. Right panels: boxplots of absolute counts of IFN γ +
840 SFUs (log scale) in untreated and RTX samples, analyzed by ratio t-test. * $p < 0.05$, ** $p < 0.01$,
841 *** $p < 0.001$.

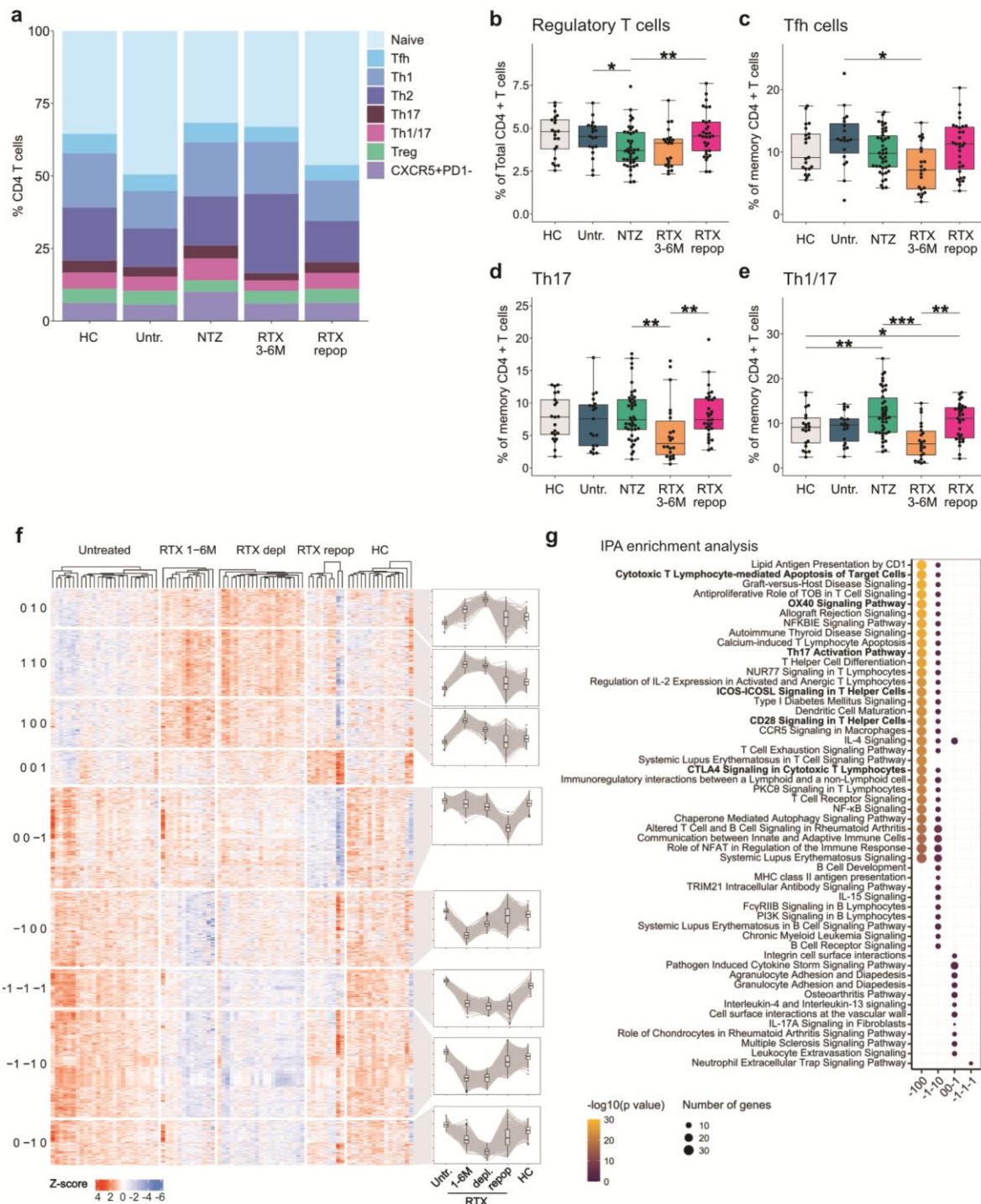


Figure 5. Reduced Th17 and Th1/17 proportion and functions during B-cell depletion are restored at B-cell repopulation. **a**, Stacked bar plot of CD4+ T-cell subtypes assessed by FACS across sample groups. **b-e**, Boxplots of percentage of regulatory T cells out of total CD4+ T cells (**b**), and percentages of T follicular helper (Tfh, panel **c**), T helper 17 (Th17, panel **d**), and T helper 1/17 (Th1/17, panel **e**) out of memory CD4+ T cells across sample groups. Data analyzed by mixed-effects models, including individual ID to account for paired data. **f**, Heatmap of differentially expressed genes in PBMC bulk RNA-seq data in RTX treated samples compared to untreated pwMS, corrected after estimation of B-cell abundance effect on gene expression. Patterns of gene expression in different gene clusters according to sample group are illustrated

852 in the boxplots on the right side of the heatmap and are named with a 3-digit label, where each
853 digit indicates the result of the comparison against untreated samples of RTX 1-6M, RTX depl
854 and RTX repop samples, respectively. **g.** Ingenuity pathway enrichment analysis of gene clusters
855 detected by expression pattern analysis corrected for B-cell effect and further filtered for genes
856 correlating with MS4A1 expression, and downregulated in RTX samples compared to untreated
857 pwMS (clusters “-100”, “-1-10”, “00-1”, “-1-1-1”).

Supplementary Files

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