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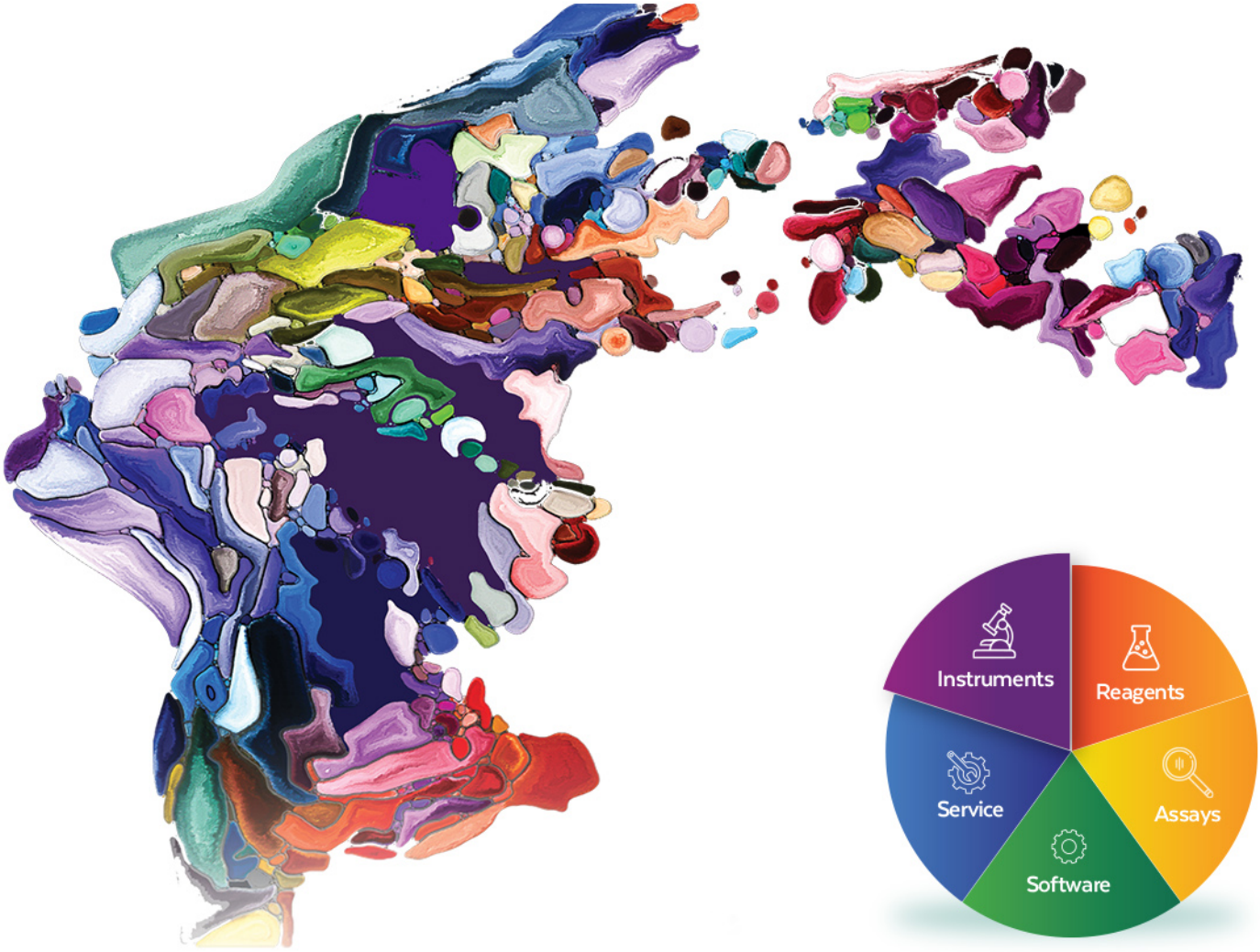

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Basic

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Immunodeficiencies and autoimmunity

Research Article

B-cell modulation with anti-CD79b antibodies ameliorates experimental autoimmune encephalitis in mice

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B cells play a major role in the pathogenesis of many autoimmune diseases like MS, rheumatoid arthritis, or systemic lupus erythematosus. Depletion of B cells with anti-CD20 antibodies is an established therapy for MS. However, total B-cell depletion will also affect regulatory B cells that are known to suppress autoimmune responses. In our studies, we describe an alternative approach based on targeting CD79b that induces only partial B-cell depletion and achieves therapeutic effects by B-cell modulation. Prophylactic and therapeutic treatment with an antibody against CD79b and also a deglycosylated variant of this antibody, lacking effector function like antibody-dependent cellular cytotoxicity or complement activation, significantly reduced the development and progression of EAE in mice. Our data show that modulation of B cells via CD79b is equally effective as almost complete B-cell depletion with anti-CD20 antibodies and may constitute an alternative approach to treat MS.

Keywords: autoimmunity · B cells · neuroimmunology

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

MS is an autoimmune disease of the CNS characterized by loss of myelin, axonal pathology, and progressive neurologic dysfunction [1, 2].

B cells play an essential role in the pathogenesis of MS. They differentiate into plasma cells which secrete autoreactive antibodies contributing to demyelination within the CNS [3]. They also function as APCs and, thus, contribute to the development and progression of MS [3, 4].

In addition, B cells are regulators of immune processes by secreting both, pro- and anti-inflammatory cytokines [3].

Based on these pathogenic B-cell properties, anti-CD20 antibodies (rituximab, ocrelizumab, ofatumumab) are of high interest in the treatment of human MS [5–7]. These antibodies deplete immature and mature B cells but spare CD20-negative plasma cells [8]. Immunological analysis revealed that anti-CD20 B-cell depletion diminished proliferation and proinflammatory differentiation of peripheral T cells [8, 9].

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However, not all CD20$^+$ B cells contribute to the progression of MS. In the mouse model of EAE, B cells regulate autoimmune responses and control proinflammatory differentiation of other APCs [8, 10] by provision of anti-inflammatory IL-10 and IL-35 [8, 11, 12]. Moreover, following depletion, reappearing B cells are stimulated by autoantigens and shape the evolving B-cell repertoire in a pathogenic manner as the emerging post-anti-CD20 population showed a substantial increase in the frequency of differentiated, antigen-experienced B cells with a reduced BCR diversity. Functionally, these activation-related changes translated into an enhanced APC function of B cells [13].

CD79a (Ig-α) and CD79b (Ig-β) are involved in the activation and anergy of B cells [14] and contribute to setting the BCR signaling threshold by regulating receptor internalization [15]. Chronic antigen stimulation of the BCR leads to dissociation of the BCR from Ig-α/-β and monophosphorylation of Ig-α and Ig-β ITAM motifs resulting in reduced binding of spleen tyrosine kinase (Syk) and induction of inhibitory mechanisms [16, 17]. Thus, not only survival of mature murine B cells [18] is dependent on signaling via Ig-α/-β heterodimers but also apoptosis can be induced via Ig-α/-β heterodimers [19].

Recent animal studies showed the therapeutic potential of antibodies against mouse CD79b in autoimmunity. In the animal model of collagen-induced arthritis (CIA) depletion and blockade of B cells with an anti-CD79b antibody improved arthritis, while depletion with an antibody against CD20 was less effective. The advantage of anti-CD79b over anti-CD20 antibodies became evident in treatment of established arthritis [20], but not in a setting with prophylactic treatment [21]. The antibody against mouse CD79b (HM79b) was shown to induce signal transduction even in anergized B cells [17].

In another study, this antibody was used in combination with an antibody against CD79a (F11–172) for depletion of B cells in the model of MRL-lpr lupus nephritis and showed beneficial effects [22].

Little is known about the therapeutic potential of depletion and blockade of CD79b in MS. We have studied a prophylactic and therapeutic approach with B-cell modulation with anti-CD79b, a deglycosylated variant of this antibody and a depleting antibody against CD20 in mice with EAE. We show that both CD79b antibodies significantly improve the development and progression of EAE. B-cell modulation with anti-CD79b is equally effective in treatment with anti-CD20 but leads to less systemic B-cell depletion.

Results

Prophylactic B-cell modulation with anti-CD79b reduces the development of EAE

EAE was induced in C57BL/6N mice by immunization with MOG-peptide 35–55 on day 0. From day 5–19, mice were treated with an intact or deglycosylated anti-CD79b antibody or purified Syrian hamster IgG (Control). Clinical symptoms of EAE were evaluated in a blinded manner and increased from day 9 to 19. B-cell depletion with intact anti-CD79b resulted in a highly significant reduction of EAE symptoms from day 9 throughout day 19. Treatment with deglycosylated anti-CD79b also reduced EAE symptoms but lost efficacy after a couple of days (Fig. 1A). On day 19, we quantified the infiltrating cells in the brain by flow cytometry. Total leukocytes, B cells, CD8$^+$ T cells, and CD4$^+$ T cells were reduced by intact anti-CD79b, while monocytes were not affected. Treatment with deglycosylated anti-CD79b was again less efficient at this time point (Fig. 1B and C). Cell counts in the spleen were quantified by flow cytometry on day 19. Intact anti-CD79b markedly reduced the number of B cells in the spleen and slightly increased monocyte counts, while deglycosylated anti-CD79b induced much less B-cell depletion and did not increase monocyte counts. Total leukocytes, CD4$^+$and CD8$^+$ T cells were not changed by either of the antibodies (Fig. 1D and E). The gating strategy is shown in Supporting information Fig. S1.

Prophylactic B-cell modulation with anti-CD79b reduces MOG-specific immune responses

Restimulation of splenocytes with MOG-peptide 35–55 was performed to quantify the cellular immune response against MOG. The MOG-peptide-specific release of IL-17, IL-6, TNF-α, and IFN-γ was significantly reduced in mice treated with intact anti-CD79b. Treatment with deglycosylated anti-CD79b was less efficient (Fig. 1F-I).

We analyzed the effect of B-cell modulation with anti-CD79b on antigen-specific T-cell proliferation in mice with EAE using in vitro restimulation of CFSE-labeled splenocytes with MOG-peptide. MOG-peptide-induced proliferation of CD4+ T cells was almost completely abrogated in anti-CD79b-treated mice compared to the control group (Fig. 1J and Supporting information Fig. S2).

Both, treatment with intact and deglycosylated anti-CD79b significantly reduced the levels of IL-17 measured in the supernatant of brain tissue and plasma (Fig. 1K and L). The levels of total IgG and MOG-peptide-specific IgG in the plasma were equally reduced by intact and deglycosylated anti-CD79b (Fig. 1M and N).

To quantify the extent to which treatment with anti-CD79b affects the number of B cells with potential regulatory properties, we treated naive C57BL/6N mice (n = 3, group) with either anti-CD79b or an isotype control antibody for 5 days and analyzed B-cell subsets in the peripheral blood and spleen on the next day (Supporting information Fig. S3). As expected, the number of mature B cells significantly decreased in the peripheral blood. The absolute number of T1 transitional B cells remained stable resulting in an increased percentage of T1 B cells in the blood. In the spleen, short-term treatment (5 days) with anti-CD79b did not reduce the number of follicular B cells and even increased the number of T1 transitional B cells and marginal zone B cells, which were recently described to have regulatory properties [23–25].

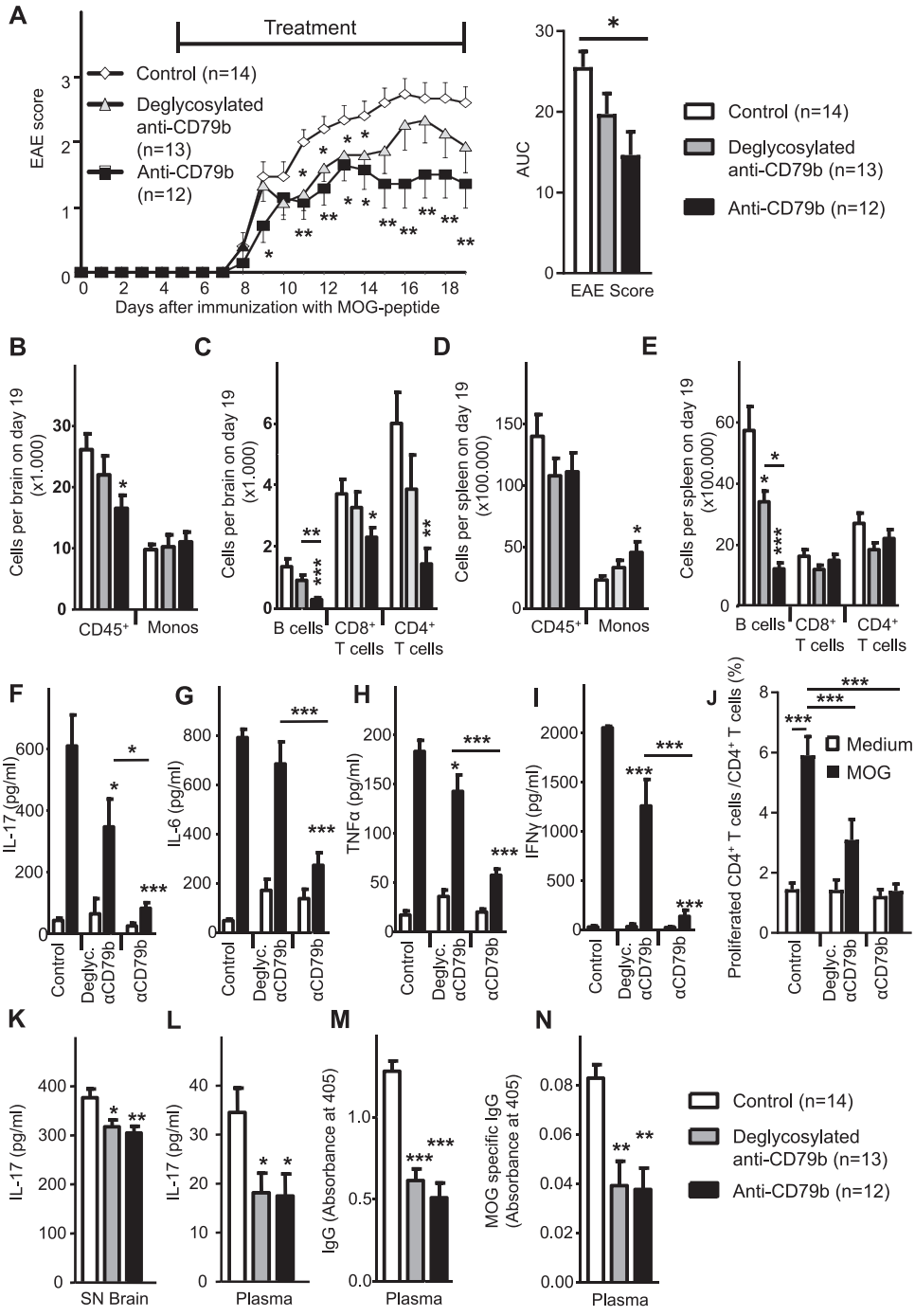

Figure 1. Prophylactic treatment with anti-CD79b reduces the development of EAE. EAE was induced with MOG-peptide 35–55 on day 0 and mice were treated with an intact (20 μg/day, n = 12), or deglycosylated anti-CD79b antibody (20 μg/day, n = 13), or purified Syrian hamster IgG (Control, 20 μg/day, n = 14) from day 5 to 19. (**A**) Clinical symptoms of EAE (EAE score) by daily monitoring and cumulative scores (AUC). (**B** and **C**) Total leukocytes (CD45$^+$), monocytes (Monos), B cells, CD8$^+$ T cells, and CD4$^+$ T cells infiltrating the brain were quantified by flow cytometry on day 19. (**D** and **E**) Cell counts in the spleen were quantified by flow cytometry on day 19. (**F-J**) On day 19, CFSE-labeled splenocytes were restimulated with MOG-peptide 35–55 or medium as control for 3 days, and the levels of IL-17, IL-6, TNF-α, and IFN-γ were measured in the supernatant by ELISA. Proliferated CD4$^+$ T cells were analyzed by flow cytometry (**K** and **L**). IL-17 was measured in the supernatant of brain tissue and in the plasma by ELISA. (**M** and **N**) The levels of total IgG and MOG-specific IgG were measured in the plasma by ELISA. Data are represented as mean ± SEM (one-way ANOVA with Bonferroni post-test). $^*p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (one-way ANOVA with Bonferroni post-test). Representative results from one out of two independently performed experiments are shown.

Prophylactic B-cell modulation with anti-CD79b inhibits cerebral leukocyte influx

EAE symptoms develop within 9–12 days after immunization with MOG-peptide 35–55 in close correlation with the cerebral influx of monocytes, T cells, and B cells. We, therefore, treated mice with intact anti-CD79b, deglycosylated anti-CD79b, or purified Syrian hamster IgG (Control) from day 5 to 10 after immunization with MOG-peptide and analyzed the cerebral infiltrate already on day 11. Total leukocytes, B cells, $CD8^+$ T cells, and $CD4^+$ T cells were significantly reduced in mice treated with intact anti-CD79b. Treatment with deglycosylated anti-CD79b was less efficient (Fig. 2A and B). To investigate whether leukocyte infiltration in the brain differs from that in the spinal cord, we analyzed also the spinal cord. The total leukocyte infiltration was lower in the spinal cord than in the brain. Both, intact and deglycosylated anti-CD79b reduced leukocyte infiltration in the spinal cord with almost equal efficacy. (Fig. 2C and D). As expected, depletion of B cells in the spleen and peripheral blood was much more pronounced with intact anti-CD79b than with deglycosylated anti-CD79b. Surprisingly, however, short-term treatment with intact and deglycosylated anti-CD79b (from day 5 to 10) also reduced the number of total leukocytes, monocytes, and T cells in the spleen and peripheral blood (Fig. 2E-H). This was not seen after long-term treatment with the antibodies (day 5–19, see Fig. 1)

To get further information about the effects of anti-CD79b on B-cell phenotypes, we analyzed the expression of CD19, IgM, and CD86 after treatment with intact or deglycosylated anti-CD79b. Surface expression of CD19 on B cells in the spleen and blood was significantly and equally reduced with both antibody variants. Surface expression of IgM was also reduced with both antibody variants; however, intact anti-CD79b was somewhat more effective. CD86 was upregulated with intact anti-CD79b, but not or only to a minor degree with deglycosylated anti-CD79b (Fig. 2I-M).

B-cell modulation with anti-CD79b and anti-CD20 reduce the development of EAE to a comparable degree

EAE was induced by immunization with MOG-peptide 35–55 on day 0. From day 5 to 14, mice were treated with intact anti-CD79b antibody, a depleting anti-CD20 antibody or purified Syrian hamster IgG (Control). Clinical symptoms of EAE were equally ameliorated with both antibodies against B cells (Fig. 3A).

Leukocyte subpopulations were quantified in the brain and spleen by flow cytometry on day 19. Anti-CD20 almost completely depleted the B cells in the brain and spleen, while intact anti-CD79 was much less effective, especially in the spleen. In contrast, anti-CD79 reduced the number of cerebral $CD8^+$ T cells and total leukocytes more strongly than anti-CD20. Cerebral monocytes or $CD4^+$ T cells remained unchanged with both treatments (Fig. 3B-E).

To get further information on B-cell phenotypes, we analyzed surface expression of CD19, IgM, and CD86 on B cells in the spleen and peripheral blood after treatment with anti-CD20 and anti-CD79b. Five days after the last injection of anti-CD20, we still observed a pronounced downregulation of CD19 and IgM from the surface of B cells. In contrast, downregulation of CD19 and IgM was much lower with intact anti-CD79b, indicating that these effects of anti-CD79b are rather quickly reversible. Anti-CD79b resulted in weak upregulation of CD86 on B cells as seen before, while anti-CD20 treatment induced a clear upregulation (Fig. 3F-J).

Treatment with anti-CD79b and anti-CD20 modulates MOG-specific immune responses

To get further information how anti-CD79b and anti-CD20 influence the MOG-peptide-specific immune response, we restimulated the splenocytes on day 19 with MOG-peptide 35–55 or medium as control for 3 days. Treatment with anti-CD20 and anti-CD79b strongly decreased the MOG-peptide-specific release of IL-17, TNF-alpha, and IFN-gamma (Fig. 4A-C). Treatment with anti-CD79b but not with anti-CD20 significantly increased MOG-peptide-specific release of IL-10 (Fig. 4D). Cerebral levels of IL-17 were decreased with anti-CD79b but upregulated with anti-CD20 (Fig. 4E). Plasma levels of IL-17 remained unchanged (Fig. 4F). The concentrations of total IgG and MOG-peptide-specific IgG in the plasma were equally reduced in anti-CD20- and anti-CD79b-treated mice (Fig. 4G-H).

Therapeutic treatment with anti-CD79b attenuates the progression of EAE

To investigate, whether therapeutic treatment with anti-CD79b improves the progression of EAE, we induced EAE by immunization with MOG-peptide 35–55 on day 0 and treated the mice only after appearance of first EAE symptoms from day 10 to 21. Already one day after the first injection of intact or deglycosylated anti-CD79b, we observed a significant reduction of EAE symptoms. The improvement continued in both groups until day 14. From day 15 to 21, only intact anti-CD79b was able to improve symptoms of EAE, while the deglycosylated anti-CD79b lost efficacy (Fig. 5A).

Leukocytes infiltrating the brain were quantified by flow cytometry on day 21. Total leukocytes, monocytes, B cells, $CD8^+$ and $CD4^+$ T cells were reduced by intact anti-CD79b but not by deglycosylated anti-CD79b (Fig. 5B and C). In the spleen only, the intact anti-CD79b reduced the number of B cells (Fig. 5D). Surface expression of CD19 on splenic B cells was significantly and equally reduced with both antibody variants. (Fig. 5E). Surface expression of IgM on splenic B cells was downregulated more strongly with intact than with deglycosylated anti-CD79b as seen before (Fig. 5F). CD86 was upregulated with intact anti-CD79b and to a minor degree with deglycosylated anti-CD79b (Fig. 5G).

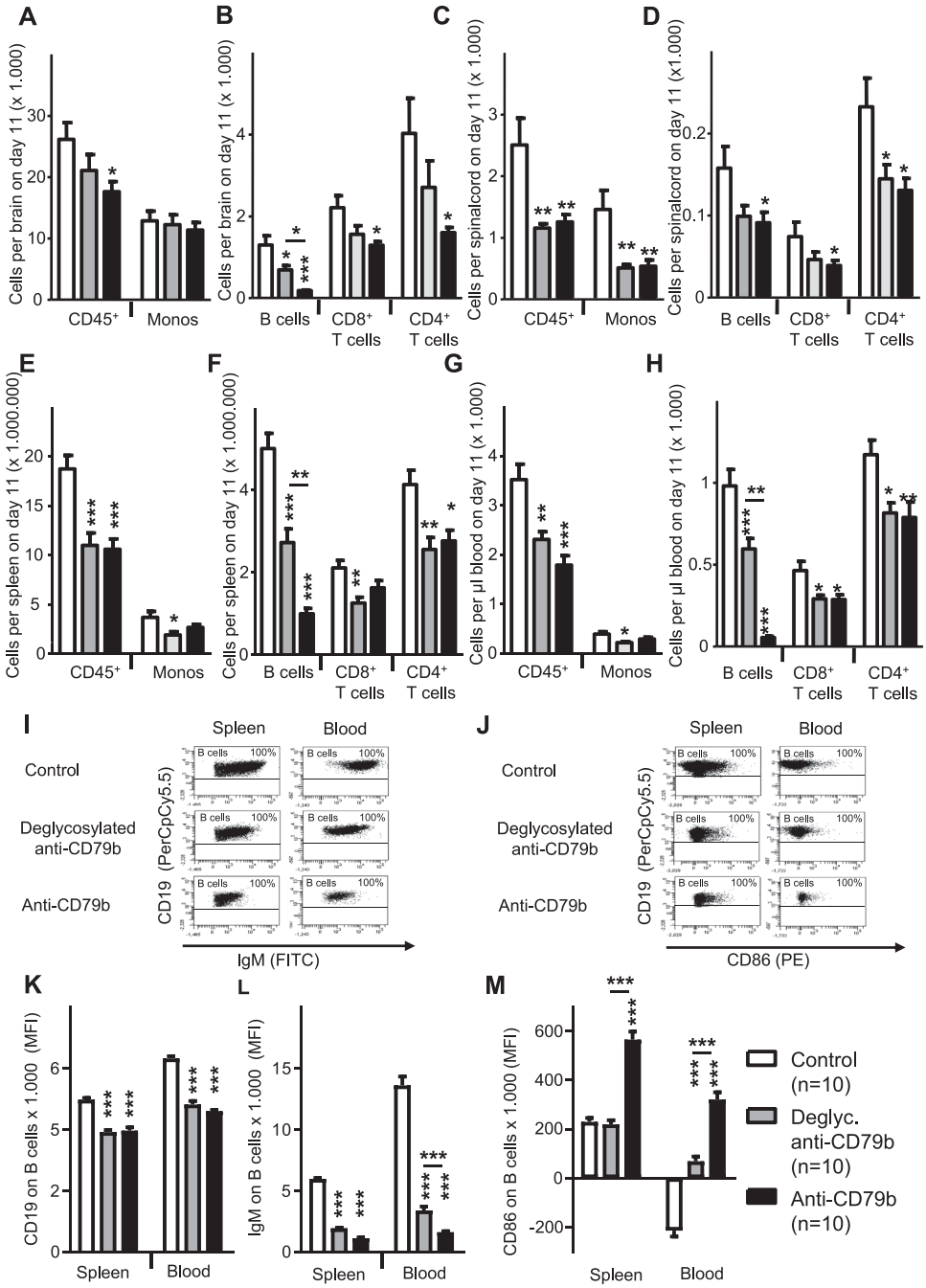

Figure 2. Treatment with anti-CD79b inhibits cerebral leukocyte influx, decreases leukocyte counts in the spleen and modulates the B-cell phenotype. EAE was induced with MOG-peptide 35–55 on day 0 and mice were treated with an intact (20 μg/day, n = 10), or deglycosylated anti-CD79b antibody (20 μg/day, n = 10). or purified Syrian hamster IgG (Control, 20 μg/day, n = 10) from day 5 to 10. Analysis was performed on day 11. (**A** and **B**) Total leukocytes (CD45$^+$), monocytes (Monos), B cells, CD8$^+$ T cells, and CD4$^+$ T cells infiltrating the brain were quantified by flow cytometry on day 11. (**C** and **D**) Leukocytes infiltrating the spinal cord on day 11. (**E-H**) Cell counts in the spleen and peripheral blood were quantified by flow cytometry on day 11. (**I-M**) Representative dot blots and quantification of surface expression of CD19, IgM, and CD86 on B cells from the spleen and blood on day 11. Data are represented as mean ± SEM. $^*p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (one-way ANOVA with Bonferroni post-test). Representative results from one out of two independently performed experiments are shown.

Furthermore, both treatments reduced the IL-17 plasma levels in a not significant manner (Fig. 5H).

Treatment with intact and deglycosylated anti-CD79b reduced the levels of IgG in the plasma, while only treatment with intact anti-CD79b significantly reduced the levels of MOG-specific IgG in the plasma (Fig. 5I and J).

To investigate the immune response after therapeutic treatment with intact or deglycosylated anti-CD79b, we restimulated

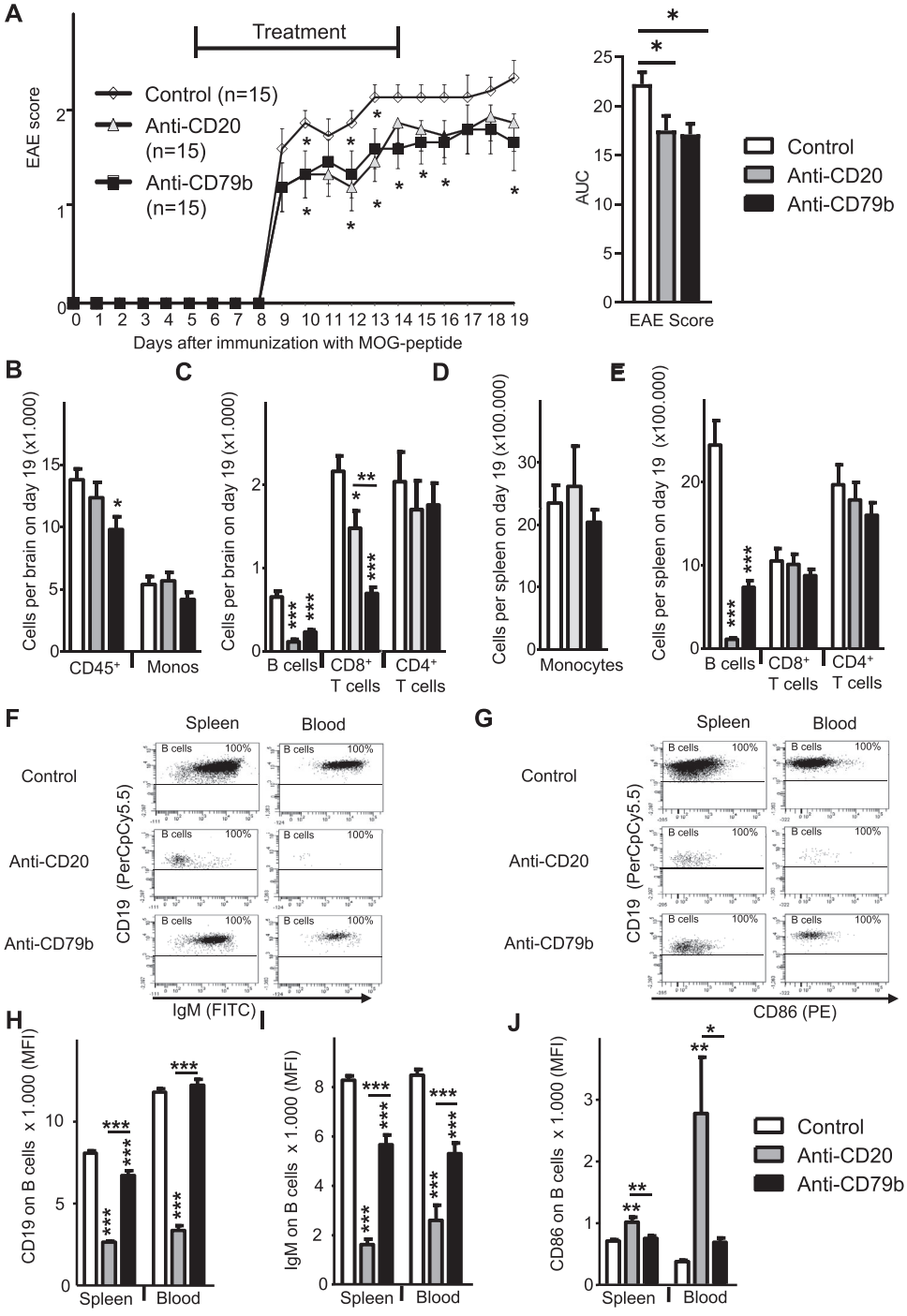

Figure 3. Prophylactic treatment with anti-CD79b and anti-CD20 reduces the development of EAE to a comparable degree. EAE was induced with MOG-peptide 35–55 on day 0. Mice were treated with an intact anti-CD79b antibody (anti-CD79b, 20 μg/day, n = 15), a depleting anti-CD20 antibody (anti-CD20, 20 μg/day, n = 15) or purified Syrian hamster IgG (Control, 20 μg/day, n = 15) from day 5 to 14. (**A**) Clinical symptoms of EAE (EAE score) by daily monitoring and cumulated scores (AUC). (**B** and **C**) Total leukocytes (CD45$^+$), monocytes (Monos), B cells, CD8$^+$ T, cells and CD4$^+$ T cells infiltrating the brain were quantified by flow cytometry on day 19. (**D** and **E**) Cell counts in the spleen on day 19. (**F-J**) Representative dot blots and quantification of surface expression of CD19, IgM, and CD86 on B cells from the spleen and blood on day 19. Data are represented as mean ± SEM. $^*p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (one-way ANOVA with Bonferroni post-test). Representative results from one out of two independently performed experiments are shown.

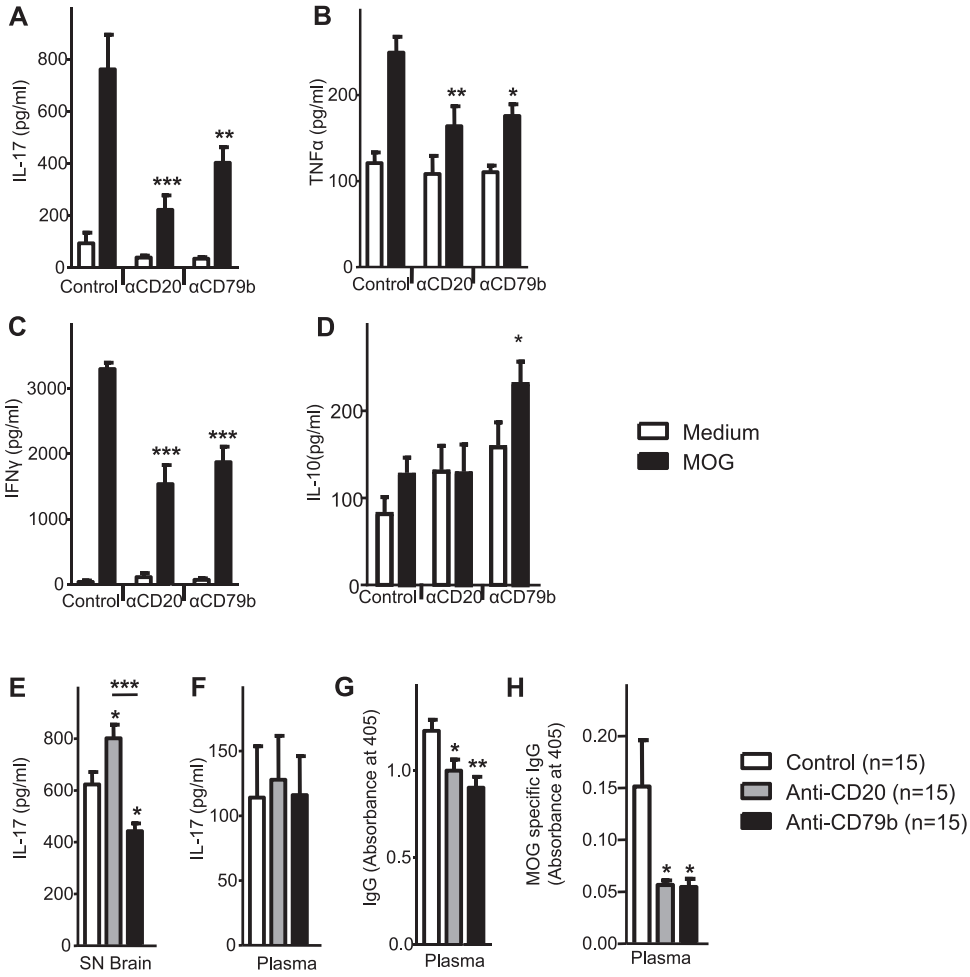

Figure 4. MOG-specific IgG and release of cytokines from restimulated splenocytes in mice treated with anti-CD79b or anti-CD20. EAE was induced with MOG-peptide 35–55 on day 0 and mice were treated with antibodies as described in Fig. 3 (n = 15/group). (**A-D**) On day 19, splenocytes were restimulated with MOG-peptide 35–55 or medium as control for 3 days, and the levels of IL-17, TNF-α, IFN-γ, and IL-10 were measured in the supernatant by ELISA. (**E** and **F**) IL-17 was measured in the supernatant of brain tissue and in the plasma by ELISA. (**G** and **H**) The levels of total IgG and MOG- specific IgG were measured in the plasma by ELISA. Data are represented as mean ± SEM, one-way ANOVA with Bonferroni post-test of control versus anti-CD79b or control versus deglycosylated anti-CD79b: $^{*}p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Representative results from one out of two independently performed experiments are shown.

splenocytes on day 19 with MOG-peptide 35–55 or medium as control for 3 days. Treatment with intact but not deglycosylated anti-CD79b significantly decreased the MOG-specific release of IL-17, TNF-alpha, and IFN-gamma (Fig. 5K-M). The release of IL-6 was not significantly reduced (Fig. 5N).

Discussion

We have analyzed the prophylactic and therapeutic potential of an intact antibody against CD79b in a model of EAE. We compared this antibody with an antibody against CD20 and a deglycosylated variant of the anti-CD79b antibody that is unable to induce depletion of B cells via recruitment of cytotoxic effector cells or activation of complement.

B cells play critical positive and negative regulatory roles in EAE pathogenesis depending on the EAE model and the timing of antibody treatment. Total B-cell depletion with anti-CD20 before disease induction with MOG-peptide35-55 exacerbated EAE most likely due to depletion of a regulatory B10-cell subset [26, 27]. In contrast, in MOG-protein-induced EAE early treatment with anti-CD20 antibodies ameliorated EAE and suppressed the development of Th1 and Th17 cells [27]. Anti-CD20 mediated B-cell depletion after onset of EAE symptoms is known to reduce clinical symptoms in both EAE models [26, 27] as well as in a model of spontaneous relapsing-remitting EAE in SJL/J mice [28].

For our studies, we have chosen the MOG-peptide 35–55 model. For the prophylactic approach, we started treatment after disease induction but before appearance of clinical EAE symptoms (day 5 after induction), while therapeutic treatment was started after appearance of EAE symptoms (day 10 after induction). Prophylactic and therapeutic treatment with the unmodified and deglycosylated variant of the anti-CD79b antibody significantly

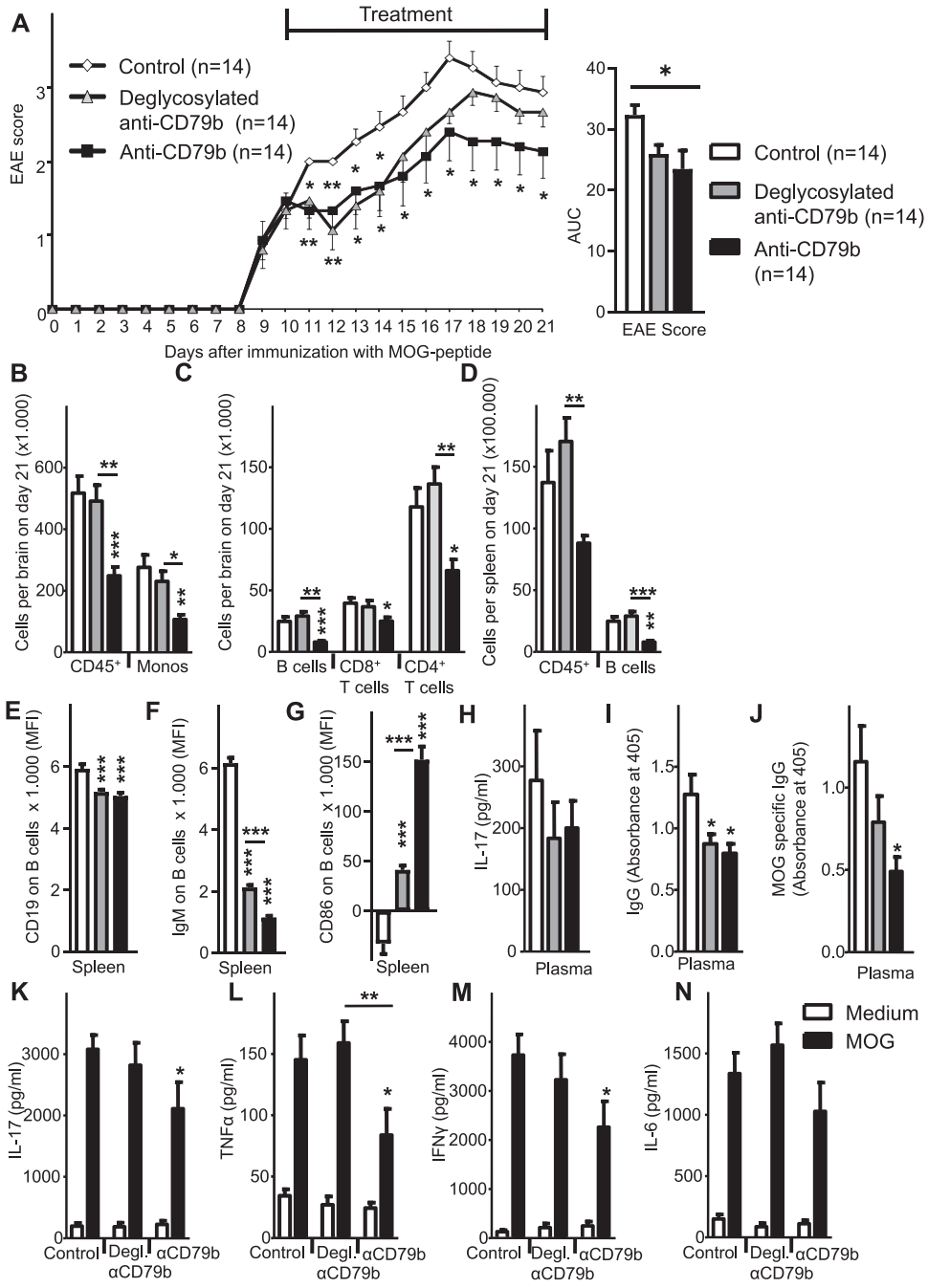

Figure 5. Therapeutic treatment with anti-CD79b attenuates the progression of EAE. EAE was induced with MOG-peptide 35–55 on day 0 and mice were treated with an intact (20 μg/day, n = 14) or deglycosylated anti-CD79b antibody (anti-CD79b, 20 μg/day, n = 14) or purified Syrian hamster IgG (Control, 20 μg/day, n = 14) from day 10 to 21. (**A**) Clinical symptoms of EAE (EAE score) by daily monitoring and cumulative scores (AUC). (**B** and **C**) Total leukocytes ($CD45^+$), monocytes (Monos), B cells, $CD8^+$ T, cells and $CD4^+$ T cells infiltrating the brain were quantified by flow cytometry on day 21. (**D**) Cell counts in the spleen were quantified by flow cytometry on day 21. (**E-G**) Quantification of surface expression of CD19, IgM, and CD86 on splenic B cells on day 21. (**H**) IL-17 was measured in the plasma by ELISA. (**I** and **J**) The levels of total IgG and MOG-specific IgG were measured in the plasma by ELISA. (**K-N**) On day 21, splenocytes were restimulated with MOG-peptide 35–55 or medium as control for 3 days, and the levels of IL-17, TNF-α, IFN-γ, and IL-6 were measured in the supernatant by ELISA. Data are represented as mean ± SEM. $^*p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (one-way ANOVA with Bonferroni post-test). Representative results from one out of two independently performed experiments are shown.

reduced the development of clinical EAE signs. Interestingly, the deglycosylated anti-CD79b antibody that is unable to interact with Fc-receptors was as effective as the intact antibody during the first days of treatment but lost beneficial activity after a couple of days. Consistent with this clinical time-course cerebral inflammation and MOG-peptide-specific immune responses measured at day 19–21 were no longer suppressed with deglycosylated anti-CD79b. However, when this analysis was performed earlier (day 11) the differences between intact and deglycosylated anti-CD79 were much lower or not existent. As expected, the intact CD79b antibody induced a much more pronounced depletion of B cells than the deglycosylated anti-CD79b, suggesting that a stronger B-cell depletion is important for prolonged beneficial activity of anti-CD79b antibodies. The clinical outcome was somewhat unexpected as the deglycosylated anti-CD79b antibody was previously described to be as effective as the unmodified antibody in reducing the onset and disease symptoms in a mouse model of CIA [20].

Prolonged treatment of mice with the deglycosylated anti-CD79b antibody induced some reduction of B cells in the peripheral blood and spleen. This was a surprising result as deglycosylated antibodies do not induce antibody-dependent cellular cytotoxicity or complement activation and cannot deplete cells by classical immunological effector mechanisms [29]. The deglycosylated anti-CD79b seems to reduce B-cell numbers by interfering with B-cell survival or B-cell proliferation. Indeed, it was recently described that anti-CD79b blocks the calcium influx into B cells activated by anti-IgM [21]. In addition, antibodies against CD79b have been described to downmodulate the BCR [30–33]. Moreover, it was shown that also the deglycosylated anti-CD79b antibody markedly interferes with survival and proliferation of cultured B cells in vitro [20].

To see what effects of anti-CD79b treatment persist and what effects are quickly reversible, we treated one group of mice from day 5 to 19 and analyzed them immediately after the last injection, while another group of mice was treated from day 5 to 14 and analyzed 5 days later. We found that anti-CD79b administration until day 19 resulted in a reduction of cerebral $CD4^+$ T-cell infiltration, IL-17 plasma levels, and total monocyte numbers in the spleen, while treatment only until day 14 leads to a loss of these effects. These data suggest that repeated and prolonged treatments with anti-CD79b antibodies are required to achieve long-term suppression of encephalitis.

Prophylactic treatment with intact anti-CD79b was equally effective as anti-CD20 to reduce clinical EAE symptoms, B-cell infiltration in the brain, and suppression of inflammatory MOG-peptide-specific cytokine release from splenocytes.

Interestingly, treatment with intact anti-CD79b was much more effective than anti-CD20 or deglycosylated anti-CD79b to reduce infiltrating leukocytes and CD8+ T cells in the brain.

Treatment with anti-CD79b but not with anti-CD20 significantly increased the release of IL-10 from MOG-peptide restimulated splenocytes.

We assume that B-cell modulation by anti-CD79b works in two ways: On the one hand, anti-CD79b treatment depletes pathogenic B cells and abrogates MOG-specific antibody production, on the other hand, it may preserve regulatory B cells and lead to an increased MOG-specific release of IL-10. The increased release of IL-10, in turn, could contribute to a reduced release of proinflammatory cytokines through direct action on T cells [34].

B cells were also found to be important for encephalitogenic T-cell activation [35] and for antigen-specific T-cell proliferation in EAE and other models [26, 36]. Recent clinical trials established B-cell depletion by the anti-CD20 chimeric antibody Rituximab as a beneficial therapy for patients with relapsing-remitting MS. In an EAE model with human-CD20 transgenic mice, Rituximab rapidly depleted peripheral B cells, strongly reduced EAE severity, and was associated with a reduction in T-cell proliferation and IL-17 production. While Rituximab is not considered to be a broad immunosuppressant, these results indicate a role for B cells as a therapeutic cellular target in regulating encephalitogenic T-cell responses in specific tissues [37]. To find out if this is also the case with B-cell modulation with anti-CD79b, we analyzed the effect of B-cell modulation with anti-CD79b on antigen-specific T-cell proliferation in mice with EAE using in vitro restimulation of CFSE-labeled splenocytes with MOG-peptide. MOG-peptide-induced proliferation of CD4+ T cells was almost completely abrogated in anti-CD79b-treated mice compared to the control group.

Short-term B-cell modulation with anti-CD79b in naive mice led to a significant increase in marginal zone B cells. It has recently been described that marginal zone B cells act as regulatory B cells and can suppress the antigen-specific CD4+ T cell and CD8+ T- cell response, by release of IL-10 as one potential suppressive mechanism. In addition, marginal zone B cells are able to induce Tr1 cells [23–25].

We also analyzed the phenotype of the remaining B cells in the spleen and peripheral blood after long-term treatment with anti-CD79b and anti-CD20. Anti-CD20 induced a strong downregulation of CD19 and IgM from the surface of B cells. Anti-CD20-mediated loss of CD19 was shown to be caused by transfer of CD19 from B cells to monocytes and neutrophils and shaving of the antibody-CD20 complex in an Fc-receptor dependent manner [38, 39]. The same may be true for surface IgM. Intact anti-CD79b induced some downregulation of CD19 and also downregulation of IgM from the surface of B cells, but not as pronounced as anti-CD20, suggesting that other mechanisms may be responsible. This is further supported by the observation that the deglycosylated anti-CD79b was almost as effective as intact anti-CD79b indicating that anti-CD79b-induced downregulation is not dependent on an Fc-receptor mediated transfer of surface molecules to neutrophils and monocytes.

Treatment with anti-CD20 resulted in a higher expression of the costimulatory molecule CD86 on the remaining B cells compared to treatment with intact anti-CD79b. The increased costimulatory activity of these remaining B cells may explain why anti-CD20- treated mice showed a markedly increased expression of IL-17 in the brain. This may weaken the beneficial effects of anti-CD20 treatment.

It was reported that the few B cells that remain or return after treatment with anti-CD20 show a substantial increase in the frequency of differentiated, antigen-experienced B cells with reduced BCR diversity. These changes translate into an enhanced APC function of B cells [13]. B-cell depletion with anti-CD79b is much less pronounced and may result in a lower expansion of potentially harmful B cells. Apart from costimulatory B cells, monocytes also may contribute to the enhanced cerebral IL-17 expression in anti-CD20-treated mice. Recent studies demonstrated that EAE exacerbation induced by depletion of B cells closely correlated with an enhanced production of proinflammatory cytokines by $CD11b^+$ APCs [8]. Also in MS patients, peripheral monocytes show signs of enhanced activation and proinflammatory differentiation after treatment with anti-CD20 [8], suggesting that B cells physiologically control the activity of myeloid cells and that this desirable B- cell property is abolished by anti-CD20 treatment [10, 13].

Our data suggest that it is unlikely that the beneficial effect of B-cell depletion/modulation with anti-CD79b can be pinpointed to a single downstream mechanism. Anti-CD79b treatment has multiple downstream effects including less cerebral leukocyte infiltration, changed cytokine release, lower MOG-specific antibodies, and lower T-cell activation. We believe that all of these effects contribute to the beneficial effects of anti-CD79b treatment. An advantage of anti-CD79b compared to anti-CD20 could be the lower degree of B-cell depletion and a direct impact of the antibodies on B cells via binding to CD79b. The latter view is supported by our finding that deglycosylated anti-CD79b also show beneficial effects in the mouse model of EAE.

In humans, a single treatment with rituximab depletes the B cells usually for 6–12 months with all the associated side-effects and risks of selective expansion of potentially harmful B cells during subsequent B-cell recovery. Treatment with anti-CD79b may, thus, be a new option to treat autoimmune diseases in the future.

Materials and Methods

Induction of EAE

C57BL/6N ($H\text{-}2^b$) mice were obtained from Charles River (Sulzfeld, Germany). On day 0, female 8–12 weeks old mice were immunized subcutaneously at both flanks with a total of 200 μL solution containing 200 μg MOG-peptide 35–55 (MEVGWYRSPFSRVVHLYRNGK) in CFA (Sigma-Aldrich F5506, St. Louis, MO) containing 1 mg *Mycobacterium butyricum* (BD Bioscience 264010, Franklin Lakes, NJ). On days 0 and 2, mice were injected i.p. with 0.25 μg pertussis toxin from *Bordetella pertussis* (Sigma-Aldrich) dissolved in 200 μL PBS containing 1% BSA. Individual mice were observed daily and clinical scores were assessed by a blinded investigator as follows: 0 = no clinical disease, 1 = loss of tail tone only, 2 = partial paralysis of hind legs, 3 = complete paralysis of one or both hind legs, 4 = complete paralysis of hind legs and partial or complete paralysis of one or both front legs, and 5 = complete paralysis of all legs or death. All experiments with mice were approved by the local authorities. Mice were kept under specific pathogen-free conditions and obtained water and food ad libitum with a 12 h light/dark cycle.

Treatment of mice

Mice were treated as indicated in the figure legends by daily i.p. injections of 20 μg purified anti-CD79b antibody (clone HM79b, hamster IgG), 20 μg purified and deglycosylated anti-CD79b antibody (clone HM79b), 20 μg purified anti-CD20 antibody (clone MB20-11) or the same amount of purified Syrian hamster IgG (Jackson ImmunoResearch). The anti-CD79b antibody (clone HM79b, hamster IgG) was obtained from Dr. John Cambier [40]. Deglycosylation of the anti-CD79b antibody was performed overnight at 37°C with peptide-*N*-glycosidase F (New England Biolabs) using 2000 U enzyme for 1 mg antibody and subsequent dialyzation against PBS. To verify complete deglycosylation by ELISA, plates were coated overnight with various concentrations of intact or deglycosylated anti-CD79b antibody, washed with PBS/0.05% Tween 20, blocked with carbo-free blocking solution, and detected with biotinylated Lens Culinaris Agglutinin followed by streptavidin-HRP (all from Vector Laboratories). The chimeric murine IgG2a anti-murine CD20 antibody derived from clone MB20-11 was produced by transient calcium phosphate transfection of 293 T cells followed by purification of secreted antibody from the culture supernatant using protein G, as described in Refs. [41, 42] and kindly provided by Dr. Falk Nimmerjahn.

Culture of splenocytes

Splenocytes were labeled for 15 min at 37°C with 5 μM CFSE. To analyze the MOG-peptide-specific release of cytokines, total splenocytes (2 Mio cells/well) were cultured for 3 days with or without MOG-peptide (20 μg/mL) in 96-well flat-bottom plates in a total volume of 250 μL medium (RPMI 1640 with 10% heat-inactivated FCS, penicillin/streptomycin, nonessential amino acids, 1 mM sodium pyruvate, and 50 μM 2-ME). The concentration of cytokines (IL-17, IL-6, TNF, IFN-γ, IL-10) in the culture supernatant was determined by ELISA (Biolegend and BD Bioscience). After staining for CD4, proliferation of $CD4^+$ T cells was analyzed by flow cytometry.

Flow cytometry

Peripheral blood was drawn from the retro-orbital venous plexus of anesthetized mice and anticoagulated with EDTA. To prepare single-cell suspensions of brain tissue, mice were sacrificed with carbon dioxide and transcardially perfused with 20 mL NaCl

0.9%. Half of the brain was cut into small pieces and pressed through a 100-μm cell strainer in a total volume of 1 mL. After centrifugation, cells were resuspended in 8 mL 40% Percoll. Percoll (2 mL, 80%) was underlayed and centrifuged for 20 min at 700 × *g*. Cells in the interphase were recovered and washed once in RPMI medium with 10% FCS. The spinal cord was flushed out with PBS and prepared in the same manner as the brain to analyze the number of infiltrating leukocytes. For flow cytometry, cells were preincubated for 10 min on ice with Fc-block (clone 2.4G2; 5 μg/mL, eBioscience) and then stained with combinations of directly labeled antibodies for 25 min: anti-CD4 (clone RM4-5, Biolegend), anti-CD8 (clone 53–6.7, Biolegend), anti-CD19 (clone eBio1D3, eBioscience), anti-CD11b (clone M1/70, eBioscience), anti-IgM (clone eB121-15F9, eBioscience), anti-Ly6C (clone AL-21, BD Bioscience), anti-Ly6G (clone 1A8, Biolegend), anti-CD86 (clone GL1, Biolegend), anti-CD23 (clone B3B4, Biolegend), anti-CD21/35 (clone eBio4E3, eBioscience), anti-CD93 (clone AA4.1, Biolegend), and anti-CD45 (clone 30-F11, BD Bioscience). Red blood cells were lysed with FACS-lysing solution (BD Biosciences) and samples analyzed on a FACSCantoII (BD Bioscience) with FACSDiva software. Leukocyte subsets were identified by their FSC-SSC properties and expression of surface markers. The number of cells was quantified using counting beads (Invitrogen). The authors adhered to the guidelines for analysis of flow cytometry analysis [43].

Brain supernatants

Half of the brain was cut into small pieces and pressed through a 100-μm cell strainer in a total volume of 1 mL. Cells were incubated for 30 min on ice. After centrifugation at 1000× *g* for 10 min, supernatants were removed and stored at −20°. The concentration of IL-17 in the brain supernatant or plasma was determined by ELISA (Biolegend) according to manufacturer's protocol.

IgG and MOG-specific IgG ELISA

Plates were either coated with rabbit anti-mouse IgG (5 μg/mL, Jackson ImmunoResearch) or MOG-peptide (10 μg/mL) overnight by room temperature, washed with PBS/0.05 Tween 20, blocked with PBS/2% BSA, and detected with HRP-labeled polyclonal rabbit anti-mouse immunoglobulins.

Statistics

Data are represented as mean. Error bars indicate the SEM. Significance was calculated with a one-way ANOVA with Bonferroni post-test or one-tailed student´s *t*-test, as indicated in the figure legends. One asterisk indicates $p < 0.05$, two asterisks $p < 0.01$, and three asterisks $p < 0.001$. A *p*-value less than 0.05 was considered significant.

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Author contributions: KR and MM designed experiments and wrote the article. KR, MM, SN, YT, SB, KS, FN, AL, FW, and JNS performed and analyzed experiments.

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Abbreviation: **CIA**: collagen-induced arthritis

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