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Bruton Tyrosine Kinase Inhibition Limits Multiple Sclerosis Disease–Driving Inflammation While Promoting Regulatory B Cells

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Abstract

Background and Objectives

In multiple sclerosis (MS), a variety of immunosuppressive treatments are available. While effective, these approaches often lead to sustained impairment of essential components of the immune system, posing long-term safety concerns. Consequently, there is a growing interest in alternative therapeutic approaches that selectively limit pathogenic B-cell functions while preserving their physiologic roles. In this study, we investigated the therapeutic potential of inhibiting the enzyme Bruton tyrosine kinase (BTK), a key signaling molecule in both B-cell and myeloid cell activation.

Methods

The effects of the BTK inhibitor evobrutinib were evaluated in various experimental in vivo models of CNS demyelination, each representing different aspects of disease pathology as well as a naïve healthy condition. The impact on disease onset and severity was determined, and phenotypical alterations in different cell populations were assessed via flow cytometry. Furthermore, functional changes in both murine and human myeloid cells induced by BTK inhibition under specific Fc receptor–dependent stimulation were analyzed in *in vitro* settings using flow cytometry.

Results

In a naïve, healthy environment, evobrutinib promoted the development of regulatory B-cell properties. In various experimental models of CNS demyelination, BTK inhibition limited the differentiation of proinflammatory B cells while supporting their regulatory properties. Beyond modulating B-cell responses, BTK inhibition also attenuated the activation of myeloid cells after Fc receptor–mediated antigen uptake, a process assumed to be of importance in conditions, such as neuromyelitis optica spectrum disorder and myelin oligodendrocyte glycoprotein–antibody associated disease. In addition, BTK inhibition was shown to suppress the secretion of proinflammatory cytokines and reduce antigen presentation, further dampening pathogenic immune responses.

Discussion

These findings highlight the potential of BTK inhibition as a selective and sustainable immunomodulatory strategy for both B cells and myeloid cells in the context of chronic CNS inflammation. Despite their efficacy, broad-spectrum immunosuppressive therapies often fail to provide targeted immune modulation. By contrast, BTK inhibition promotes regulatory B-cell properties while leaving other B-cell functions intact, providing the basis for its broad use—potentially in combination with established anti-inflammatory agents.

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Supplementary Material

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Glossary

BCR = B-cell receptor; **BMDM** = bone marrow–derived macrophage; **BTK** = Bruton tyrosine kinase; **BTKi** = BTK inhibition; **FcR** = Fc receptor; **Ig** = immunoglobulin; **MOG** = myelin oligodendrocyte glycoprotein; **MOGAD** = MOG antibody–associated disease; **MS** = multiple sclerosis; **NMOSD** = neuromyelitis optica spectrum disorder.

Introduction

Multiple sclerosis (MS) is the most common cause of nontraumatic disability in young adults, characterized by inflammation-induced damage within the CNS.[1,2] Therapeutic approaches have been mainly designed to permanently limit disease-driving components of the immune system. Of these approaches, anti-CD20–mediated depletion of B cells is likely the most powerful strategy to limit acute MS relapses.[3] However, increasing evidence amasses that the nonspecific obliteration of this integral part of the immune system entails undesirable consequences. These repercussions include a slow but continuous decline in the humoral compartment—as evidenced by decreasing levels of circulating immunoglobulins (Igs)—which increases the risk of secondary immunodeficiencies.[4] In addition, responses to novel immune challenges, e.g., in the context of vaccinations, were shown to be impaired.[5,6] Furthermore, the broad and unselective depletion of all B cells also erases their regulatory activities, with the risk of increasing the proinflammatory and potentially pathologic functions of other immune cells, such as cells of myeloid origin.[7,8] While there is currently no consensus on the definition of regulatory B cells by surface markers, the expression of various molecules (including PD-L1, CD39, CD73, FasL, CD1d, and TIM-1) has been associated with regulatory properties. Functionally, all identified B-cell subsets with regulatory properties are capable of producing immune system–controlling cytokines, with interleukin (IL)-10 as the most prominent cytokine released.[9]

Based on these limitations, alternative strategies specifically targeting pathologic B-cell functions while sustaining or even promoting immune system–controlling properties are currently being explored. One such approach is the inhibition of Bruton tyrosine kinase (BTK), a nonreceptor tyrosine kinase expressed in cells of hematopoietic origin, including B cells and myeloid cells. Functionally, BTK is central in relaying extracellular signals from the B-cell receptor (BCR), but it is also critically involved in Fc receptor (FcR), Toll-like receptor, and cytokine receptor pathways.[4,10,11] While BTK inhibition (BTKi) was historically developed to treat B-cell malignancies,[12] the tremendous short-term success of B cell–targeting therapies in MS has sparked interest in the deployment and testing of BTK inhibitors for this disease.[13,14] Moreover, the dual mechanism of BCR and FcR inhibition by BTK-targeting small molecules carries potential beyond controlling pathogenic B-cell function and MS relapse biology. In this regard, its limiting effect on FcR-mediated activation of myeloid cells may be of particular interest for decelerating chronic progression of MS, a process that appears to perpetuate independently of acute relapses.[15] Furthermore, this mechanism is of particular relevance in other CNS demyelinating diseases driven by antibody-mediated activation of myeloid cells, as is the case in neuromyelitis optica spectrum disorder (NMOSD) and myelin oligodendrocyte glycoprotein (MOG) antibody–associated disease (MOGAD). To date, BTK inhibitors have shown efficacy in limiting other antibody-driven autoimmune diseases, such as systemic lupus erythematosus.[16]

The goal of our work was to assess the consequences of the interference with BTK signaling in B cells and myeloid cells. Therefore, we used a multitude of animal models of CNS demyelination, each representing a specific facet of MS, NMOSD, or MOGAD-driving inflammation and inhibited BTK using evobrutinib.[17] Most strikingly, we report that (1) BTK inhibition promotes regulatory B-cell properties—even in a setting without induced inflammation—while limiting proinflammatory B-cell activation and differentiation. In addition, (2) independent of B cells, BTKi impedes proinflammatory activation and antigen presentation of murine and human myeloid cells, while enhancing phagocytosis. Mechanistically, these features combine and translate into reduced pathologic T-cell responses and an amelioration of disease in various clinically relevant settings.

Methods

Standard Protocol Approvals, Registrations, and Participant Consents

After obtaining written informed consent, healthy donors and patients with positive serum MOG antibodies presenting with various neurologic manifestations were enrolled at Hospital Clinic Barcelona, Spain; University Medical Center Düsseldorf, Germany; and University Medical Center Göttingen, Germany. The ethics committees of the University Medical Center Göttingen approved the study protocol (protocol number #03/04/14). Detailed characteristics of the study participants are provided in eTable 1 in the Supplement. Blood samples were collected during routine clinical assessment and were kindly provided by Albert Saiz (Barcelona, Spain) and Marius Ringelstein (Düsseldorf, Germany).

Mice

Wild-type mice of the C57BL/6J strain were obtained from Charles River Laboratories. Dr. Kuchroo (Boston, USA) kindly provided MOG p35-55 TCR transgenic TCR^{MOG} mice. Dr. Wekerle (Munich, Germany) kindly provided MOG Ig heavy chain knockin (IgH^{MOG}) mice. B cell–deficient μMT mice and TCR transgenic OT II mice were obtained from Jackson Laboratory (California, USA). All procedures involving animals followed the regulations of the Central Department for Animal Experiments at the University Medical Center, Göttingen, and were authorized by the State Office for Consumer Protection and Food Safety of Lower Saxony (protocol numbers 33.9-42502-04-16/2267, 33.9-42502-04-21/3680, and 33.9-42502-04-17/2745). Sample size was calculated a priori according to the approved animal experiment application, and exclusion criteria were predefined. No further exclusions were made. Purchased animals of the same sex and age were randomly assigned to treatment and control groups. For in-house–bred animals, sex and age were considered during randomization. To reduce potential confounders such as cage effects, each cage contained one animal from each treatment group. All personnel involved were aware of group allocation at all stages (allocation, conduct, outcome assessment, data analysis). Experiments and analyses were nevertheless performed as objectively and unbiased as possible. Overall, 257 animals were used across all experiments. Detailed information on the experiments can be found in the corresponding approved animal protocol.

Further description of the experiments is provided in eMethods in the Supplement. Additional data are presented in eTable 1 and eFigures 1–8 in the Supplement.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8. Tests for normality of data were performed using the D'Agostino-Pearson test ($n \geq 8$) or the Shapiro-Wilk normality test ($n < 8$). Sample sizes for animal experiments were chosen with adequate statistical power based on the literature and previous experiments. All ex vivo data were tested for outliers using the Grubbs test with $\alpha = 0.05$. Two-sided unpaired *t* tests with the Welch correction or Mann-Whitney *U* test were used for statistical comparison. Analysis of in vitro data was performed using one-way analysis of variance corrected by the Holm-Sidak or Kruskal-Wallis test with the Dunn multiple-comparison test between all groups. Associated statistics are indicated in the respective figure legend. Data are presented as the arithmetic mean with the corresponding SEM. A value of $p < 0.05$ was considered significant.

Data Availability

All data associated with this study are included in the main figures or the supplementary figures/tables (online resources).

Results

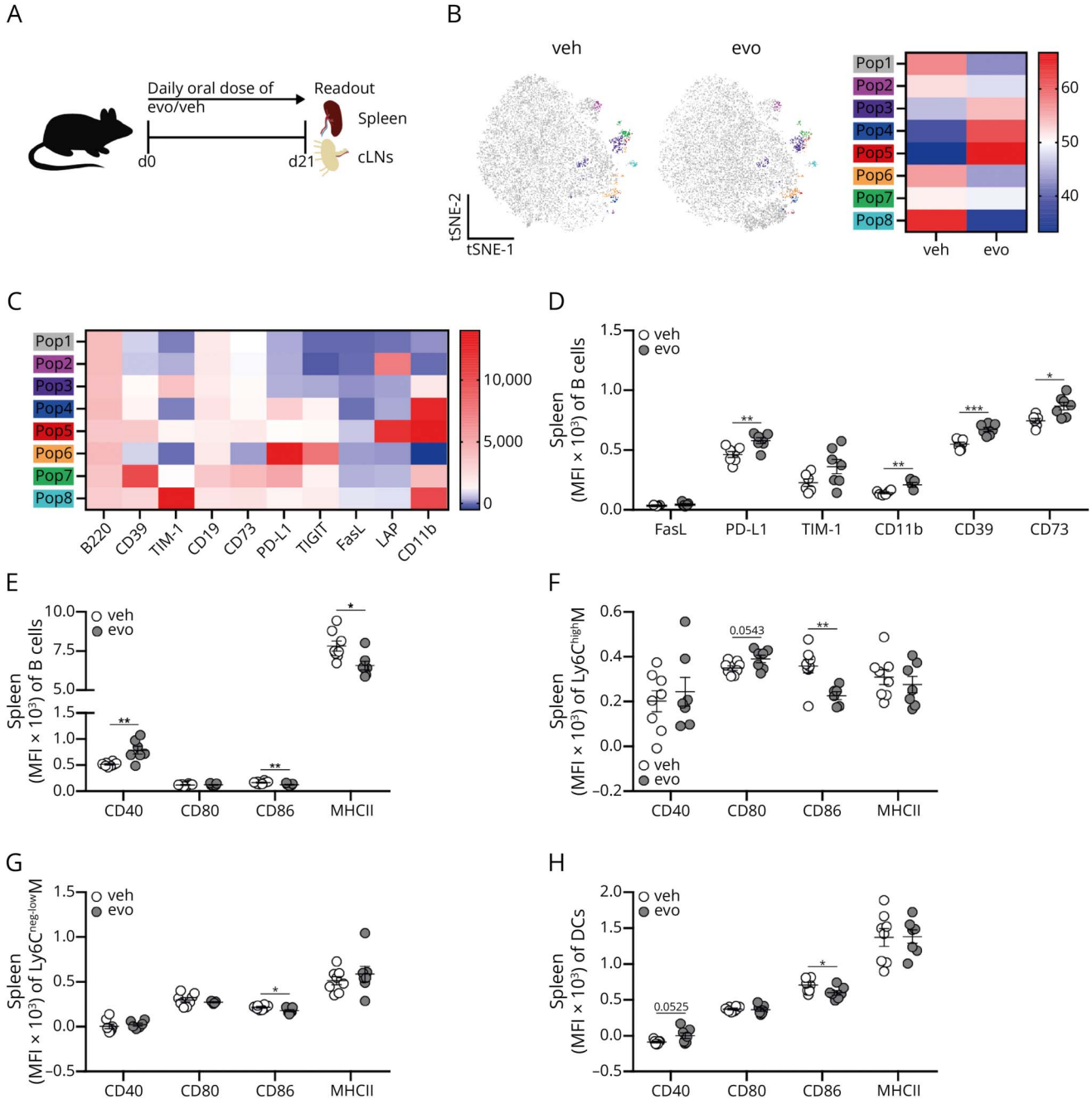
Therapeutic BTK Inhibition Enhances Regulatory Properties of B Cells and Myeloid Cells Independent of Inflammation

We recently reported that the BTK inhibitor evobrutinib prevents antigen-driven activation of B cells, resulting in a decreased disease severity in a B cell–driven model of CNS inflammation.[18] To investigate the potential of evobrutinib on a broader basis and independent of inflammation, we first analyzed the impact of BTKi in naïve healthy mice (Figure 1A). After 21 days of evobrutinib treatment, the cell frequencies of B cells, T cells, monocytes, and dendritic cells were unaffected (eFigure 1, A–D, and eFigure 3). B cells are known to be the most affected cell type after BTKi treatment. Notably, we observed significant changes in the maturation state of B cells. Specifically, there was an increased frequency of follicular II (FOII) B cells accompanied by a pronounced reduction in the BCR-BTK–dependent follicular I (FOI) B-cell population, in both the spleen and cervical lymph nodes (cLNs) (eFigure 1, E and F, and eFigure 4).

While transcriptional markers such as FoxP3 are well established for distinguishing T-cell subtypes, the identification of B-cell subtypes in different inflammatory states remains less clear. To address this, we analyzed B-cell populations in the spleen and cLNs using a collection of published B-cell markers with established regulatory function. Strikingly, BTKi treatment reduced the proportion of B cells without expression of regulatory molecules (Figure 1B). In addition, co-expression of multiple regulation-associated molecules was observed, with an enhanced population of B cells expressing high levels of LAP and CD11b, as well as $PD\text{-}L1^{+}CD11b^{high}$, under BTKi treatment (Figure 1C). B cells also exhibited increased expression of regulatory markers such as PD-L1 and TIM-1 (Figure 1D). Notably, this upregulation of regulatory molecules was also evident in the cervical lymph nodes (eFigure 1G). While the overall population of B cells with regulatory properties remains relatively small, their role in controlling CNS inflammation is well established and considered essential.[8,19] Of interest, we also observed an upregulation of CD40 expression, while the expression of antigen presentation–associated markers, such as CD86 and MHCII, was reduced. Although CD40 is traditionally involved in antigen presentation, its upregulation—paired with reduced CD86 and MHCII expression—may suggest a compensatory mechanism or functional shift toward regulatory properties rather than classical antigen presentation (Figure 1E and eFigure 1H).

Besides these alterations in B cells, a similarly altered expression pattern was observed in monocytes as well as dendritic cells (Figure 1, F–H, and eFigure 1, I-K). In the cLNs, the composition of monocytes was notably changed with a reduced frequency of inflammatory $Ly6C^{high}$ monocytes correlating with an increased frequency of anti-inflammatory $Ly6C^{neg\text{-}low}$ monocytes (eFigure 2, A and B). Furthermore,

Figure 1 BTKi Prevents the Maturation of B Cells and Promotes the Development of B Cells and Myeloid Cells With Regulatory Properties in Naïve Animals



(A) Naïve wild-type mice were treated daily with 3 mg/kg of evobrutinib (evo) or vehicle control (veh) over a period of 21 days. Spleens and cervical lymph nodes (cLNs) were analyzed via flow cytometry. (B) *t*-distributed stochastic neighbor embedding (tSNE) visualization of flow cytometry analysis based on $B220^{+}CD19^{+}$ B cells. (C) Heat map of defined populations (Pop) between treatment and different molecules. Mean fluorescence intensity (MFI) of (D) molecules associated with regulatory properties of B cells, analyzed on $B220^{+}$ B cells in the spleen, and of (E) surface molecules with antigen presentation in the spleen. (F–H) MFI of surface molecules associated with antigen presentation on $Ly6C^{high}$ and $Ly6C^{neg\text{-}low}$ monocytes (M) and dendritic cells (DCs) in the spleen. Representative data of 2 independent experiments are shown as mean ± SEM; n = 7–8 mice; overall 28 animals. Asterisks indicate significant difference calculated using unpaired *t* tests with the Welch correction or Mann-Whitney *U* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

BTKi treatment resulted in an enhanced frequency of plasmacytoid dendritic cells in both lymphatic organs (eFigure 2, C-E). Given that T cells do not express BTK to a biologically relevant amount[15] and can only be affected indirectly by BTKi, their activation state remained unaltered in the absence of an inflammatory stimulus (eFigure 2, F and G). These findings indicate that evobrutinib treatment promotes regulatory properties in both B-cell

and myeloid cell compartments. Importantly and of great value for its possible translation to chronic human diseases, BTKi induces these therapeutically desirable modifications independently of cellular stimulation within an inflammatory context.

BTKi Prevents Spontaneous CNS Autoimmune Disease by Limiting the Activation of Proinflammatory Autoreactive B Cells

Having established that BTKi promotes the development of immune cells with regulatory properties in a naïve setting, we next studied the therapeutic potential of BTKi in a model focusing on the pathogenic role of self-reactive B cells. In the double-transgenic $IgH^{MOG} \times TCR^{MOG}$ mouse line characterized by high frequencies of MOG-specific B cells (IgH^{MOG}) and MOG-specific T cells (TCR^{MOG}), symptoms of experimental autoimmune encephalomyelitis (EAE) develop spontaneously around 4–6 weeks of age.[17] Prophylactic treatment with evobrutinib, starting at 3.5 weeks of age, resulted in a significant reduction in overall EAE incidence, reducing the risk of disease development by 55% (Figure 2, A and B). At 10 weeks of age, analysis of spinal cord pathology revealed a marked reduction in both demyelination and CNS immune cell infiltration (Figure 2C and eFigure 5, A–C). To determine whether these protective effects were mediated by suppression of inflammatory immune responses, we analyzed the B-cell activation status. While the frequency of B cells in the spleen remained unchanged after BTKi treatment (eFigure 5D), their expression of markers of activation and antigen presentation was significantly reduced (Figure 2, D and E). Similar effects could be observed in the cLNs (eFigure 5, E and F). Notably, BTKi again led to an increased expression of regulatory molecules such as PD-L1 and TIGIT on splenic B cells (Figure 2F), as well as FasL expression in the draining cLNs (eFigure 5G).

In addition to B-cell modulation, T cells in the spleen and cLNs were indirectly affected by BTKi treatment, showing reduced expression of activation markers (Figure 2G and eFigure 5, H and I). We also observed changes in BTK-expressing myeloid cells. In evobrutinib-treated mice, frequencies of monocytes and dendritic cells in the spleen were elevated (eFigure 6, A and B). However, these cells displayed a significant reduction in activation and antigen presentation–associated markers in both the spleen and cLNs (Figure 2, H–K, and eFigure 6, C and D). Further examination of monocyte subpopulations revealed no alteration in Ly6C expression (eFigure 6, E and F). Nonetheless, the $Ly6C^{high}$ monocyte subset exhibited a reduced expression of activation and antigen presentation molecules in both lymphatic organs after BTKi treatment (eFigure 6, G and H).

These findings highlight the multifaceted therapeutic effects of BTKi, demonstrating its capacity to promote regulatory B-cell phenotypes while simultaneously suppressing the activation of self-reactive B cells and limiting proinflammatory differentiation of myeloid cells. It is important to note that these effects again occurred in the absence of an external inflammatory stimulus. This highlights the potential of BTKi as a therapeutic strategy for modulating immune cell function in both inflammatory and non-inflammatory conditions, with implications for the treatment of autoimmune diseases and other immune-mediated disorders.

BTKi Mitigates EAE Severity by Modulating Proinflammatory Myeloid Cells

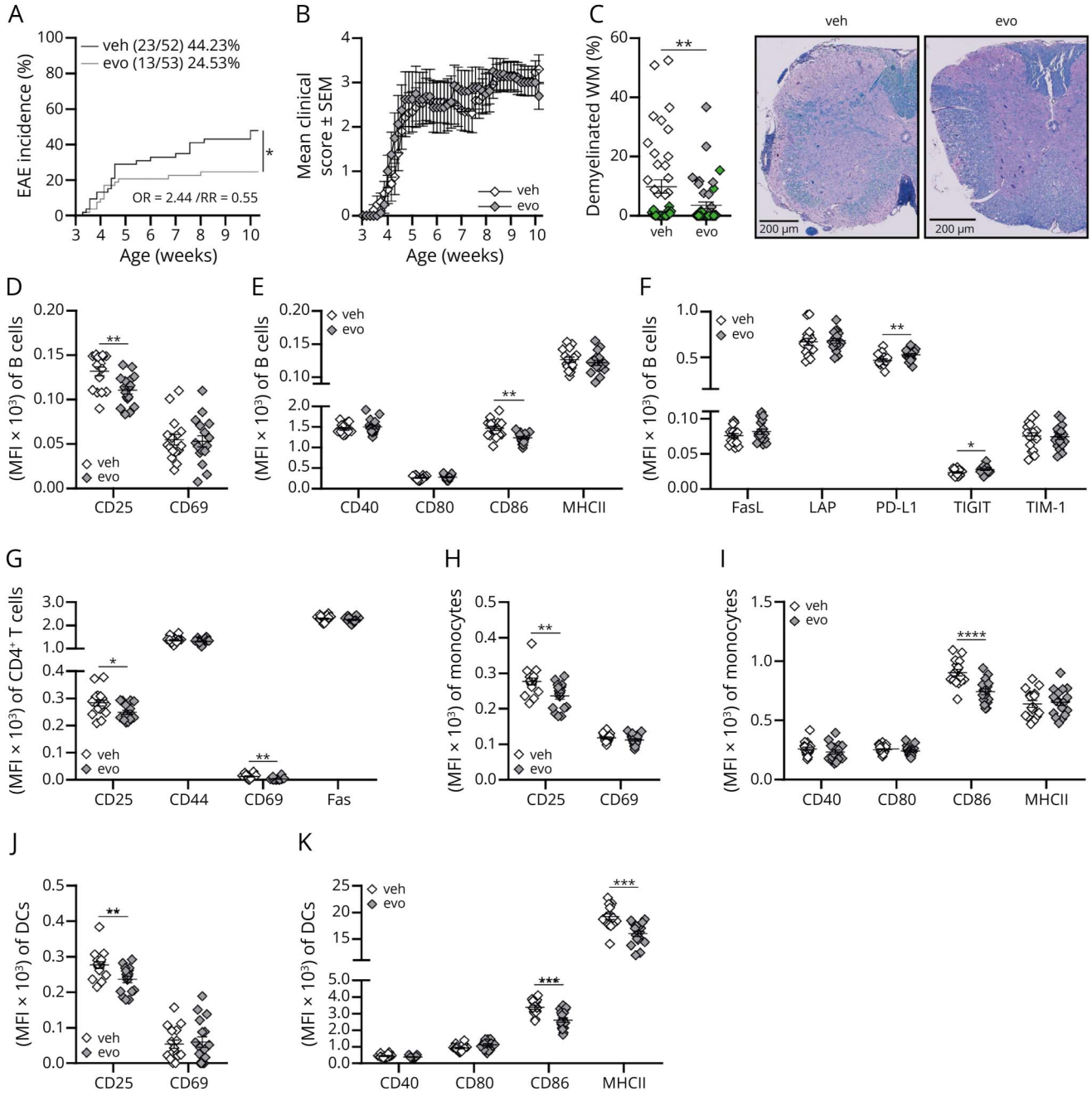
To further investigate whether BTKi affects BTK-expressing myeloid cells in a clinically meaningful manner, we used an EAE model focusing on myeloid cell interactions with T cells, namely T cell–driven EAE induced by immunization with the MOG_{35-55} peptide (Figure 3A). Of note, this model develops independently of B cells, and previous studies have shown that B-cell depletion exacerbates clinical disease severity in this setting.[8]

In this model, BTKi treatment resulted in a significant reduction in clinical severity during disease onset, translating into a cumulative reduction in overall disease severity (Figure 3, B and C). Notably, the distribution of immune cells in the spleen remained unchanged (eFigure 7A). However, phenotypic analysis of B cells again revealed an upregulation of regulatory markers including FasL and PD-L1 in the spleen (Figure 3D), and also CD11b and CD39 in cLNs (Figure 3E). BTKi also influenced B-cell maturation states. In the spleen, transitional 1 (T1) B cells accumulated while anergic transitional 3 (T3) B cells, known for their unique signaling profile and potential autoreactivity,[20] were significantly reduced (eFigure 7B). In addition, there was an increased frequency of FOII B cells alongside a pronounced reduction in the BCR-BTK–dependent FOI subset (eFigure 7C). A similar pattern of altered maturation was observed in marginal zone precursor B cells, which accumulated in the spleen (eFigure 7D).

Shifting the focus to myeloid cells, we observed a BTKi-induced reduction in the proinflammatory $Ly6C^{high}$ monocyte subset, accompanied by an expansion of the anti-inflammatory $Ly6C^{neg\text{-}low}$ population (Figure 3F). In addition, splenic monocytes displayed a significantly diminished expression of activation and antigen presentation markers (Figure 3, G and H).

Given the pronounced clinical effects during disease onset, we sought to determine whether the reduced severity in this T cell–driven EAE model was primarily due to the regulation of B cells or myeloid cells. To this end, we induced EAE in B cell–deficient mice using the MOG_{35-55} peptide and examined myeloid cell activation (Figure 3I). Inflammatory conditions led to an expansion of the $Ly6C^{high}$ monocyte subset,

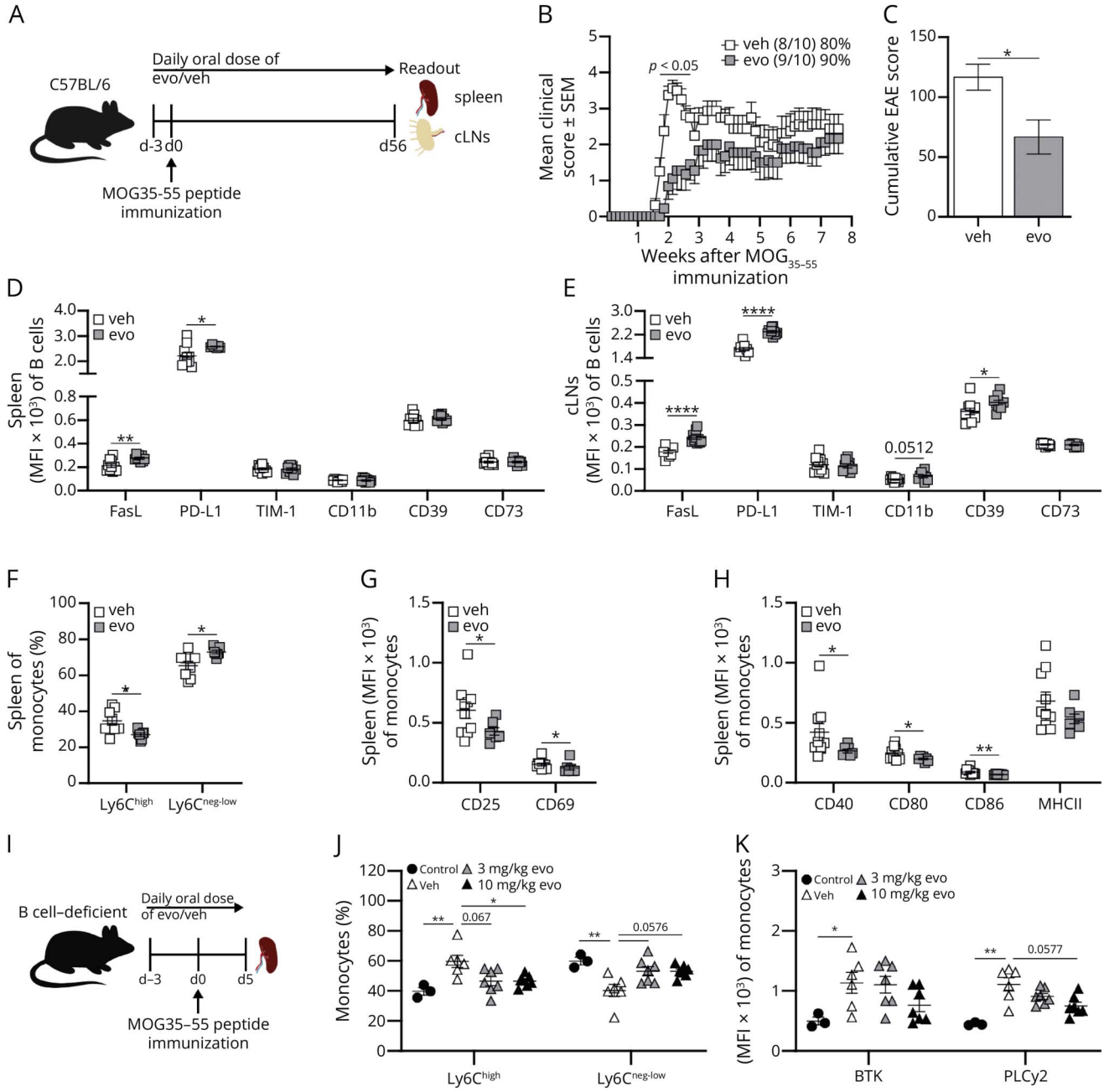
Figure 2 BTKi Reduces the Incidence of Spontaneous EAE by Limiting Proinflammatory Activation of B Cells and Myeloid Cells



Transgenic IgH^{MOG} x TCR^{MOG} mice were treated daily with 3 mg/kg of evobrutinib (evo) or vehicle control (veh) starting at the age of 3.5 weeks. At the age of 10 weeks, histologic analysis of the spinal cords was performed and spleens were assessed using flow cytometry. (A) Frequency of EAE incidence. (B) Mean clinical score ± SEM of mice with EAE symptoms. (C) Demyelinated white matter (WM) areas of the spinal cord were assessed by Luxol fast blue/periodic acid-Schiff (LFB/PAS) staining. Histologic analysis (left) and representative sections (right); scale bar = 200 µm. Mice without EAE symptoms are indicated by green symbols. (D–F) Mean fluorescence intensity (MFI) of surface molecules associated with activation, antigen presentation, and regulatory functions, analyzed on CD19^{+}B220^{+} B cells in the spleen. (G) MFI of surface molecules associated with activation analyzed on CD4^{+} T cells in the spleen. (H–K) MFI of surface molecules associated with activation and antigen presentation of CD11b^{+} monocytes and CD11b^{var}CD11c^{+} dendritic cells (DCs) in the spleen. Data are shown as mean ± SEM; histologic analysis of n = 41–53 mice; flow cytometry analysis of n = 17 mice; representative data of 2 independent experiments; overall 105 animals. Asterisks indicate significant difference calculated using unpaired *t* tests with the Welch correction or Mann-Whitney *U* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

which was substantially reduced with BTKi treatment, returning to near-baseline levels (Figure 3J). Furthermore, analysis of BTK and PLCγ2 expression confirmed an inflammatory activation of these signaling pathways after immunization, which was dose-dependently suppressed by BTKi (Figure 3K). This suppression was evident in both Ly6C^{high} and Ly6C$^{neg\text{-}low}$

Figure 3 BTKi Treatment Ameliorates T Cell–Mediated CNS Autoimmune Disease in Wild-Type as Well as in B Cell–Deficient Mice and Dampens the Proinflammatory Activity of Myeloid Cells



(A–H) C57BL/6 mice were immunized with the MOG_{35-55} peptide, n = 10 mice; overall 20 animals. Mice were treated daily with either 3 mg/kg of evobrutinib (evo) or vehicle (veh) starting 3 days prior to immunization. (B and C) Mean clinical score ± SEM of mice with EAE symptoms and the cumulative EAE score measured using the area under the curve. (D and E) Mean fluorescence intensity (MFI) of surface molecules associated with regulatory functions analyzed on $CD19^+B220^+$ B cells in the spleen and cLNs. (F) Frequency of $Ly6C^{high}$ and $Ly6C^{neg\text{-}low}$ expression of monocytes in the spleen. (G and H) MFI of surface molecules associated with activation and antigen presentation of $CD11b^+$ monocytes. (I–K) B cell–deficient μMT mice were immunized with the MOG_{35-55} peptide, n = 7 mice and representative data of 2 independent experiments; overall 39 animals. Mice were treated daily with either 3 mg/kg and 10 mg/kg of evo or veh starting 3 days prior to immunization. On Day 5 after immunization, the spleen was harvested and the activation of myeloid cells was analyzed. (J) Frequency of $Ly6C^{high}$ and $Ly6C^{neg\text{-}low}$ monocytes of living cells in the spleen. (K) MFI of BTK and PLCy2 expression of $CD11b^+$ monocytes in the spleen. (C–H) Unpaired *t* tests with the Welch correction or Mann-Whitney *U* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). (B, J, K) One-way analysis of variance corrected by the Holm-Sidak or Kruskal-Wallis test with the Dunn multiple-comparison test, comparing the treated groups with the vehicle control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

monocytes (eFigure 7, E and F). Of interest, neutrophils also exhibited similar BTKi-induced reductions in activation markers (eFigure 7G).

In conclusion, this set of experiments suggests that BTKi treatment exerts broad regulatory effects on myeloid cells, which translate into suppression of T cell–mediated

experimental CNS autoimmunity independent of B cells. These findings highlight the therapeutic potential of BTKi not only in modulating regulatory B cells but also in attenuating pathogenic myeloid cell contributions.

BTKi Modulates Myeloid Cell Activity by Directly Targeting BTK-Dependent Signaling Pathways

To dissect the extent to which myeloid cells are directly influenced by BTKi, we generated murine bone marrow–derived macrophages (BMDMs) (Figure 4A). On treatment with evobrutinib, we observed a marked reduction in BTK expression (Figure 4B). In addition, BTKi induced FcR internalization in these cells (eFigure 8, A–C). Notably, after FcR engagement, BTKi significantly reduced BTK autophosphorylation (Figure 4C), demonstrating a direct impact on the BTK signaling cascade. To exclude any potential cytotoxicity of evobrutinib, a WST-1 assay confirmed no effects on BMDM viability (Figure 4D). In addition, evobrutinib treatment attenuated the production of proinflammatory cytokines (Figure 4E), further supporting its role in modulating inflammatory signaling pathways in myeloid cells. Consistent with this reduced inflammatory activation, BTKi-treated BMDMs exhibited diminished expression of antigen presentation–associated molecules (Figure 4F). We further assessed whether these phenotypic changes translate into functional modifications by evaluating the phagocytic capacity of BMDMs. Of interest, the overall phagocytosis rate was enhanced after BTKi treatment, irrespective of the presence of antigen-specific antibodies (Figure 4G).

To explore how these phenotypic and functional alterations influence adaptive immunity, we analyzed the capability of BTKi-treated BMDMs to activate encephalitogenic T cells (Figure 4H). Remarkably, BTKi-treated BMDMs demonstrated a reduced ability to stimulate T-cell proliferation and differentiation into inflammatory subsets (Figure 4, I–K).

These results highlight the direct impact of BTKi on myeloid cells by modulating BTK-dependent signaling pathways. Evobrutinib diminishes proinflammatory activation, impairs antigen presentation capabilities, and reduces the ability of macrophages to drive T-cell activation. Together with the findings from our EAE models, this underscores the pivotal role of BTKi in regulating both innate and adaptive immune responses.

BTKi Reduces Anti-MOG Antibody–Mediated EAE

MOG-specific antibodies facilitate opsonization of CNS antigens, enabling myeloid antigen-presenting cells to recognize low antigen concentrations via FcR-dependent internalization. This process plays a key role in the pathogenesis of MOGAD and can be modeled in an established EAE setting that focuses on anti-MOG antibodies.[21] To investigate whether BTKi can mitigate the development of anti-MOG-antibody–mediated EAE, we used a transgenic TCR^{MOG} mouse model. Repeated injections of anti-MOG antibodies induced EAE in untreated mice, which were 3.4 times more likely to develop clinical EAE symptoms compared with BTKi-treated mice (Figure 5, A and B). Evobrutinib treatment reduced EAE severity, as evidenced by a seemingly decreased CNS demyelination (Figure 5C) and reduced infiltration and activation of myeloid cells, including macrophages (Figure 5D) and microglia (Figure 5E). Notably, the infiltration of T cells into the CNS remained unchanged under BTKi treatment (Figure 5F). Furthermore, monocytes isolated from the spleen demonstrated a tendency toward reduced antigen presentation capability (Figure 5G), although their activation status was not affected by BTK inhibition (Figure 5H). The anti-MOG antibody–mediated EAE model depends on the activation of MOG-specific T cells. While T cells do not express BTK to a biologically relevant amount,[15] they are indirectly influenced by altered monocyte function. Consequently, the composition of $CD4^+$ and $CD8^+$ T cells remained unaltered under BTKi treatment (Figure 5I). However, owing to reduced monocyte antigen-presenting abilities, the activation of both $CD4^+$ (Figure 5J) and $CD8^+$ (Figure 5K) T cells showed a trend toward reduction.

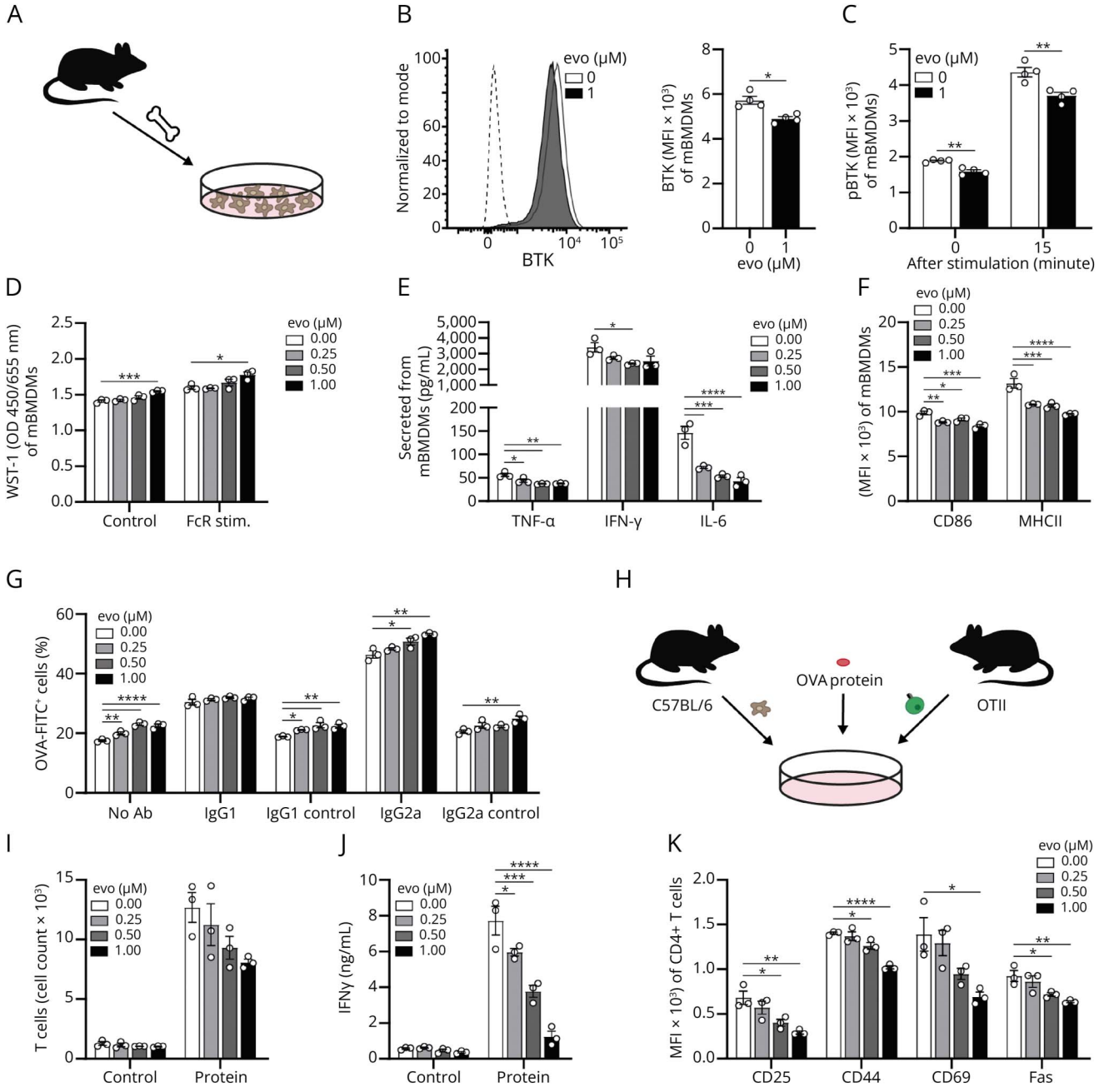
These findings highlight the pivotal role of FcR-mediated myeloid activation in anti-MOG antibody–driven CNS inflammation and demonstrate that BTKi can effectively attenuate this process. By targeting BTK-dependent signaling, BTKi reduces myeloid cell activation and the associated proinflammatory cascade, culminating in reduced demyelination and disease severity in antibody-driven experimental CNS autoimmune disease.

BTKi Inhibits Antigen Presentation and Cytokine Secretion by Human Macrophages

Antigen recognition and presentation by myeloid cells play a critical role in antibody-mediated demyelinating disorders such as MOGAD.[22] Accordingly, we investigated whether BTKi could modulate myeloid cell functions in the context of antibody-mediated demyelination. As expected, serum analysis of MOG antibody–positive patients revealed the presence of anti-MOG antibodies (Figure 6A and eTable 1). When exposed to patient serum, human monocyte-derived macrophages (MDMs) generated from healthy donors displayed enhanced uptake of MOG, indicating increased antigen recognition facilitated by the antibodies (Figure 6B). This mirrors the observations in the aforementioned antibody-mediated EAE model, which closely resembles the clinical manifestations of human MOGAD, emphasizing the pivotal role of antigen presentation pathways in progression of such diseases.[23]

Given the heightened phagocytic activity induced by anti-MOG antibodies, we examined whether BTKi could interfere with this process. After evobrutinib exposure, MDMs exhibited reduced BTK expression, paralleling findings in murine BMDMs (Figure 6C). The activation of BTK triggers autophosphorylation, and although the baseline expression of phosphorylated BTK was significantly reduced, 15 minutes after FcR engagement, there was no difference in the amount

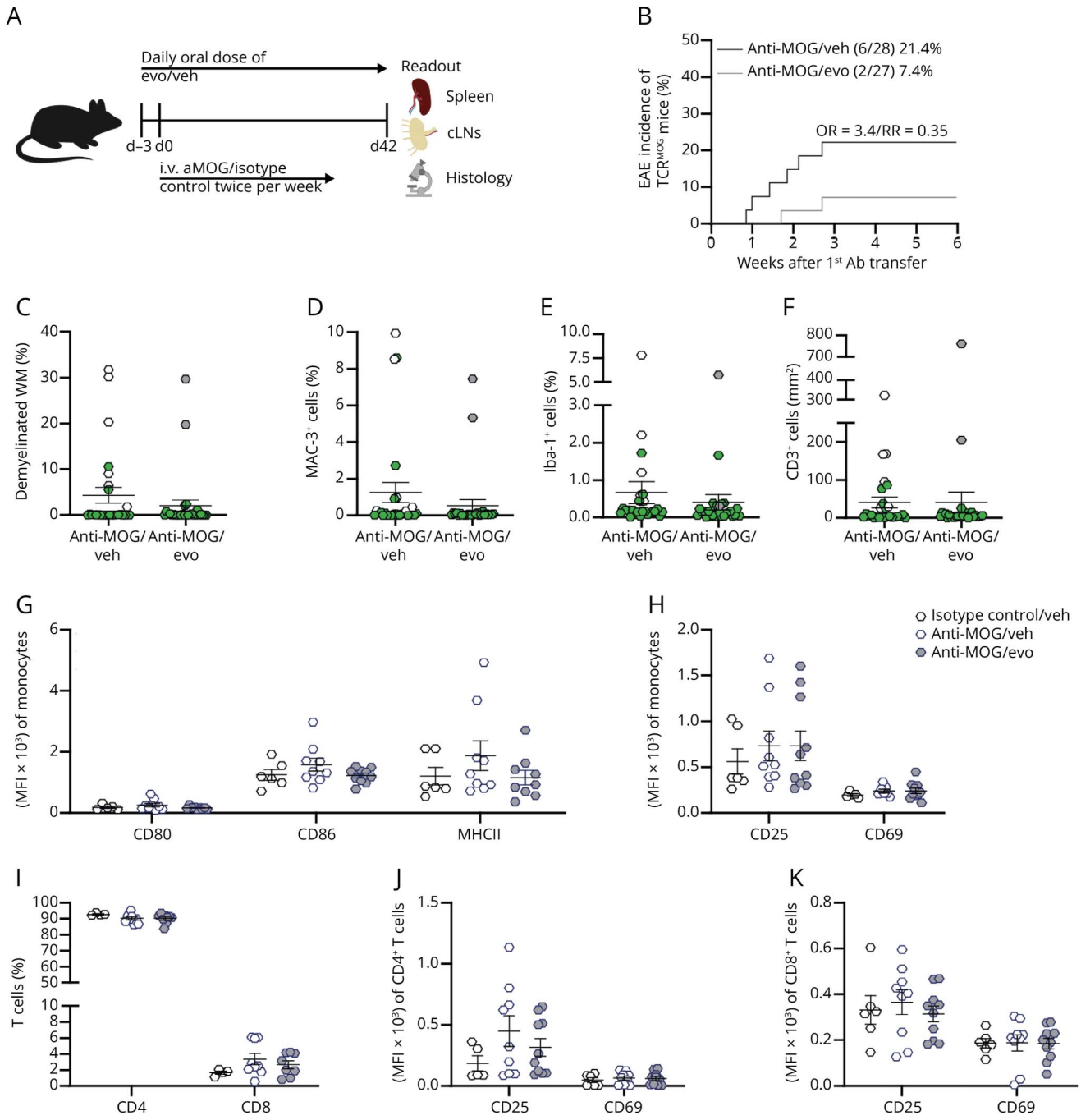
Figure 4 BTKi Treatment Reduces BTK Phosphorylation and Consequently Cytokine Secretion by Murine Macrophages as Well as Their Capacity to Present Antigen to T Cells



(A–F) Murine bone marrow–derived macrophages (BMDMs) were preactivated with IFN-γ for 6 hours. Afterward, they were cultured either alone (ctrl), with evo for 18 hours, or by FcR stimulation and analyzed via flow cytometry and ELISA. (B) Mean fluorescence intensity (MFI) of BTK expression after 18 hours of evo treatment. Example histogram (dotted line = isotype ctrl), median fluorescence intensity (MFI). (C) MFI of BTK phosphorylation after FcR stimulation. (D–F) FcR stimulation for 18 hours with evo. (D) Cell viability was determined using the WST-1 assay. (E) Cytokine production in cell supernatants. (F) MFI of CD86 and MHCII. (G) After overnight preincubation with LPS, BMDMs were cultured with FITC-labeled ovalbumin (OVA), (1) without antibodies, (2) IgG1 anti-OVA, (3) isotype control (IgG1 ctrl), (4) IgG2a anti-OVA, (5) isotype control (IgG2a ctrl), and evo for 2.5 hours. (H–K) BMDMs were preactivated with IFN-γ for 6 hours, followed by OVA and evo overnight. Later, BMDMs were cocultured with OVA-specific T cells for 72 hours. (I) Count of living T cells. (J) IFN-γ concentrations in cell supernatants. (K) MFI of T-cell surface molecules. Data sets are representative of at least 3 independent experiments, n = 3. The mean ± SEM is indicated in all graphs. (B and C) Unpaired *t* tests with the Welch correction or Mann-Whitney *U* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). (D–K) One-way analysis of variance corrected by the Holm-Sidak or Kruskal-Wallis test with the Dunn multiple-comparison test, comparing treated groups with the control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

of phosphorylated BTK between the evobrutinib-treated and control-treated groups (Figure 6D and eFigure 7D-F). Of interest, BTKi-treated macrophages demonstrated increased CD40 expression, suggesting enhanced phagocytic potential (Figure 6E). It is important to note that viability assays confirmed that evobrutinib had no toxic effects on human MDMs (eFigure 7 G). BTKi also influenced cytokine secretion profiles. Evobrutinib dose-dependently reduced the production

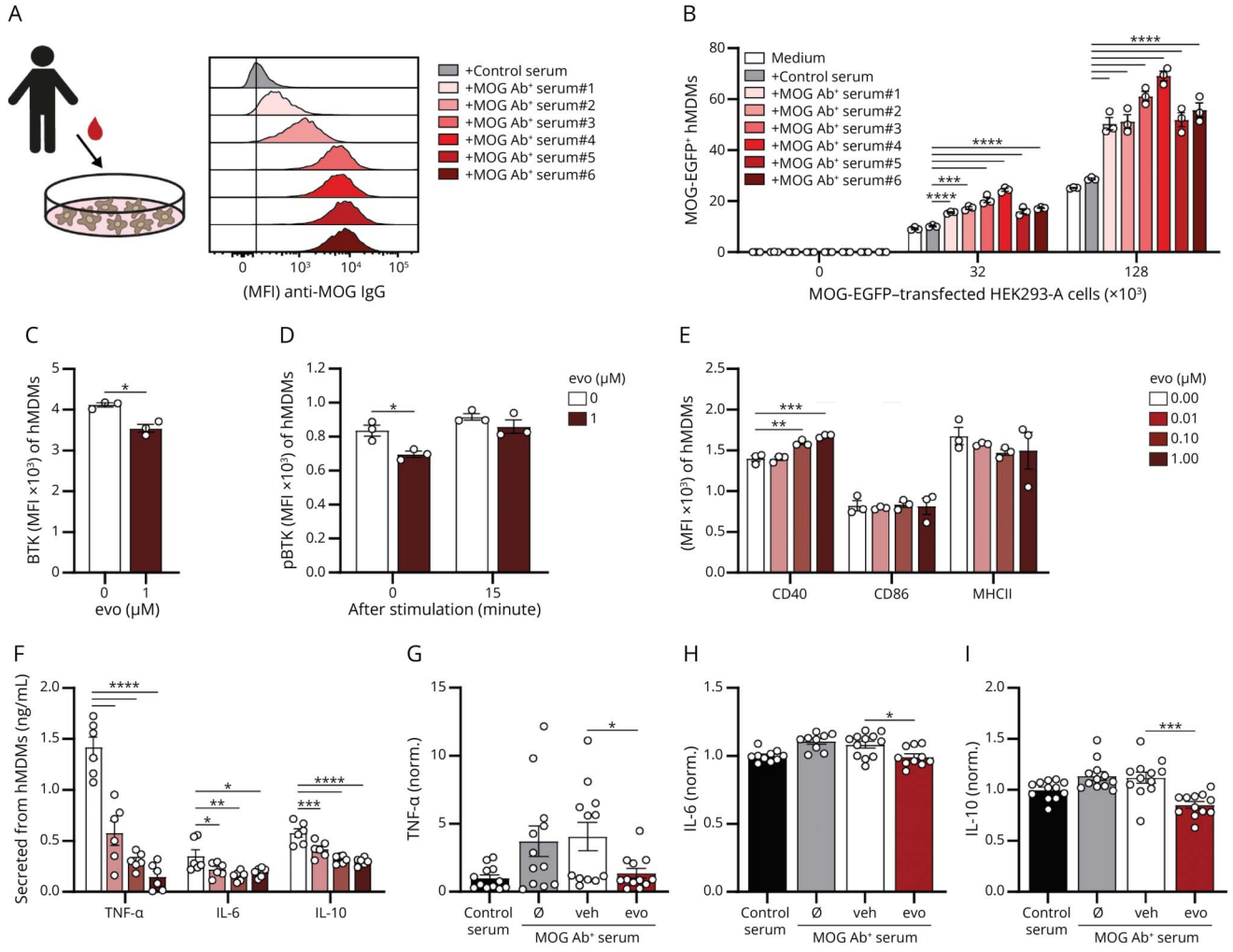
Figure 5 BTKi Reduces the Incidence of Anti-MOG Antibody–Triggered EAE



(A–F) Naïve transgenic TCR^{MOG} mice received i.v. injections of 8.18C5 starting 3 days prior to first antibody injection with either 3 mg/kg of evobrutinib (evo) or vehicle (veh), n = 27–28 mice; overall 65 animals. (C–F) Histologic analysis of the white matter (WM) in the spinal cord. Mice without EAE symptoms are indicated by green symbols. (C) Demyelinated WM areas of the spinal cord were assessed by Luxol fast blue/periodic acid-Schiff (LFB/PAS) staining. (D) Infiltration of macrophages (MAC-3$^+$) into the spinal cord. (E) Frequency of activated microglia (Iba-1$^+$). (F) Infiltration of T cells (CD3$^+$) into the spinal cord. Mean fluorescence intensity (MFI) of molecules associated with antigen presentation (G) and activation (H) on monocytes isolated from the spleen. (I) Frequency of the splenic T-cell composition. MFI of activation-associated molecules on CD4$^+$ T cells (J) and CD8$^+$ T cells (K) isolated from the spleen. Data are shown as mean ± SEM. (B–F) Unpaired *t* tests with the Welch correction or Mann-Whitney *U* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). (G–K) Representative data of at least 4 independent experiments, n = 6–10. One-way analysis of variance corrected by the Holm-Sidak or Kruskal-Wallis test with the Dunn multiple-comparison test, comparing treated groups with the control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

of the proinflammatory cytokines TNF-α and IL-6 in human MDMs, even in the presence of patient serum containing anti-MOG antibodies (Figure 6, F–H). Of interest, the secretion of the anti-inflammatory cytokine IL-10 was also reduced in both experimental setups (Figure 6, F and I), indicating a broad dampening of macrophage activity.

Figure 6 Anti-MOG Antibodies Isolated From Patients Facilitate Recognition of MOG by Human Macrophages Triggering Their Activation—BTKi Treatment Can Neutralize This Proinflammatory Cascade



(A and B) Human monocyte-derived macrophages (MDMs) were cultured with MOG-EGFP–transfected HEK293-A cells in the presence of control serum or anti-MOG antibody serum for 2 hours. The frequency of MOG-EGFP$^+$ MDMs was quantified via flow cytometry. (C) Mean fluorescence intensity (MFI) of BTK expression after 18 hours of evo treatment in human MDMs. (D) MFI of phosphorylated BTK after FcR-dependent stimulation and evo treatment for 2.5 hours. (E and F) MDMs were cultured alone, with evo for 18 hours, and by FcR stimulation. (E) MFI of surface molecules. (F) Cytokine secretion in cell supernatants. (G–I) MDMs cultured with MOG-EGFP–transfected HEK293-A cells in the presence of control or anti-MOG antibody serum with either evo or veh for 18 hours. (G) TNF-α secretion, (H) IL-6 secretion, and (I) IL-10 secretion were normalized to control serum, respectively. Data sets are representative of at least 3 independent experiments, n = 3–6. The mean ± SEM is indicated in all graphs. (B, C, G–I) Unpaired *t* tests with the Welch correction or Mann-Whitney *U* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). (A, D, E, F) One-way analysis of variance corrected by the Holm-Sidak or Kruskal-Wallis test with the Dunn multiple-comparison test, comparing treated groups with the control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

These findings demonstrate that BTKi exerts a direct impact on both murine and human macrophages by modulating their antigen presentation and cytokine secretion profiles. By inhibiting FcR-mediated BTK signaling, BTKi reduces the inflammatory potential of macrophages while enhancing their phagocytic capabilities. This dual regulatory effect emphasizes the therapeutic potential of BTKi in mitigating antibody-mediated CNS demyelination, such as in MOGAD, by directly targeting pathogenic myeloid cell functions.

Discussion

In this study, we investigated the immunologic and clinical translational properties of a novel approach in treatment of chronic CNS inflammation, the therapeutic inhibition of the enzyme BTK. Using evobrutinib[24] as a proof of principle, we demonstrated its ability to induce profound immunologic changes in peripheral immune cells, targeting pathways where BTK is functionally involved. Other BTK inhibitors such as tolebrutinib, fenebrutinib, and remibrutinib, which are currently being evaluated in clinical trials for MS, have shown effects on EAE outcomes comparable to those of evobrutinib.[18,25–28] Furthermore, in vitro investigations of these inhibitors in isolated myeloid cells resulted in, among others, reduced cytokine production.[15,29,30] Therefore, we are convinced that using evobrutinib as a proof of principle gives valuable insights into the mood of action of BTK inhibition on B cells and macrophages.

Our findings establish that evobrutinib modulates the phenotype and function of B cells, which are central players in the pathogenesis of MS, NMOSD, and MOGAD. These diseases share a reliance on pathogenic B-cell activity and autoantibodies in driving acute relapses. In all 3 disease entities, anti-CD20 treatment has been shown to be effective, highlighting the importance of CD20-positive immune cells, presumably B cells, as crucial drivers in propagation of chronic CNS inflammation. Of note, we recently expanded this concept to the concomitant depletion of CD20-positive T cells,[31] which may also contribute to the broad effectiveness of anti-CD20 in these and other diseases. Furthermore, recent experimental and clinical observations suggest that regulatory properties exist within the population of CD20-positive B cells,[8,32] which should be preserved or even therapeutically fostered. In this context, our results pioneer the concept that BTKi can serve as an alternative or complementary strategy for controlling pathogenic B-cell activity without broadly depleting the entire B-cell compartment. Specifically, evobrutinib treatment promoted the emergence of regulatory B cells while simultaneously inhibiting antigen-mediated activation and pathogenic differentiation of B cells. These findings represent a significant step toward developing targeted therapies that selectively modulate harmful immune responses while preserving or fostering protective regulatory mechanisms. This conceptional notion may be of particular value when such a B cell–modulating therapy follows initial eradication of established pathogenic B cells via short-term anti-CD20 induction therapy.

Beyond B cells, we focused on alterations within the myeloid cell compartment, which are key activators of T cells through recognition of small amounts of antibody-opsonized self-antigens[21] and presentation. Using EAE models focused on myeloid cells, we demonstrated that BTKi suppresses T cell–driven disease independently of its effects on B cells, as evidenced by its efficacy in B cell–deficient mice. Dissecting its effect on myeloid cells, we observed that BTKi controlled the production of proinflammatory cytokines on activation. These findings indicate that BTKi affects both B and myeloid cells, with its impact on myeloid cells alone being clinically meaningful. Of note, these properties are not reflected by anti-CD20 treatment, where instead an activation within the myeloid cell compartment can be observed.[7]

In our final set of experiments, we focused on the role of BTKi in antibody-driven CNS inflammation, which is especially relevant to conditions such as NMOSD and MOGAD.[21] In this regard, evobrutinib treatment prevented development of spontaneous EAE triggered by autoantibodies in combination with preexisting myelin-recognizing T cells. In this model, myeloid cells detect minute amounts of CNS-drained autoantigen through FcR when antigen is opsonized by anti-myelin antibodies. Subsequent internalization and digestion of the antigen-antibody complex lead to antigen presentation of myelin peptide to myelin-reactive T cells. However, antigen presentation of myeloid cells was blocked by BTKi, ultimately reducing EAE incidence. Of note, the phagocytic capability was not reduced, but in fact, it was enhanced by BTK inhibition. This is an important finding regarding the efficient removal of myelin debris by myeloid cells as a prerequisite for subsequent remyelination. While it has been formally proven that CNS-intrinsic microglia increase the removal of myelin debris on BTK inhibition,[15] we here report the parallel statement for peripheral immune subsets such as myeloid cells. These findings align with in vitro data showing that human phagocytes enhanced antigen recognition when exposed to MOG-specific antibodies from patients. It is important to note that evobrutinib inhibited this process within biologically achievable concentration range,[33] reducing the subsequent proinflammatory cytokine response.

Collectively, our results highlight the dual action of BTKi on B cells and myeloid cells, disrupting the pathogenic interaction between these immune compartments. These observations suggest that BTKi may offer a unique therapeutic advantage over existing treatments by simultaneously attenuating antibody production, antigen presentation, and T-cell activation. Although evobrutinib was not able to meet its primary outcome in the investigated Phase 3 trial for relapsing MS, a post hoc analysis of its Phase 2 trial revealed effectiveness in reducing the volume of slow-expanding lesions. This could indicate a beneficial effect of BTK inhibition on nonrelapsing progressive diseases,[34] which would be in line with the observed immunomodulatory effects of evobrutinib. Therefore, the effectiveness of BTKi in MS clinical trials may extend to antibody-driven diseases such as NMOSD and MOGAD, broadening its potential therapeutic scope. In the broader context, BTKi represents a promising approach for treating immune-mediated diseases characterized by interactions among B cells, myeloid cells, and pathogenic antibodies.

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Author Contributions

S. Dybowski: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data. J. Thode: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data. M. Freier: major role in the acquisition of data. D. Saberi: major role in the acquisition of data. S. Nessler: major role in the acquisition of data. A. Geladaris: major role in the acquisition of data. S. Häusser-Kinzel: drafting/revision of the manuscript for content, including medical writing for content. M. Ringelstein: drafting/revision of the manuscript for content, including medical writing for content. S. Torke: drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data. M.S. Weber: drafting/revision of the manuscript for content, including medical

writing for content; study concept or design; analysis or interpretation of data.

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Disclosure

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