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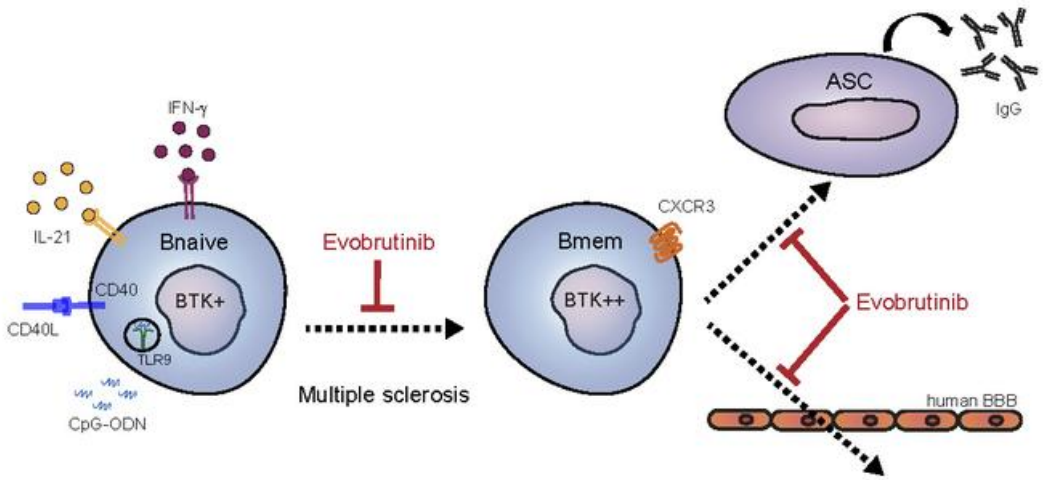
Graphical abstract



Human T-bet^{+} B cell development is associated with BTK activity and suppressed by evobrutinib

Liza Rijvers[1,2,*], Jamie van Langelaar[1,2,*], Laurens Bogers[1,2,¶], Marie-José Melief[1,2,¶], Steven C. Koetzier[1,2], Katelijn M. Blok[2,3], Annet F. Wierenga-Wolf[1,2], Helga E. de Vries[4], Jasper Rip[1,2], Odilia B.J. Corneth[5], Rudi W. Hendriks[5], Roland Grenningloh[6†], Ursula Boschert[7,§], Joost Smolders[1,2,3,§], Marvin M. van Luijn[1,2]

[1]Department of Immunology, MS Center ErasMS, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands. [2]MS Center ErasMS, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands [3]Department of Neurology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands. [4]Department of Molecular Cell Biology and Immunology, Amsterdam UMC, MS Center Amsterdam, Amsterdam Neuroscience, Amsterdam, The Netherlands. [5]Department of Pulmonary Medicine, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands. [6]EMD Serono, Billerica, MA, USA. [7]Ares Trading SA, Eysins, Switzerland, an affiliate of Merck KGaA, Darmstadt, Germany. [*,¶,§] Shared contribution. [†]Affiliation at the time the research was conducted.

Corresponding author: Marvin M. van Luijn, PhD, Erasmus MC, MS Center ErasMS, Department of Immunology, Wytemaweg 80, 3015 CN, Rotterdam, The Netherlands. Phone: +31107043190; fax: +31107044760; e-mail: m.vanluijn@erasmusmc.nl

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Keywords: BTK, next-generation inhibitor, B cells, CXCR3, class switching, transmigration, antibody-secreting cells

Abstract

Recent clinical trials show promising results for the next-generation Bruton's tyrosine kinase (BTK) inhibitor evobrutinib in the treatment of multiple sclerosis (MS). BTK has a central role in signaling pathways that govern the development of B cells. Whether and how BTK activity shapes B cells as key drivers of MS is currently unclear. In contrast to BTK protein, we found higher levels of phospho-BTK in *ex vivo* blood memory B cells of relapsing and secondary progressive MS patients versus controls. In these MS groups, BTK activity was induced to a lesser extent after anti-IgM stimulation. BTK positively correlated with CXCR3 expression, both of which were increased in blood B cells of clinical responders to natalizumab (anti-VLA-4 antibody) treatment. Under *in vitro* T_{FH}-like conditions, BTK phosphorylation was enhanced by T-bet-inducing stimuli IFN-γ and CpG-ODN, whilst the expression of T-bet and T-bet-associated molecules CXCR3, CD21 and CD11c were affected by evobrutinib. Furthermore, evobrutinib interfered with *in vitro* class switching as well as memory recall responses, and disturbed CXCL10-mediated migration of $CXCR3^+$ switched B cells through human brain endothelial monolayers. These findings demonstrate a functional link between BTK activity and disease-relevant B cells and offer valuable insights into how next-generation BTK inhibitors could modulate the clinical course in MS patients.

Introduction

B cells play a detrimental role in the pathophysiology of autoimmune and chronic inflammatory diseases including multiple sclerosis (MS) (1). In MS, this is exemplified by the presence of unique oligoclonal IgG fractions in the cerebrospinal fluid and the high efficacy of anti-CD20 monoclonal antibody B cell-depleting therapies (2). Since B cells are required for protective immune responses, their long-term depletion by such therapies puts patients at risk of infectious complications. More functionally restricted therapeutic strategies are warranted to target B cell subsets that contribute to diseases such as MS. To accomplish this, it is critical to understand how extrinsic and intrinsic signals instigate such B cells in humans.

Probably after their escape from peripheral tolerance checkpoints (3), naive B cells of MS patients differentiate into memory populations capable of stimulating pro-inflammatory $CD4^+$ T cells to enter the central nervous system (CNS) (4). These CNS-homing $CD4^+$ T cells respond to B cell-tropic Epstein-Barr virus (EBV) (5) and produce high levels of interferon (IFN)-γ, but not interleukin (IL)-17 (4, 6, 7). Concomitantly, peripheral interaction between B and T cells likely contributes to the enhanced CNS recruitment of B cells expressing CXCR3 in MS (8).

CXCR3 expression on B cells is triggered by T-box transcription factor T-bet (9). In mice, IFN-γ production by $CD4^+$ T cells is essential for the induction of T-bet in autoreactive B cells within germinal centers (10). IFN-γ synergizes with pathogen-associated Toll-like receptor 9 (TLR9) ligands to induce T-bet expression and autoimmune-like responses (11). B cells of MS patients are highly sensitive to both triggers (12), resulting in enhanced class switching (8). Moreover, $CXCR3^+$ memory B cells are triggered by EBV (13) and show pronounced CNS-infiltrating ability and recall responses *in vitro* (8, 13, 14). This points to a tendency of such B cells to further mature into antibody-secreting cells after entering the CNS, a process associated with less favorable outcomes in MS (15).

Bruton's tyrosine kinase (BTK) inhibitors, which are currently first-line treatments for chronic lymphocytic leukemia (16), represent an emerging class of compounds for the treatment of autoimmune diseases (17, 18). BTK is known for its role as a key signaling molecule downstream of the B cell receptor (BCR) to regulate cell proliferation and survival. Additionally, it is involved in other pathways including TLR9 signaling to stimulate B cell-driven autoimmunity (11, 19). Evobrutinib is a novel, highly selective and covalent BTK inhibitor (20), with promising results in a positive phase 2 clinical trial for relapsing MS (21) and suppressive effects on B-cell maturation and antigen-presenting function in experimental autoimmune encephalomyelitis (EAE) (22). How BTK activity corresponds to CXCR3(T-bet)$^{+}$ B cell differentiation in MS and whether evobrutinib targets their brain-homing and antibody-producing potential is unknown.

In this study, we compared BTK expression and phosphorylation in blood B cell subsets from control and different clinical MS groups, including clinically isolated syndrome (CIS), relapsing-remitting MS (RRMS) before and after natalizumab (NTZ; anti-VLA-4 antibody) treatment, secondary progressive MS (SPMS) and primary progressive MS (PPMS). In human B cells, BTK phosphorylation was triggered by anti-immunoglobulin (Ig)M, CD40L, IL-21, IFN-γ and TLR9 ligand CpG-ODN and correlated with T-bet and CXCR3 expression levels. Class switching, brain endothelial transmigration and memory recall responses were addressed in the context of CXCR3(T-bet)-expressing B cells with and without the use of evobrutinib *in vitro*.

Results

BTK activity is increased and less inducible in blood B cells from distinct MS subgroups

To address whether BTK is differentially regulated in B cells during the pathogenesis of MS, we compared BTK protein expression and phosphorylation in *ex vivo* peripheral blood B cells between HC, CIS, RRMS, SPMS and PPMS subgroups (Fig. 1A-C and Supplementary Table 1). Transitional ($IgM^+CD27^-CD38^{high}$), naive mature ($IgM^+CD27^-CD38^{-/dim}$) and both non-class switched (IgM^+CD27^+) and class-switched (IgM^-IgD^-) subsets were analyzed separately (Fig. 1A). In contrast to comparable levels of BTK protein (Fig. 1B), levels of phospho-BTK were elevated in the RRMS and SPMS group, which was highest and significant compared with both the CIS and HC group for class-switched B cells (Fig. 1C). For RRMS, this was primarily observed for class-switched subsets, while this was the case for all subsets except transitional B cells in SPMS. BTK protein and phospho-BTK levels did not correlate with age (Supplementary Fig. 1) or clinical parameters such as disease duration, time to and between first and second attack, MRI activity and time to conversion to secondary progression (data not shown) in these MS subgroups. Ratios of phospho-BTK and BTK protein expression showed similar, but less significant differences (Supplementary Fig. 2). We next compared the inducibility of phospho-BTK in B cells by stimulating with anti-IgM $F(ab')_2$ fragments for 5 min. Although phospho-BTK was induced in all groups, this was significantly reduced in RRMS and SPMS (Fig. 1D). This attenuated inducibility was similar for all IgM^+ subsets (transitional, naive mature and non-class switched memory B cells; Fig. 1E). These differences in anti-IgM-induced phospho-BTK were not explained by changes in IgM expression prior to stimulation (Supplementary Fig. 3). These data reveal that phospho-BTK levels and inducibility are altered in B cells of patients with the relapsing to progressive form of MS.

BTK protein and phosphorylation levels correspond to CXCR3 expression in B cells from MS patients

To explore if BTK plays a role in the differentiation of B cells that are poised to infiltrate the MS brain (8), we first determined its association with CXCR3 expression in *ex vivo* B cells of the patient groups. Both BTK protein and phospho-BTK positively correlated with CXCR3 levels, which was seen across all patient groups and HCs (Fig. 2A and B) and thus likely represents a generic feature of B cells. Overall, weaker but similar trends were found for VLA-4, but not for CXCR4 and CXCR5 (Supplementary Fig. 4A). Given that NTZ (anti-VLA-4 antibody) is an effective MS drug causing peripheral accumulation of possibly brain-homing $CXCR3^+$ B cells (Fig. 2C) (8), we next studied how BTK protein levels and phosphorylation coincided with CXCR3 expression in B cells from NTZ-treated MS patients. In 1 year post-treatment blood samples, we found a positive correlation of BTK protein and phospho-BTK with CXCR3 levels (Fig. 2D). This was not seen for CXCR4 and CXCR5 (Supplementary Fig. 4B). CXCR3 expression levels were higher in post-treatment B cells of clinical responders compared with non-responders (Fig. 2E). In addition, CXCR3 was upregulated in post- versus pre-treatment B cells of clinical responders only (Fig. 2E), which further underlined our previous findings (8). This was also the case for BTK protein and, to a lesser extent, phospho-BTK (Fig. 2F). Finally, phospho-BTK was less inducible by anti-IgM stimulation in B cells of non-responders (Fig. 2G), which supported earlier observations (Fig. 1D). These results imply that BTK acts as a regulator of $CXCR3^+$ B cell differentiation.

BTK activity contributes to T-bet expression and *in vitro* use of evobrutinib attenuates class switching in human B cells

We have previously found that IFN-γ and TLR9 signaling pathways synergize to trigger human $CXCR3(T\text{-}bet)^+$ class-switched B cells under *in vitro* T_{FH}-like conditions, i.e. in the presence

of CD40L and IL-21 signals (8). To determine whether BTK is actively involved in this process, we analyzed phospho-BTK in blood B cells from 6 healthy donors after stimulation with IL-21, soluble CD40L, IFN-γ and/or CpG-ODN for 5, 10, 20 and 40 min. Although anti-IgM was a more robust trigger of BTK activity (Supplementary Fig. 5A and B), IFN-γ alone and/or IFN-γ together with CpG-ODN induced phospho-BTK under IL-21/CD40L-containing conditions at most time points tested, albeit with differences between individual donors (Fig. 3A and B). Additionally, we found a positive correlation between phospho-BTK and T-bet levels in B cells after 48 h stimulations with IFN-γ and IFN-γ with CpG-ODN (Fig. 3C), which was also influenced by the additional presence of anti-IgM $F(ab')_2$ fragments (Supplementary Fig. 5C). Both IFN-γ- and CpG-ODN-mediated upregulation of T-bet in B cells was attenuated by BTK inhibitor evobrutinib (20) (Fig. 3D), pointing to direct or indirect involvement of BTK activity in T-bet induction.

To study further the impact of BTK on *in vitro* T-bet$^+$ B cell differentiation, we cultured naive mature B cells with IL-21, irradiated CD40L-3T3, IFN-γ and CpG-ODN for 11 days to induce Ig class switching (8) (Fig. 4A). The addition of evobrutinib to these cultures diminished T-bet induction as well as class switching, leaving the formation of antibody-secreting cells (ASCs; $CD38^{high}CD27^{high}$) intact (Fig. 4B). This was accompanied by an increase in CD21 and a decline in CD11c surface levels (Fig. 4C; T-bet-associated markers) (23), thereby verifying the impact of evobrutinib on T-bet$^+$ B cells. These findings indicate that differentiation of naive into T-bet$^+$ class-switched memory B cells involves BTK activity and is suppressed *in vitro* by the use of evobrutinib.

The *in vitro* brain endothelial transmigration capacity of CXCR3$^+$ class-switched memory B cells is disrupted by evobrutinib

Under T_{FH}-like conditions, IFN-γ- and IFN-γ/CpG-induced CXCR3 expression on B cells was significantly reduced by evobrutinib (48 h; Fig. 5A). CXCR4 and CXCR5 remained unaffected, while VLA-4 showed a trend towards decreased expression (Fig. 5A and Supplementary Fig. 6A). The evobrutinib-mediated reduction in CXCR3 seemed to be less in the presence of anti-IgM (Supplementary Fig. 6B). This implies a selective impact of evobrutinib on CXCR3 and supports the additive effect of IFN-γ/CpG on T-bet and phospho-BTK levels without anti-IgM (Fig. 3C and D, Fig. 4B). To study the potential of evobrutinib to inhibit brain-homing CXCR3$^+$ memory B cells, we purified CD27$^+$ memory B cells from healthy blood and analyzed subsets for their ability to cross brain endothelial monolayers *in vitro* (Fig. 5B). Overall, class-switched B cells were better able to migrate through these monolayers than non-class switched B cells, irrespective of CXCL10 and CXCL12 supplementation (Fig. 5B). As expected, CXCR3-expressing class-switched memory (Fig. 5C) and especially IgG1$^+$ (Fig. 5D and Supplementary Fig. 6C) cells were most attracted to CXCL10 (8). Although this effect was small, CXCL10-mediated transmigration of class-switched subsets was reduced by evobrutinib for all donors tested, an effect that was hardly encountered in the presence of CXCL12 (Fig. 5C and D). This impact of evobrutinib on the brain-homing capacity of CXCR3$^+$ B cells supported the *ex vivo* results obtained with blood samples of NTZ-treated MS patients (Fig. 2C-G). Although outnumbered by BTK$^{low/neg}$ T cells, B cells expressed high levels of BTK and CXCR3 in MS CSF (Supplementary Fig. 7) (8). Together, this indicates that therapeutic targeting of BTK likely halts CXCR3$^+$ B cells from infiltrating the CNS in MS.

Evobrutinib interferes with *in vitro* development of CXCR3^{+} memory B cell populations into antibody-secreting cells

Evobrutinib attenuated T-bet-associated class switching, but not plasmablast induction *in vitro* using human naive B cells (Fig. 4B). Nonetheless, upon reaching the MS brain, CXCR3^{+} class-switched memory B cells are likely reactivated to further develop into ASCs (13-15). To explore if such a recall response is affected by BTK activity, we cultured CD27^{+} memory B cells under IL-21/CD40L-stimulating conditions for 6 days with and without evobrutinib (Fig. 6A). Both T-bet expression and class switching were induced by IFN-γ and CpG-ODN (Fig. 6B), which was also seen during naive B cell cultures. In the presence of evobrutinib, memory B cells had slightly lower T-bet levels and were less able to develop into ASCs (CD38^{high}CD27^{high}), while class switching remained unaltered (Fig. 6B and C). This suppressive effect on ASC formation was supported by a marked reduction in IgG secretion (Fig. 6D). IgM secretion was not affected by evobrutinib under these circumstances (Fig. 6D).

Discussion

For optimal use of newly identified immunotherapies in diseases such as MS, it is important to uncover how biological targets contribute to its immunopathogenesis. In the current study, we investigated the association of BTK and its suppression by the highly selective and clinically effective drug evobrutinib with the development of pathogenic human B cells. We demonstrate that BTK activity is enhanced and less inducible in circulating B cells of MS patients. In addition, the association of BTK with CXCR3 and T-bet upregulation as well as the reducing effects of evobrutinib *in vitro* indicate that BTK promotes the development of class-switched memory B cells in a T-bet-related manner. The accumulation of *ex vivo* BTK^+CXCR3^+ B cells in the blood of clinical NTZ responders as well as the impairment of *in vitro* $CXCR3^+$ memory B cell migration across the blood-brain barrier and their maturation into ASCs by evobrutinib further empowers BTK as a therapeutic target in MS.

The reduced risk of side effects and their high potential to penetrate the CNS make next-generation BTK inhibitors promising candidates for treatment of MS (24). Such inhibitors could affect local interaction between B and T cells (22, 25), which accumulate within the perivascular space and meninges (26). Since intrathecal production of oligoclonal IgG is one of the hallmarks of MS, regardless of the pathogenicity of these antibodies, interference of BTK inhibitors with local differentiation of memory B cells into ASCs could be pathologically meaningful. The latter is especially true for patients in the advanced, progressive stages of MS where the disease has been postulated to be compartmentalized within the CNS, while targeting of circulating lymphocytes could be more relevant in the early phases of relapsing MS. This assumption is supported by the current selective upregulation of phospho-BTK in circulating B cells of patients with the RRMS to SPMS and not the PPMS form. This could be relevant and should be further investigated for determining which types of patients would benefit from treatment with BTK inhibitors. Results from ongoing clinical trials with various BTK inhibitors

in SPMS and PPMS have to be awaited, but the successful phase II evobrutinib trial in MS at least included SPMS cases with superimposed relapses (NCT02975349) (21). Although we did not find a direct association with disease parameters, our findings support the notion that altered BTK activity influences the function of pathogenic B cells interacting with T cells as driver of MS (21, 22, 25).

Irrespective of their mode of action, the efficacy of BTK inhibitors such as evobrutinib at least partly relies on how BTK in B and myeloid cells is regulated and triggers pathogenic subsets, which may differ between autoimmune diseases. Despite enhanced BTK expression in B cells of systemic lupus erythematosus (SLE) and anti-citrullinated protein antibodies (ACPA)-positive but not -negative rheumatoid arthritis (RA) patients (27, 28), evobrutinib only showed mild effects in SLE and RA clinical trials (NCT02975336 and NCT03233230, respectively). This is in sharp contrast to MS patients, who had markedly less gadolinium-enhancing lesions after receiving evobrutinib (NCT02975349) (21). There could be several reasons for these contradicting observations. We found that phospho-BTK, but not BTK protein expression was increased and correlated with CXCR3 expression in *ex vivo* B cells from MS patients. In B cells of ACPA-positive RA patients, both phospho-BTK (Y551) and BTK protein levels were found to be higher (27). This implies that in MS, alternative mechanisms underlie BTK dysregulation in B cells as compared with primarily antibody-driven autoimmune diseases. This is supported by observations that central B cell tolerance mechanisms are defective in RA, SLE and neuromyelitis optica spectrum disorder (NMOSD), but not MS (29). Moreover, BCR signaling molecules upstream of BTK may be differentially controlled in B cells between diseases, e.g. PTEN and LYN in SLE (30, 31) and SYK and CBL-B in MS (32-34). The increased BTK activity across all B cell subsets from MS patients could indicate either a cause or consequence of their escape from peripheral tolerance (3), which seems to depend more on BTK than central tolerance (35) and needs more investigation

in the future. It has already been shown that evobrutinib is effective in attenuating BTK activity in BCR-induced human B cells (22). We found reduced IgM-specific stimulation of phospho-BTK in B cells from MS patients, suggesting that maximum phosphorylation levels are reached earlier. In another report, a similar trend was observed after triggering B cells of MS patients with both anti-IgM and -IgG (22). This implies that from the naive stage onwards, B cells need less external signals to differentiate into CXCR3(T-bet)$^{+}$ memory and ASC populations in MS (8, 12).

Phospho-BTK levels were further upregulated in *ex vivo* class-switched memory B cells, which is in line with previous studies (22, 27). Before memory differentiation occurs, naive B cells take up, process and present antigen via the BCR to interact with T_{FH} cells at follicular borders within secondary lymphoid organs. Not only costimulatory molecules such as CD40L, but also third signals such as T_{FH} cell-derived IL-21 further guide B cell differentiation. In this phase, B cell-intrinsic T-bet induction seems to be mediated by concomitant IL-21, IFN-γ and TLR9 signaling pathways (36-38). Our results indicate that IFN-γ and TLR9 signals, at least partly, depend on BTK activity for upregulating T-bet, thereby triggering CXCR3 expression and Ig class switching *in vitro*. Such a role of BTK was also found for autoreactive B cells in SLE mice (39). Although the underlying molecular mechanism remains to be defined, proto-oncogene ETS-1 may be one of the determining factors. ETS-1 is downregulated after TLR9 ligation, a process that is controlled by BTK (40). Since ETS-1 plays a role in T-bet induction (41, 42), IFN-γ stimulation possibly overcomes TLR9-mediated reduction in ETS-1 so that it can act together with T-bet to promote Ig class switching. The synergy between IFN-γ and TLR9 responses in B cells has been proposed to regulate peripheral tolerance (43), which is disturbed in MS (3). The observed inhibitory effects of evobrutinib on IFN-γ- and CpG-ODN-mediated class switching in B cells is relevant given their enhanced responsiveness to such signals and capacity to serve as potent antigen-presenting cells in MS (4, 8, 12). This is

supported by the fact that the antigen-presenting cell function of B cells is reinforced by T-bet (8, 44) as well as BTK (45, 46), and is impaired in EAE mice following evobrutinib treatment (22).

One limitation of our *in vitro* experiments is that we did not take into account a possible survival advantage of CXCR3(T-bet)$^+$ class-switched B cells and ASCs. The observed *in vitro* effects of evobrutinib also need to be confirmed *in vivo*. The suppressive effect of evobrutinib on class-switched B cells crossing blood-CNS barriers *in vitro* is at least in line with the role of BTK in B cell tissue homing (47). BTK is known to mediate CXCR4 and CXCR5 signaling (48), but our current finding that it can control the CXCR3-CXCL10 axis is new. With CXCL10, CXCL12 and CXCL13 being locally enriched, CXCR4 and CXCR5 can be expected to be more involved in the organization, while CXCR3 may be more related to the recruitment of B cells in the MS brain (8). The increased BTK activity in CXCR3$^+$ class-switched memory B cells of MS patients puts this pathogenic subset forward as a candidate target of evobrutinib to prevent B cell recruitment in the CNS. This increase was found in B cells of both RRMS and SPMS but not PPMS patients, pointing to a direct relation with local inflammation rather than neurodegeneration. CNS-infiltrating CXCR3$^+$ memory B cells are also poised to mature into ASCs *in vitro* (13, 14), which seems to be a process that can be affected by evobrutinib (current study). Hence, the potential of next-generation BTK inhibitors to penetrate the CNS may not only give an opportunity to prevent, but also target (clinically relevant) local antibody production in MS patients (15), which should be investigated in the near future. Such inhibitors may also be feasible in patients who have to discontinue natalizumab treatment. By exposing the disease-relevant mechanisms involved, this work reveals that BTK inhibition is a therapeutic approach that meets the need for selectively targeting pathogenic B cells in MS and other types inflammatory and autoimmune disorders (17, 21, 49).

Methods

Patients and sampling

In a first screen, we analyzed peripheral blood mononuclear cells (PBMCs) from 29 clinically isolated syndrome (CIS), 30 relapsing-remitting MS (RRMS), 15 secondary progressive MS (SPMS) and 15 primary progressive MS (PPMS) patients as well as 30 healthy controls (HC). None of these patients received any disease-modifying treatment or methyl prednisolone for at least 3 months before sampling. In a second screen, we included 15 RRMS patients who were all treated with natalizumab (NTZ) for 12 months (responders, n=10; non-responders, n=5). Responders to natalizumab were defined as treated patients who remained free of clinical attacks, i.e. subacute worsening of existing symptoms, or new symptoms after at least 30 days of improvement or stable disease (50). For one MS patient, we were able to analyze B cells in both CSF and blood. Patients and controls were included at the MS Center ErasMS, Erasmus MC (Rotterdam, The Netherlands). Clinical characteristics are shown in Supplementary Table 1. CIS was defined as a first clinical attack of demyelination in the CNS. All CIS patients were sampled within 4 months after their first attack. MS patients were diagnosed according to the McDonald 2017 criteria (51). For our functional studies, buffy coats (Sanquin, Amsterdam, The Netherlands) were obtained from healthy volunteers.

Peripheral blood was collected using Cell Preparation Tubes (CPT™; BD Biosciences, Erembodegem, Belgium) containing sodium heparin. PBMCs were isolated according to the manufacturer's instructions. PBMCs were taken up in RPMI 1640 (Lonza, Verviers, Belgium) containing 20% fetal calf serum (FCS; Thermo Fisher Scientific, Landsmeer, The Netherlands) and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) and stored in liquid nitrogen until further use.

Antibodies and flow cytometry

The details of anti-human monoclonal antibodies used for flow cytometric analysis of B cells are included in Supplementary Table 2. For our first *ex vivo* screen, we measured B cells from HC and CIS, RRMS, SPMS and PPMS patients within the same experiment, divided equally over three experiments. B cells from NTZ-treated RRMS patients were measured in a separate experiment. For the induction of phosphorylated BTK (phospho-BTK; Y223) in B cells, PBMCs were treated for 5 min with F(ab')$_2$ anti-human IgM (20µg/ml; Southern Biotech, AL, Birmingham, USA). For *in vitro* differentiation, total ($CD19^+CD3^-$), naive ($CD19^+CD3^-CD27^-$) and memory ($CD19^+CD3^-CD27^+$) B cells were purified using a FACSAria III sorting machine (BD Biosciences). For extracellular staining, cells were incubated with antibody mix for 30 min at 4°C. For intracellular staining of pBTK and T-bet, cells were fixed and permeabilized using the eBioscience FoxP3/Transcription Factor Staining Buffer Kit (Thermo Fisher Scientific) and incubated with an antibody mix for 30 minutes (min) at 4°C. For intracellular BTK staining, cells were first incubated for extracellular marker staining as described above. After extracellular staining, cells were fixed with 2% PFA for 10 min at 4°C, permeabilized in 0,5% saponine buffer for 10 min at 4°C, followed by intracellular staining in 0,5% saponine buffer for 60 min at 4°C. After staining, cells were suspended and measured on an LSRFortessa flow cytometer. Data were analyzed using FACSDiva 8.1 and FlowJo v10 software (both BD Biosciences). Dependent on the distribution per marker, we used mean (non-skewed) or median (skewed) to indicate fluorescence intensity (MFI).

Short-term B cell stimulations

For the 5-40 min *in vitro* stimulation experiments, the following B cell stimuli were used: F(ab')$_2$ goat-anti-human IgM (20µg/ml; Southern Biotech), rhIL-21 (50ng/ml; Thermo Fisher Scientific), soluble CD40L (0.1µg/ml; Enzo Life Sciences, Farmingdale, NY, USA), rhIFN-γ

(50ng/ml; Peprotech, Rocky Hill, NJ, USA) and CpG-ODN (10μg/ml; Invivogen, San Diego, California, USA). For these stimulations, we used and adapted a protocol described in a recent study (52). In short, PBMCs (5×10^5) were thawed and put on ice in RPMI containing 2% FCS using 96-wells round-bottom plates. For 5 and 10 min, live/dead (L/D) viability stain was added together with the stimuli. For 20 and 40 min, cells were first incubated with a live/dead viability stain for 15 min at 4°C, washed and then suspended with stimuli. After stimulation at 37°C, cells were placed on ice, suspended in cold eBioscience Fix/Perm buffer (Thermo Fisher Scientific) and fixed for 15 min (4°C) prior to the staining according to above described protocols. For the 48 hour (h) stimulations, the following B cell stimuli were used: $F(ab')_2$ goat-anti-human IgM (10μg/ml; Jackson ImmunoResearch, Uden, The Netherlands), rhIL-21 (50ng/ml; Thermo Fisher Scientific), soluble CD40L (0.1μg/ml; Enzo Life Sciences), rhIFN-γ (50ng/ml; Peprotech) and CpG-ODN (10μg/ml; Invivogen). 5×10^5 B cells were suspended in RPMI containing 5% FCS, β-mercaptoethanol (Sigma-Aldrich), L-glutamin (Lonza) and apo-transferrin (Sigma-Aldrich) and treated with and without evobrutinib (1μM; EMD Serono, Billerica, MA, USA) in 48-well plates.

Long-term B cell cultures

In vitro naive and memory B cell differentiation assays were performed as reported earlier (8, 13). In short, irradiated 3T3 fibroblasts expressing human CD40L were co-cultured with purified naive ($CD19^+CD38^{-/dim}CD27^-$; primary response) or memory ($CD19^+CD38^{-/dim}CD27^+$; recall response) B cells in the presence of rhIL-21 (50ng/ml; Thermo Fisher Scientific). For naive B cell cultures, rhIFN-γ (50ng/ml; Peprotech) was added with and without CpG-ODN (10μg/ml; Invivogen) to induce class switching and antibody-secreting cell (ASC) development, respectively (8). After 6 (recall response) or 11 (primary response) days of

culturing, viable (L/D^-) $CD19^+$ cells were examined using flow cytometry. The supernatants were collected and stored at -80°C until further use.

IgM and IgG ELISA

Flat-bottom 96-well plates (Corning, Tewksbury, MA, USA) were coated overnight with goat anti-human Ig (1mg/ml; Southern Biotech) at 4°C, washed with PBS/0.05%Tween-20 and blocked with PBS/5% FCS for 2 h at room temperature. Samples were added for 1.5 h. After washing, peroxidase-conjugated goat anti-human IgG (Thermo Fisher Scientific) or rabbit anti-human IgM (Jackson ImmunoResearch) were used to detect bound antibody. TMB Substrate (Thermo Fisher Scientific) was used to reveal peroxidase activity. Reactions were stopped with sulfuric acid and optical densities were measured at 450 nm using a BioTek Synergy 2 reader (Winooski, VT, USA). Concentrations were calculated using standard curves that were generated for each assay.

B cell transmigration assay

For transmigration experiments, $2.5\text{-}5\times10^5$ $CD27^+$ memory B cells isolated from buffy coats were placed on confluent monolayers of human brain endothelial cells (hCMEC/D3) (53) on 5 μm pore size transwell plates (Corning Life Sciences, Amsterdam, The Netherlands) coated with collagen. B cell migration towards medium, CXCL10 (900 ng/ml; R&D Systems, Abingdon, UK) (8), or CXCL12 (100ng/ml; R&D Systems) was analyzed after 5 h at 37°C. Percentages of memory B cell subsets were compared before and after transmigration using flow cytometry.

Statistics

Statistical analyses were performed using Graphpad Prism 9 (GraphPad Software Inc., San Diego, CA, USA) and are described in each figure legend. Both percentages and MFI are depicted as individual data points together with the corresponding mean. *P* values < 0.05 were considered statistically significant.

Study approval

All patients and controls gave written informed consent and study protocols were approved by the medical ethics committee of Erasmus MC.

Author contributions

LR performed experiments, analyzed data, interpreted results and revised the manuscript. JL set up *in vitro* B-cell differentiation and transmigration assays, interpreted results, generated displayed items and revised the manuscript. The order of LR and JL regarding the shared contribution has been determined by time of involvement in the research: LR initiated and JL finished the study. LB and MM performed experiments. LB, MM and SK analyzed data. LB and SK interpreted data. KB coordinated clinical studies and looked up patient characteristics. AW performed experiments. HV provided material and input for the migration assay. JR, OC and RH shared optimal protocols for BTK detection and revised the manuscript. RG and UB provided the BTK inhibitor. RG, UB and JS gave intellectual input. UB and JS critically revised the manuscript. JS coordinated the outpatient clinic, supervised the clinical studies and obtained funding. ML designed the research, obtained funding, discussed results, supervised the project team and wrote the manuscript.

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References

1. Sabatino JJ Jr, et al. B cells in autoimmune and neurodegenerative central nervous system diseases. *Nat Rev Neurosci.* 2019;20(12):728-45.
2. Cencioni MT, et al. B cells in multiple sclerosis - from targeted depletion to immune reconstitution therapies. *Nat Rev Neurol.* 2021.
3. Kinnunen T, et al. Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J Clin Invest.* 2013;123(6):2737-41.
4. Jelcic I, et al. Memory B cells activate brain-homing, autoreactive CD4(+) T cells in multiple sclerosis. *Cell.* 2018;175(1):85-100 e23.
5. Bjornevik K, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science.* 2022;375(6578):296-301.
6. Lunemann JD, et al. EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2. *J Exp Med.* 2008;205(8):1763-73.
7. Wang J, et al. HLA-DR15 molecules jointly shape an autoreactive T cell repertoire in multiple sclerosis. *Cell.* 2020;183(5):1264-81 e20.
8. van Langelaar J, et al. Induction of brain-infiltrating T-bet-expressing B cells in multiple sclerosis. *Ann Neurol.* 2019;86(2):264-78.
9. Piovesan D, et al. c-Myb regulates the T-bet-dependent differentiation program in B cells to coordinate antibody responses. *Cell Rep.* 2017;19(3):461-70.
10. Rawlings DJ, et al. Altered B cell signalling in autoimmunity. *Nat Rev Immunol.* 2017;17(7):421-36.
11. Rivera-Correa J, et al. Plasmodium DNA-mediated TLR9 activation of T-bet(+) B cells contributes to autoimmune anaemia during malaria. *Nat Commun.* 2017;8(1):1282.

12. Bar-Or A, et al. Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Ann Neurol.* 2010;67(4):452-61.
13. van Langelaar J, et al. The association of Epstein-Barr virus infection with CXCR3(+) B-cell development in multiple sclerosis: impact of immunotherapies. *Eur J Immunol.* 2021;51(3):626-33.
14. Janssen M, et al. Pregnancy-induced effects on memory B-cell development in multiple sclerosis. *Sci Rep.* 2021;11(1):12126.
15. Fransen NL, et al. Absence of B cells in brainstem and white matter lesions associates with less severe disease and absence of oligoclonal bands in MS. *Neurol Neuroimmunol Neuroinflamm.* 2021;8(2).
16. Timofeeva N, Gandhi V. Ibrutinib combinations in CLL therapy: scientific rationale and clinical results. *Blood Cancer J.* 2021;11(4):79.
17. Neys SFH, et al. Targeting bruton's tyrosine kinase in inflammatory and autoimmune pathologies. *Front Cell Dev Biol.* 2021;9:668131.
18. Crofford LJ, et al. The role of Bruton's tyrosine kinase in autoimmunity and implications for therapy. *Expert Rev Clin Immunol.* 2016;12(7):763-73.
19. Rip J, et al. Toll-like receptor signaling drives Btk-mediated autoimmune disease. *Front Immunol.* 2019;10:95.
20. Haselmayer P, et al. Efficacy and pharmacodynamic modeling of the BTK inhibitor evobrutinib in autoimmune disease models. *J Immunol.* 2019;202(10):2888-906.
21. Montalban X, et al. Placebo-controlled trial of an oral BTK inhibitor in multiple sclerosis. *N Engl J Med.* 2019;380(25):2406-17.
22. Torke S, et al. Inhibition of Bruton's tyrosine kinase interferes with pathogenic B-cell development in inflammatory CNS demyelinating disease. *Acta Neuropathol.* 2020;140(4):535-48.

23. Karnell JL, et al. Role of CD11c(+) T-bet(+) B cells in human health and disease. *Cell Immunol.* 2017;321:40-5.
24. Contentti EC, Correale J. Bruton's tyrosine kinase inhibitors: a promising emerging treatment option for multiple sclerosis. *Expert Opin Emerg Drugs.* 2020;25(4):377-81.
25. Bhargava P, et al. Imaging meningeal inflammation in CNS autoimmunity identifies a therapeutic role for BTK inhibition. *Brain.* 2021;144(5):1396-408.
26. Jain RW, Yong VW. B cells in central nervous system disease: diversity, locations and pathophysiology. *Nat Rev Immunol.* 2021.
27. Corneth OBJ, et al. Enhanced Bruton's tyrosine kinase activity in peripheral blood B lymphocytes from patients with autoimmune disease. *Arthritis Rheumatol.* 2017;69(6):1313-24.
28. Kong W, et al. Increased expression of Bruton's tyrosine kinase in peripheral blood is associated with lupus nephritis. *Clin Rheumatol.* 2018;37(1):43-9.
29. Cotzomi E, et al. Early B cell tolerance defects in neuromyelitis optica favour anti-AQP4 autoantibody production. *Brain.* 2019;142(6):1598-615.
30. Brodie EJ, et al. Lyn, lupus, and (B) lymphocytes, a lesson on the critical balance of kinase signaling in immunity. *Front Immunol.* 2018;9:401.
31. Wu XN, et al. Defective PTEN regulation contributes to B cell hyperresponsiveness in systemic lupus erythematosus. *Sci Transl Med.* 2014;6(246):246ra99.
32. Sohn HW, et al. Cbl-b negatively regulates B cell antigen receptor signaling in mature B cells through ubiquitination of the tyrosine kinase Syk. *J Exp Med.* 2003;197(11):1511-24.
33. van Langelaar J, et al. B and T cells driving multiple sclerosis: identity, mechanisms and potential triggers. *Front Immunol.* 2020;11:760.

34. Yasuda T, et al. Cbl-b positively regulates Btk-mediated activation of phospholipase C-gamma2 in B cells. *J Exp Med.* 2002;196(1):51-63.
35. Satterthwaite AB. Bruton's tyrosine kinase, a component of B cell signaling pathways, has multiple roles in the pathogenesis of lupus. *Front Immunol.* 2017;8:1986.
36. Knox JJ, et al. T-bet(+) memory B cells: generation, function, and fate. *Immunol Rev.* 2019;288(1):149-60.
37. Naradikian MS, et al. Cutting Edge: IL-4, IL-21, and IFN-gamma interact to govern T-bet and CD11c expression in TLR-activated B cells. *J Immunol.* 2016;197(4):1023-8.
38. Xu X, et al. Phosphorylation-mediated IFN-gammaR2 membrane translocation is required to activate macrophage innate response. *Cell.* 2018;175(5):1336-51 e17.
39. Halcomb KE, et al. Btk regulates localization, in vivo activation, and class switching of anti-DNA B cells. *Mol Immunol.* 2008;46(2):233-41.
40. Luo W, et al. A balance between B cell receptor and inhibitory receptor signaling controls plasma cell differentiation by maintaining optimal Ets1 levels. *J Immunol.* 2014;193(2):909-20.
41. Grenningloh R, et al. Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses. *J Exp Med.* 2005;201(4):615-26.
42. Nguyen HV, et al. The Ets-1 transcription factor is required for Stat1-mediated T-bet expression and IgG2a class switching in mouse B cells. *Blood.* 2012;119(18):4174-81.
43. Sindhava VJ, et al. A TLR9-dependent checkpoint governs B cell responses to DNA-containing antigens. *J Clin Invest.* 2017;127(5):1651-63.
44. Rubtsov AV, et al. CD11c-expressing B cells are located at the T cell/B cell border in spleen and are potent APCs. *J Immunol.* 2015;195(1):71-9.
45. Li R, et al. BTK inhibition limits B-cell-T-cell interaction through modulation of B-cell metabolism: implications for multiple sclerosis therapy. *Acta Neuropathol.* 2022.

46. Sharma S, et al. Btk regulates B cell receptor-mediated antigen processing and presentation by controlling actin cytoskeleton dynamics in B cells. *J Immunol.* 2009;182(1):329-39.
47. Hendriks RW, et al. Targeting Bruton's tyrosine kinase in B cell malignancies. *Nat Rev Cancer.* 2014;14(4):219-32.
48. de Gorter DJ, et al. Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity.* 2007;26(1):93-104.
49. Lehmann-Horn K, et al. Deciphering the role of B cells in multiple sclerosis - towards specific targeting of pathogenic function. *Int J Mol Sci.* 2017;18(10).
50. van Langelaar J, et al. T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention. *Brain.* 2018;141(5):1334-49.
51. Thompson AJ, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol.* 2018;17(2):162-73.
52. Rip J, et al. Phosphoflow protocol for signaling studies in human and murine B cell subpopulations. *J Immunol.* 2020;204(10):2852-63.
53. Weksler BB, et al. Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J.* 2005;19(13):1872-4.

Figure 1

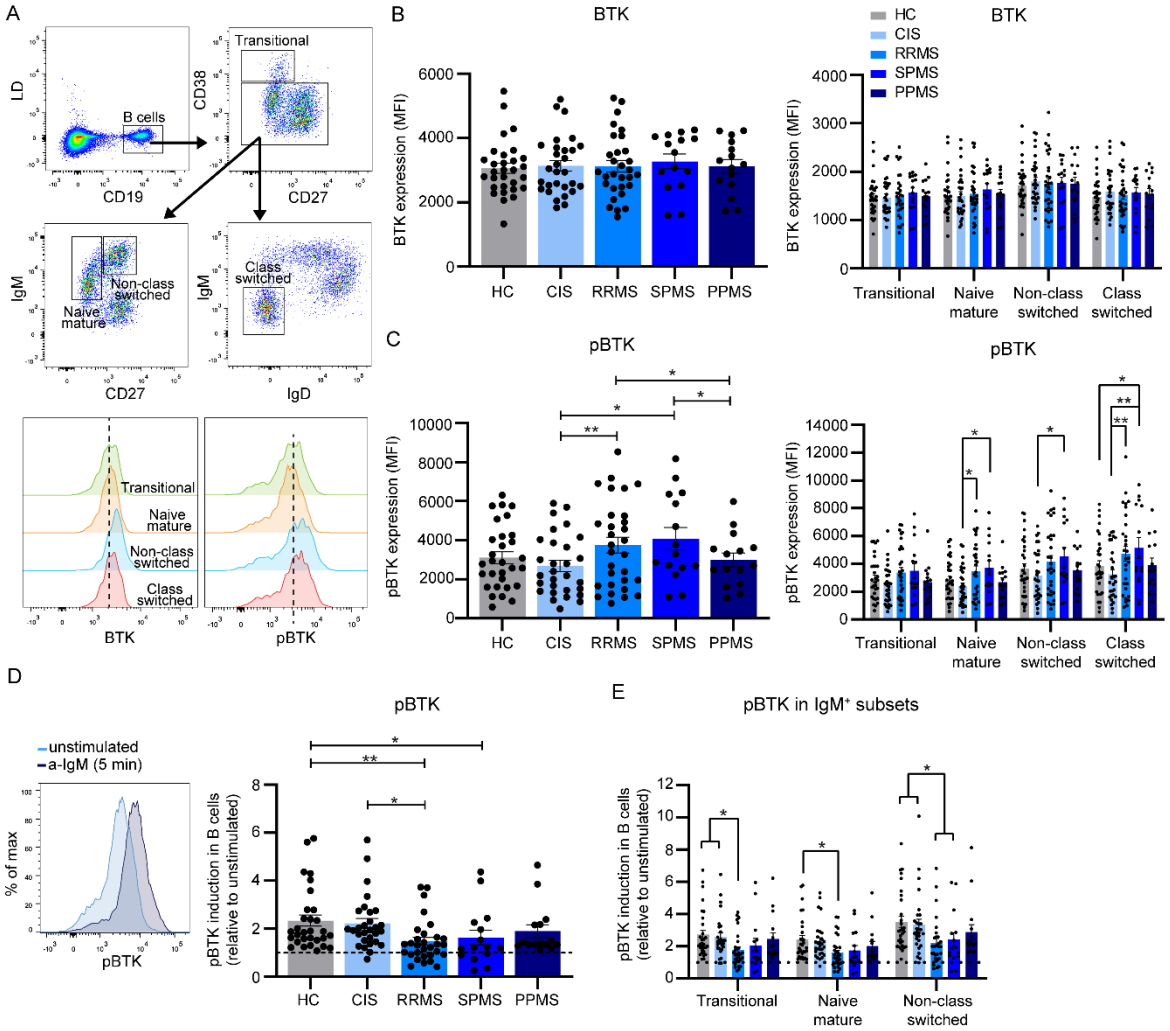

Figure 1. Phospho-BTK is upregulated and less induced in B cells of RRMS and SPMS patients. (**A**) FACS gating strategy used to analyze total BTK protein and phospho-BTK (pBTK) expression levels (MFI) in transitional ($CD38^{high}CD27^{-}$), naive mature ($CD38^{dim/-}IgM^{+}CD27^{-}$), non-class switched ($CD38^{dim/-}IgM^{+}CD27^{+}$) and class-switched ($CD38^{dim/-}IgM^{-}IgD^{-}$) B-cell subsets. Total BTK protein (**B**) and phospho-BTK (**C**) levels were studied in blood B cells (*left*: total*; right*: subsets) from healthy controls (HC; n=30) and different MS patient groups, including clinically isolated syndrome (CIS; n=29), relapsing-remitting MS (RRMS; n=30), secondary progressive MS (SPMS; n=15) and primary progressive MS (PPMS; n=15). The inducibility of phospho-BTK in total B cells (**D**) and IgM^{+} subsets (**E**) were compared between patient and control groups using anti-IgM for 5 min. These FACS data were collected in three independent experiments, with 5–10 samples from controls and each patient group per experiment. Data are presented as the mean ± SEM. Two-way ANOVA with Fisher's LSD post-hoc test was performed. $^{**}p < 0.01$ and $^{*}p < 0.05$.

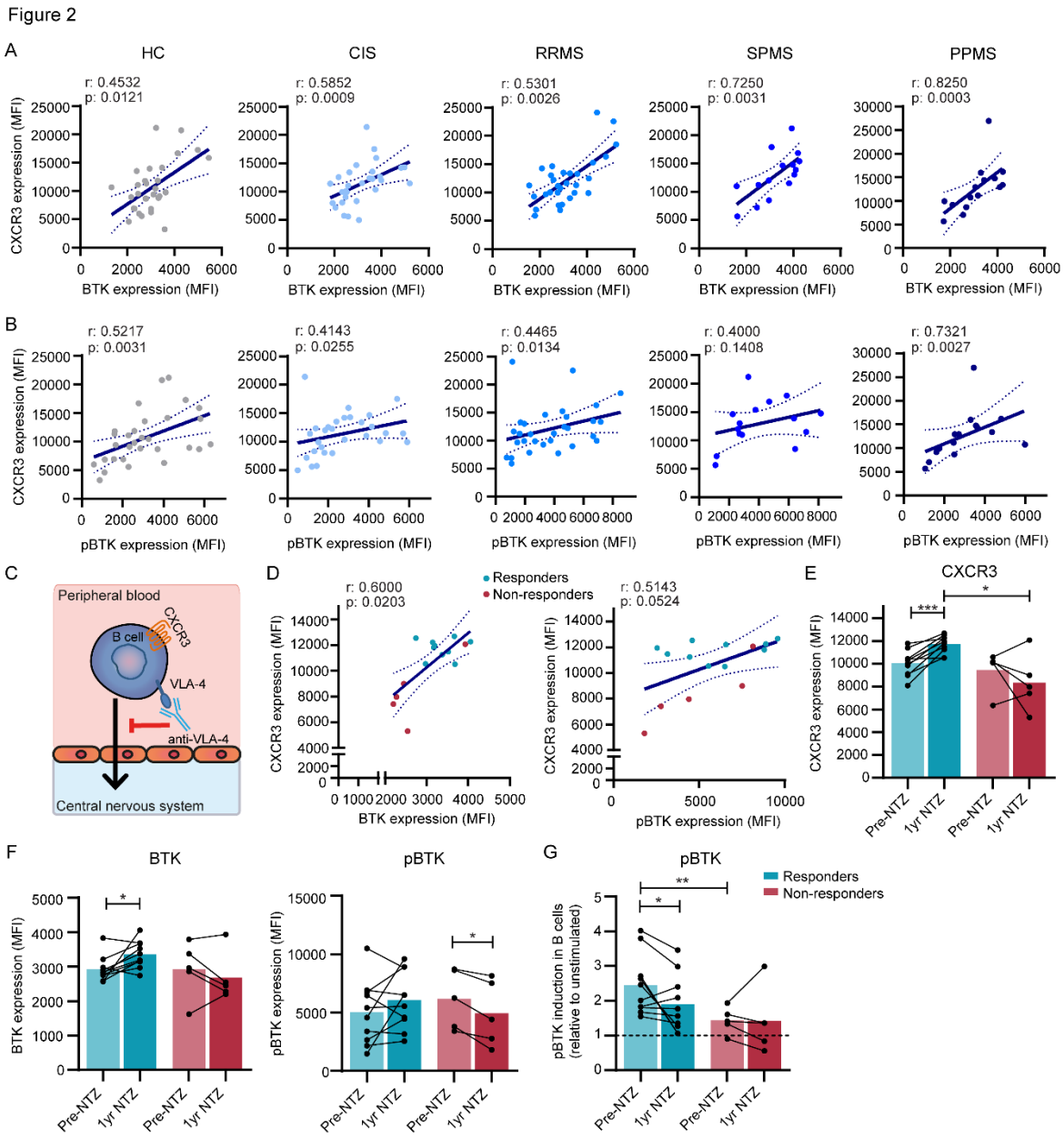

Figure 2. Correlation of BTK and phospho-BTK with CXCR3 expression levels in B cells from MS patients. The association of total BTK protein (**A**) and phospho-BTK (**B**) with CXCR3 expression was analyzed for blood B cells from healthy control (HC; n=30), clinically isolated syndrome (CIS; n=29), relapsing-remitting MS (RRMS; n=30), secondary progressive MS (SPMS; n=15) and primary progressive MS (PPMS; n=15) groups. FACS data were collected in the same number of experiments as depicted in Figure 1. (**C**) Graphical illustrations of natalizumab (NTZ; anti-VLA-4 monoclonal antibody) treatment blocking the migration of $CXCR3^+$ B cells into the central nervous system. (**D–F**) Total BTK protein, phospho-BTK and CXCR3 expression levels were assessed in *ex vivo* blood B cells from NTZ-treated MS patients. These were compared between pre- and 1 year (yr) post-treatment samples as well as clinical responders (n=10) and non-responders (n=5). (**G**) The inducibility of phospho-BTK in B cells from responders and non-responders before and after NTZ treatment. For the NTZ-treated MS cohort, FACS data were collected in one experiment with all patients included. Spearman correlations (**A, B** and **D**) and both paired t-test and Mann-Whitney U tests (**E–G**) were performed. ***p <0.001, **p <0.01 and *p <0.05.

Figure 3

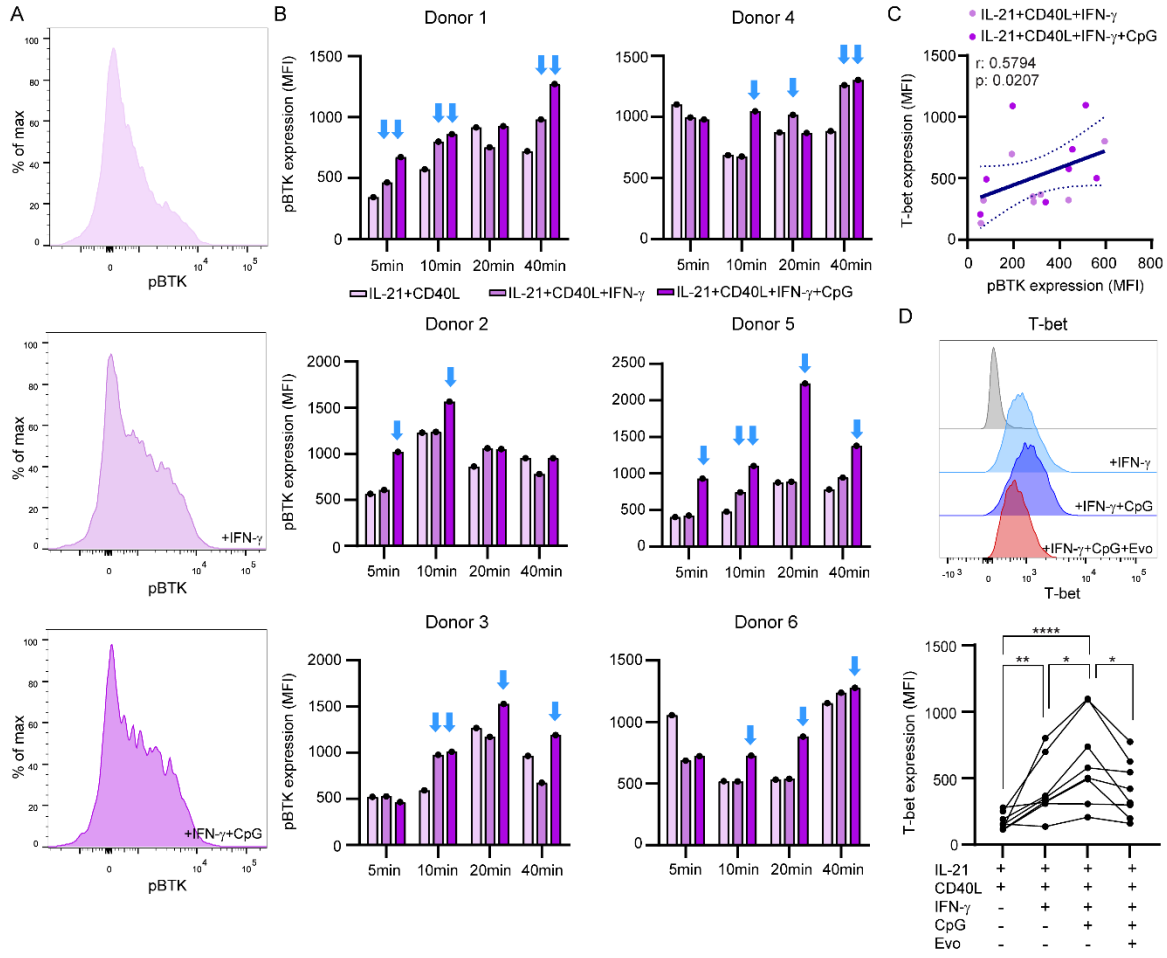

Figure 3. BTK activity is associated with IFN-γ- and CpG-mediated T-bet induction in human B cells. (**A**) Representative histograms of phospho-BTK expression in healthy blood-derived B cells stimulated with IL-21+CD40L, IL-21+CD40L+IFN-γ and IL-21+CD40L+IFN-γ+CpG for 10 min. (**B**) Donor-specific phospho-BTK induction in blood B cells under the same conditions for 5, 10, 20 and 40 min (n=6). The blue arrows indicate time points at which phospho-BTK is upregulated by IFN-γ with and without CpG. These FACS data were collected in four independent experiments, with 1–2 donors per experiment. (**C**) Correlation between phospho-BTK and T-bet levels in B cells under IL-21/CD40L/IFN-γ-inducing conditions with and without CpG for 48 h. (**D**) The impact of evobrutinib (Evo) on IFN-γ- and CpG-induced T-bet expression in B cells (48 h). These data were collected in three independent experiments, with 2–3 donors per experiment. Spearman correlation (**C**) and RM one-way ANOVA with Fisher's LSD post-hoc test (**D**) were performed. ****p <0.0001, **p <0.01 and *p <0.05.

Figure 4

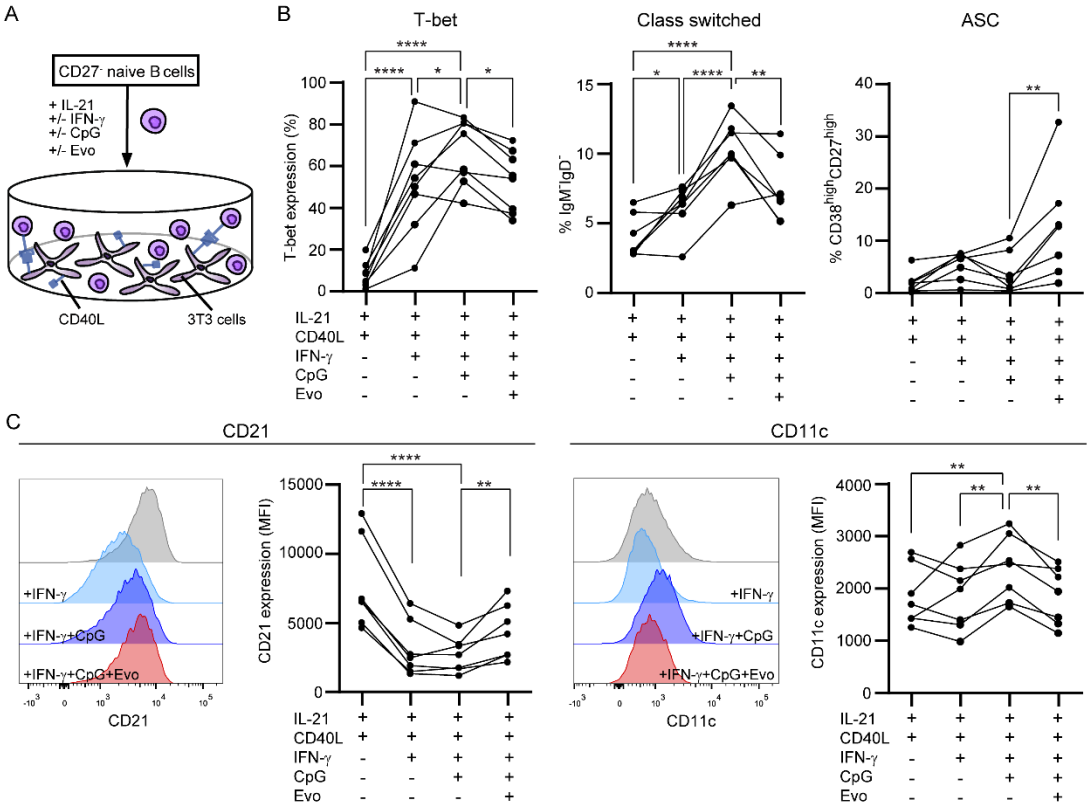

Figure 4. Evobrutinib interferes with the differentiation of T-bet$^+$ class-switched memory subsets in naive B-cell cultures. (**A**) CD27$^-$ naive B cells were purified from healthy donor blood and cultured under T_{FH}-like conditions using IL-21 and CD40L-3T3 cells for 11 days (n=7–8). IFN-γ and CpG were added with and without evobrutinib (Evo). (**B**) Proportions of *in vitro*-induced T-bet$^+$ B cells, class-switched (IgM$^-$IgD$^-$) memory B cells and antibody-secreting cells (CD38^{high}CD27^{high}; ASC). (**C**) Histogram overlays and quantification of CD21 and CD11c expression (MFI) by these cultured B cells. Cultures were performed in eight independent experiments, with 1 donor per experiment. RM one-way ANOVA with Fisher's LSD post-hoc test were used. ****p <0.0001, **p <0.01 and *p <0.05.

Figure 5

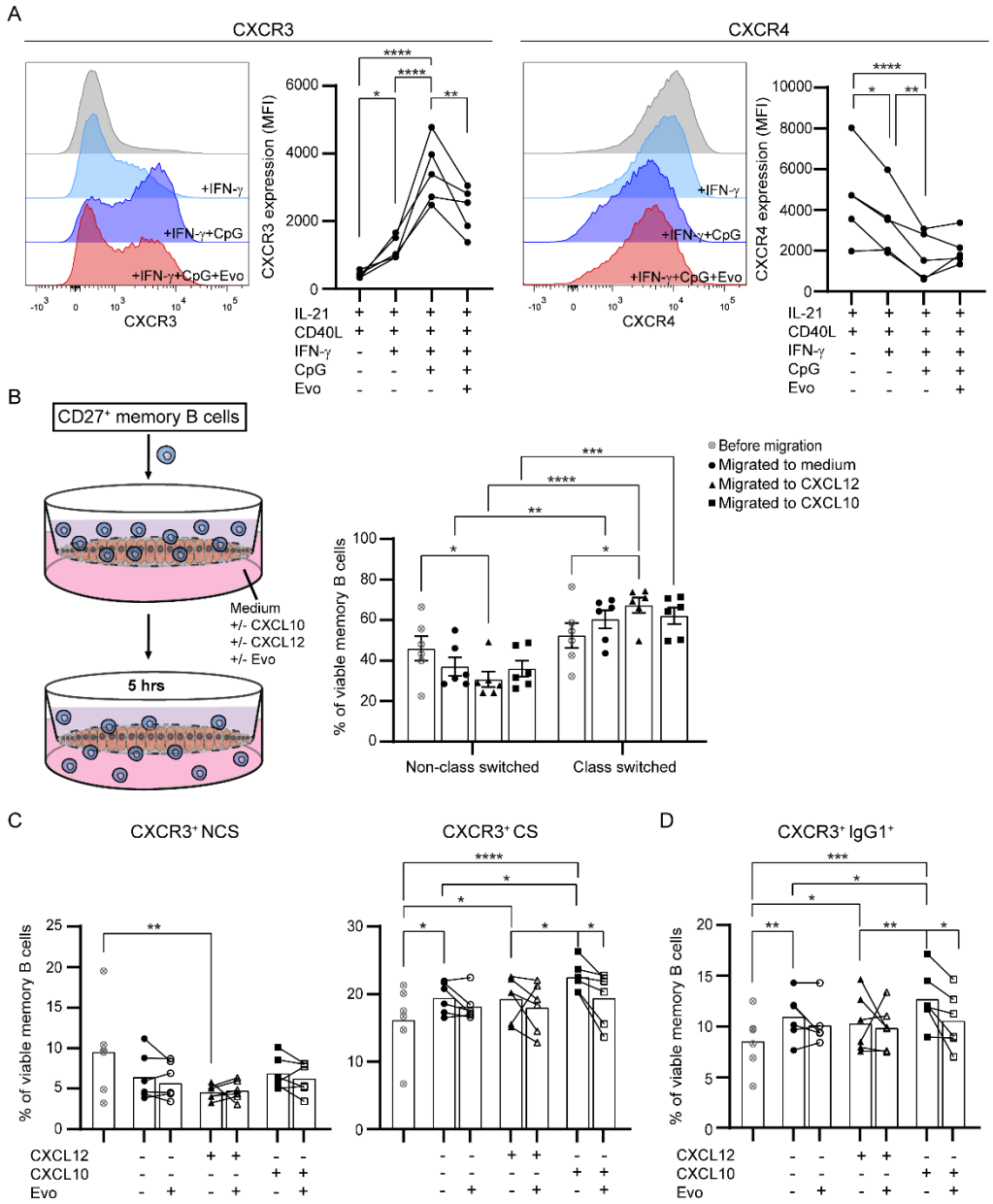

Figure 5. CXCL10-mediated migration of CXCR3$^+$ class-switched B cells through human brain endothelial monolayers is attenuated by evobrutinib. (**A**) Histogram overlays and quantification of CXCR3 and CXCR4 expression (MFI) by B cells from 5 healthy blood donors after stimulation with IL-21, CD40L, IFN-γ and CpG for 48 h. Evobrutinib (Evo) was added to IFN-γ and CpG-inducing conditions. Data were collected as described for Fig. 3C–D. (**B**–**D**) Purified CD27$^+$ memory B cells from 6 healthy blood donors were assessed for their selective migration across human brain endothelial monolayers *in vitro*. The proportions of viable non-class switched (IgM$^+$CD27$^+$; NCS), class-switched (IgM$^-$CD27$^+$; CS) and IgG1$^+$ B cells were studied before and after migration to medium, CXCL12 and CXCL10 in the context of CXCR3 expression. These FACS data were obtained from five independent experiments, with 1–2 healthy donors per experiment. Data are presented as the mean ± SEM. RM one-way ANOVA with Fisher's LSD post-hoc test were performed. ****p <0.0001, ***p <0.001, **p <0.01 and *p <0.05.

Figure 6

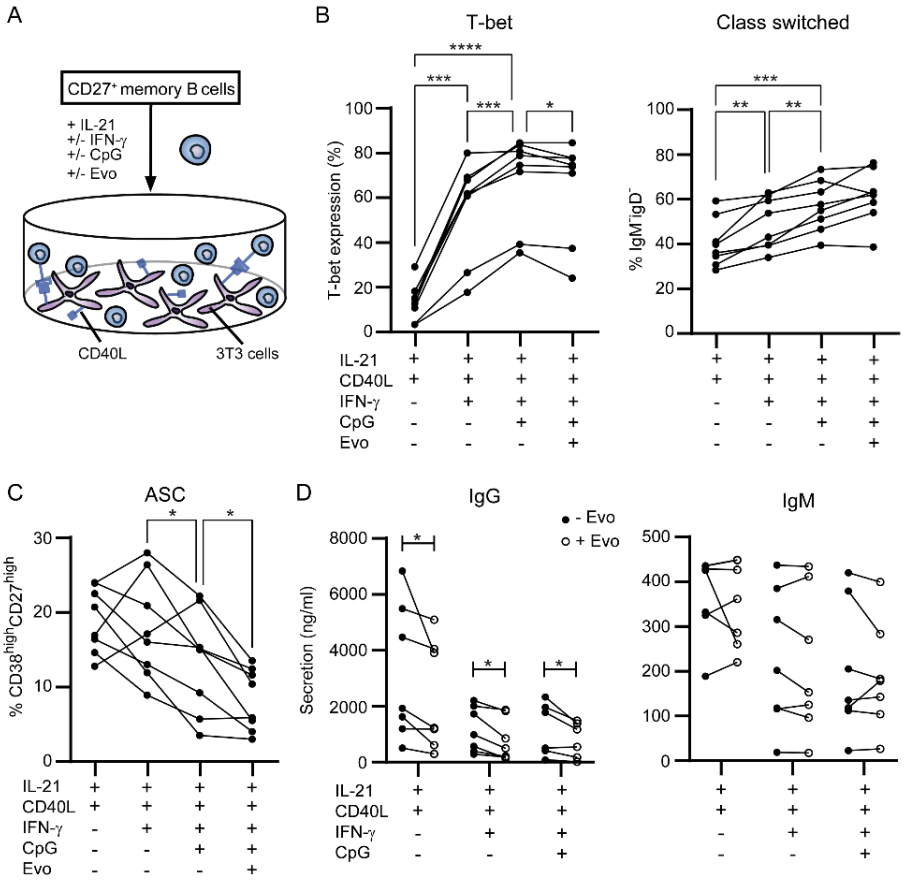

Figure 6. ***In vitro*** **memory B-cell differentiation into antibody-secreting cells is reduced by evobrutinib.** (**A**) Purified $CD27^+$ memory B cells from healthy blood donors were cultured under T_{FH}-like conditions using IL-21 and CD40L-3T3 with and without IFN-γ, CpG and/or evobrutinib (Evo) for 6 days. The percentages of T-bet^+ and class-switched (IgM^-IgD^-) memory B cells (**B**) as well as antibody-secreting cells ($CD38^{high}CD27^{high}$; ASC) (**C**) were analyzed for 8 donors. FACS data were collected as described for Fig. 4. (**D**) IgG and IgM secretion within the supernatants of these cultures as determined by ELISA (n=7). ELISA measurements were performed in two independent experiments, with 3-4 samples per experiment. RM one-way ANOVA with Fisher's LSD post-hoc tests (**B**–**C**) and Wilcoxon signed-rank tests (**D**) were used. ****p <0.0001, ***p <0.001, **p <0.01 and *p <0.05.