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Immune cell derived BDNF does not mediate neuroprotection of the murine anti-CD52 antibody in a chronic autoimmune mouse model

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ABSTRACT

The murine anti-CD52 antibody, an equivalent of the humanized antibody alemtuzumab, which is successfully used in the treatment of multiple sclerosis, was used to explore a potential neuroprotective effect driven by immune cell derived brain-derived neurotrophic factor (BDNF). Therefore, lineage specific constitutive knock-out mice with a BDNF deficiency in T cells and macrophages were used and compared to treated wildtype mice. Neither therapeutic nor preventive application of the murine anti-CD52 antibody in an animal model of multiple sclerosis, the $MOG_{35–55}$ EAE, revealed a beneficial contribution of immune cell derived BDNF to the disease outcome. Furthermore, preventive application of the murine anti-CD52 antibody worsened the clinical EAE disease course and could only be overcome by a prolonged recovery phase after treatment and before disease induction.

1. Introduction

The humanized monoclonal antibody alemtuzumab (Lemtrada ®) targets the CD52 antigen, which is expressed on lymphocytes and monocytes. It acts by depleting circulating lymphocytes and monocytes with just a single treatment course. These cells recover at different rates, especially $CD4^+$ cells may need several years to recover after a single 5 day exposure (Coles et al., 2006), whilst B cells may recover already after 6 months and may form the basis for unwanted secondary autoimmune problems (Coles et al., 2017). In phase II-III clinical studies of relapsing-remitting multiple sclerosis (MS) patients a strong reduction of relapse rate of about 74% was observed after alemtuzumab treatment compared to interferon-β 1a treatment (Coles et al., 2008; Cohen et al., 2012). Besides its role in disability improvement a role as a mediator of "neuroprotective autoimmunity" is also suggested for alemtuzumab. The concept of neuroprotective autoimmunity is based on the assumption that autoreactive T cells are not only detrimental but have also a beneficial role by expressing neurotrophic factors e.g. (Moalem et al., 2000). In this context it was shown that twelve months after alemtuzumab treatment peripheral blood mononuclear cells of multiple sclerosis patients produce increased concentrations of the neurotrophins brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) (Jones et al., 2010). Here, especially T cells displayed a significant increase in BDNF expression after alemtuzumab treatment. To be able to explore anti-CD52 treatment in a murine experimental model, a murine monoclonal anti-CD52 antibody (anti-muCD52) was generated (Turner et al., 2015). The observed expression pattern of anti-muCD52 on immune cells was similar to the human variant, with the highest level expressed on T cells, a slightly diminished expression on B cells and only little expression on NK cells. Anti-muCD52 treated mice showed a similar immune cell depletion as observed in human MS patients, whereas the pattern of immune cell repopulation differed in the way that T and B lymphocytes recovered to baseline levels at the same rate, while in humans B cells were shown to recover more rapidly than other cell types (Thompson et al., 2010). A single treatment course of the anti-muCD52 antibody in a murine experimental multiple sclerosis model, the myelin oligodendrocyte glycoprotein induced experimental autoimmune encephalomyelitis (MOG-EAE), was highly effective and resulted in a reduction of disease severity (Turner et al., 2015).

A possible BDNF mediated neuroprotective effect of alemtuzumab, was the aim of this study and was conducted with the help of lineage specific constitutive BDNF knock-out mice (C57BL/6 background) in a $MOG_{35–55}$-EAE model. Since conventional BDNF knock-out mice die

Abbreviations: BDNF, brain-derived neurotrophic factor; MOG, Myelin oligodendrocyte glycoprotein; EAE, experimental autoimmune encephalomyelitis; Anti-muCD52, anti- murine CD52; Lys M, lysozyme M; WT, wild type; CLF, lineage specific constitutive knock-out mice with a targeted BDNF deficiency T cells and macrophages; PBS, phosphate buffered saline; PCR, polymerase chain reaction; MS, multiple sclerosis

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soon after birth lineage specific constitutive knock-out mice with a targeted BDNF deficiency preferably in T cells and macrophages (CLF mice) were used, which were generated based on the Cre/loxP system (Sauer, 2002). Therapeutic application of the anti-muCD52 antibody did not indicate a neuroprotective role mediated by immune cell derived BDNF. Moreover, a preventive approach even worsens the disease symptoms in the anti-muCD52 treated animals, while a longer recovery phase before immunization leads to an approximation of the clinical courses of anti-muCD52 treated and untreated mice.

2. Material and methods

2.1. Animals

C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). In lineage specific constitutive $BDNF_{flox/flox}$ mice, the target gene (BDNF) is flanked by two loxP sequences, which is recognized by the Cre recombinase, a bacteriophage derived DNA splicing enzyme. The Cre gene is introduced under a tissue specific mouse promoter. In this case the lymphocyte-specific CD4 promoter and the myeloid specific macrophage lysozyme M (LysM) promoter (kindly provided by Prof. Linker from the Department of Neurology, Friedrich Alexander University of Erlangen-Nuremberg, Germany) were used. Crossing Cre expressing mice with mice in which the BDNF gene is floxed results in deletion of BDNF in T cells and macrophages (CLF mice). Mice were bred at the in-house animal care facilities of the Department of Medicine, Ruhr University Bochum, Germany. All animal experiments were performed in accordance with the North-Rhine-Westphalia regulations for animal welfare (AZ. 84–02.04.2013.A109).

2.2. Induction and clinical evaluation of $MOG_{35–55}$-EAE and anti-muCD52 therapy

For active induction of MOG-EAE, mice received a subcutaneous injection of 100 µg $MOG_{35–55}$ peptide (Institute of Medical Immunology, Charité, Berlin, Germany) in phosphate-buffered saline emulsified in complete Freund's adjuvant containing 100 µg *Mycobacterium tuberculosis* H37RA (Difco, Detroit MI, USA). On Days 0 and 2 post immunization, 200 ng pertussis toxin (List/Quadratech, UK) were applied by intraperitoneal injection. The monoclonal anti-muCD52 antibody was applied by subcutaneous injection at a dose of 10 mg/kg bodyweight on 5 consecutive days (between day 12 and 16). Animals were weighed and scored for clinical signs of disease in a blinded manner on a daily basis using a scale ranging from 0 to 10: 0, normal; 1, reduced tone of tail; 2, limp tail, impaired righting; 3, absent righting; 4, gait ataxia; 5, mild paraparesis of hindlimbs; 6, moderate paraparesis; 7, severe paraparesis or paraplegia; 8, tetraparesis; 9, moribund; 10, death. In accordance to North-Rhine-Westphalia animal protection laws, mice were sacrificed in case of paraplegia (score 7 or higher).

2.3. Fluorescence-activated cell-sorting analyses

Single-cell suspensions of spleen from $MOG_{35–55}$ immunized and anti-muCD52 or phosphate buffered saline (PBS) treated CLF mice were prepared on days 10, 25 and 50 after immunization. The following antibodies were used for analysis: anti-mouse CD4 (clone GK 1.5), anti-mouse CD8 (clone 53–6.7), anti-mouse CD19 (clone 1D3), and anti-mouse CD11b (clone M1/70, all from Becton Dickinson, Heidelberg, Germany). For surface stainings single-cell suspensions (1×10^6 cells) from individual organs were stained for 15 min on ice in the dark and washed with phosphate-buffered saline. Analysis was performed using a FACS Canto II (BD) and CellQuest software.

2.4. Immunohistochemistry

Immunohistochemistry was performed on 7 µm thick Paraffin embedded spinal cord cross-sections. Primary antibodies included anti-CD3 for T cells (Serotec; Wiesbaden, Germany; 1:100) and anti-Mac-3 for macrophages (Pharmingen; Heidelberg, Germany 1:100) and were combined with rabbit-anti-rat secondary antibodies (Vector via Linaris; Wertheim, Germany; 1:200). Axonal damage was assessed with the help of Bielschowsky silver impregnation. Quantitative evaluation of immune cells was performed on coded sections by a blinded observer. Therefore, six independent spinal cord cross sections were analyzed per mouse and immune cells were counted by means of overlaying a stereological grid within 6 visual fields with the intense pathology (cellular infiltrates) under a 400-fold magnification. Demyelinated areas were quantified semiautomatically with the help of CellD Software (Olympus, Hamburg, Germany) on Luxol Fast Blue stained sections.

2.5. Reverse transcriptase-polymerase chain reaction protocol

Total RNA from spinal cord and spleen was purified with RNeasy columns (Qiagen, Hilden, Germany). Reverse transcription was performed with 1 µg of purified RNA with Quanta qScript cDNA SuperMix (VWR, Langenfeld, Germany). Quantification of beta-actin was achieved with a predeveloped assay from Thermo Fisher Scientific (Mm00607939_s1, Darmstadt, Germany). BDNF mRNA expression was measured with mBDNF S (5´-GGGCCGGATGCTTCCTT-3′), BDNF AS (5´-GCAACCGAAGTATGAAATAACCATAG-3′), and mBDNF Son (5´-FAM-TTCCACCAGGTGAGAAGAGTGATGACCAT-TAMRA-3′) as primers. All PCR reactions were performed on a 7500 Real Time PCR System (Applied Biosystems) in triplicates; relative quantification was performed according to Livak und Schmittgen (Livak and Schmittgen, 2001).

2.6. Statistical analyses

Analysis of the clinical course was performed using the One-Way ANOVA followed by Kruskall-Wallis Multiple Comparison Test and for histological quantification a Mann-Whitney *U* test was used (GraphPad Prism program, California, USA). All data are given as mean ± SEM. *P*-values were considered significant at $*P < .05$ and highly significant at $**P < .01$ or $***P < .001$.

3. Results

3.1. Clinical EAE course of $MOG_{35–55}$-EAE mice treated therapeutically with anti-muCD52

In the therapeutic approach, anti-muCD52 or PBS treatment was started at the onset of disease, when the animals displayed the first clinical EAE signs, which was between day 12 and day 16 after immunization. The mean clinical score at the beginning of the therapy varied from 2.6 (CLF) to 3.6 (WT), which displays a disability range between loss of tone of tail and absent righting on a ten-point disease score (Table 1). While all of the PBS treated WT and CLF mice suffered from EAE, only 74–88% of the anti-muCD52 treated WT and CLF mice displayed any disease symptoms, which were in addition less pronounced. The disease course of anti-muCD52 treated WT as well as treated CLF mice improved immediately after therapy by 3 score points (mild paraparesis of hindlimbs vs. limp tail) (Fig. 1). This significant amelioration in the anti-muCD52 treated CLF mice was stable until the end of the observation time (Day 50), while the disease course of anti-CD52 treated WT mice improved directly after therapy but approached to the PBS treated WT disease course at Day 50 (Fig. 1).

Table 1
Summarized data of the clinical $MOG_{35–55}$-EAE course after therapeutic administration 10 mg/kg anti-muCD52 or PBS.

	Incidence N/Tot	Incidence %	Mean day of onset	Mean clinical score at the beginning of therapy	Mean max clinical score
WT PBS	22/22	100%	12.1 ± 0.6	3.5 ± 0.6	6.7 ± 0.4
WT anti-muCD52	22/25	88%	10.8 ± 0.3	3.6 ± 0.4	4.3 ± 0.3
CLF PBS	12/12	100%	11.8 ± 0.8	2.6 ± 0.6	5.9 ± 0.5
CLF anti-muCD52	17/23	74%	11.9 ± 0.4	2.6 ± 0.5	3.3 ± 0.6

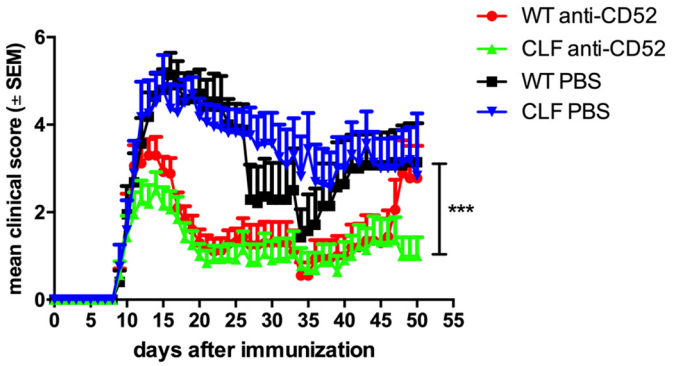

Fig. 1. Clinical disease course of WT and CLF mice immunized with 100 µg $MOG_{35–55}$ and treated therapeutically with 10 mg/kg anti-muCD52 or PBS. While all of the PBS treated WT and CLF mice suffered from EAE, only 74–88% of the anti-muCD52 treated WT and CLF mice displayed any disease symptoms, which were in addition less pronounced. n = 12–25 of 4 independent experiments. One-Way ANOVA followed by Kruskall Wallis Multiple Comparison Test. Data represent the mean ± SEM. P-values were considered significant at $^{***}P < .001$. (n number of each group, WT anti-CD52: 25, WT PBS: 22, CLF anti-CD52: 23, CLF PBS: 12).

3.2. Lymphocyte status in the spleen after therapeutic administration of anti-muCD52

The analysis of immune cells was performed on day 10, 25 and 50 after immunization. Spleen cells were used to detect for differences in immune cell populations after anti-muCD52 therapy by flow cytometry analysis. On day 10, before any EAE signs occur and before anti-muCD52 treatment was started, we observed similar ratios of CD4, CD8 and CD19 positive cells in WT and CLF mice in the peripheral compartment (Fig. 2). Only the amount of CD11b positive cells was reduced in CLF mice before disease onset and start of the antibody treatment. Directly after anti-CD52 treatment (Day 25), we could detect a significantly diminished CD4 and CD8 positive cell population in the anti-CD52 treated WT and CLF groups of about 92% (Fig. 2). The number of depleted immune cells started to recover again 50 days after immunization. The percentage of CD19 positive cells showed no remarkable differences among the groups even after anti-CD52 treatment, while the expression of CD11b cells was slightly enhanced after anti-muCD52 therapy in both treated groups of the acute and chronic phase (Fig. 2).

3.3. Immunohistochemistry of the spinal cord after therapeutic administration of anti-muCD52

Histological analysis of spinal cord sections revealed a diminished expression of macrophages (Mac3 staining) and T cells (CD3 staining) in the anti-muCD52 treated WT and CLF groups compared to the PBS treated WT control directly after treatment (Day 25). Here, anti-muCD52 treated CLF mice even displayed a stronger reduction of infiltrated T cells than the anti-muCD52 treated WT control (Fig. 3). Immune cell staining performed in the chronic phase of disease (Day 50) displayed still lower numbers of T cells in the spinal cord of anti-muCD52 treated CLF and WT mice compared to the PBS treated counterparts. Whereas on the other hand, the infiltration of macrophages in the chronic phase was slightly increased in anti-muCD52 treated WT mice compared to PBS treated WT controls, maybe representing the worsened EAE disease course of the anti-muCD52 treated WT mice in the chronic phase (Figs. 1 and 3). The analysis of axonal densities in the lesions of the spinal cord showed the highest axonal preservation in anti-muCD52 treated CLF mice in the acute as well as the chronic phase. The extent of demyelination was significantly reduced by almost 90% in anti-muCD52 treated CLF as well as WT mice directly after therapy (Day 25), whereas myelin preservation in the chronic phase was less pronounced in the treated animals (Fig. 3).

3.4. BDNF mRNA expression after therapeutic administration of anti-muCD52

The Real-Time PCR analysis of the BDNF mRNA expression in spleen and spinal cord performed directly after therapy (Day 25) showed contrary results. While the BDNF mRNA expression in the spleen was highly reduced after anti-CD52 treatment in WT as well as CLF mice, the expression of BDNF mRNA in the spinal cord was enhanced in anti-CD52 treated WT mice (Fig. 4). When the WT PBS control of the spleen was set as the reference for the relative expression of BDNF mRNA then it was clearly shown that the BDNF expression in the spinal cord of anti-CD52 treated WT mice is higher than all the other groups and even twice as high as in the spleen of the WT PBS control. The analysis of BDNF mRNA expression in the chronic phase (Day 50) showed again a reduced BDNF mRNA expression in the spleen of anti-CD52 treated WT mice compared to the PBS control (Fig. 4). Anti-CD52 and PBS treated CLF mice displayed an overall diminished BDNF mRNA expression in the spleen compared to WT mice, but a differentiated expression was not detectable between the two CLF groups. The expression levels of BDNF mRNA in the spinal cord analyzed at day 50 revealed no notably difference in anti-CD52 versus PBS treated WT and CLF mice, only a slightly reduced expression in CLF mice compared to WT mice was detected.

3.5. Clinical EAE course of $MOG_{35–55}$-EAE mice treated preventively with anti-muCD52

Since therapeutic administration of the anti-muCD52 antibody did not reveal a neuroprotective effect mediated by immune-cell derived BDNF, we chose a preventive approach which should display more the application procedure in humans. Therefore, CLF and WT mice were pretreated with the anti-muCD52 antibody or PBS and $MOG_{35–55}$ immunization followed after repopulation of the immune cells. Recovery of immune cells were followed up by control animals, which were treated at the same time point. Flow cytometry analysis showed a gradually reconstitution of CD4, CD8 and CD19 positive cells and approached to the baseline level 42 days after anti-muCD52 treatment (Fig. 5). An immunization followed immediately on the next day and the animals were observed until the chronic phase (day 50). Here, anti-muCD52 pretreated WT and CLF mice showed an enhanced EAE severity in the acute and chronic phase as compared to PBS treated controls (Fig. 6). The differences in the clinical course of anti-muCD52 treated WT mice (mean max. Clinical score: 4.2) as compared to the PBS control (mean max. Clinical score: 1.8) was much more pronounced

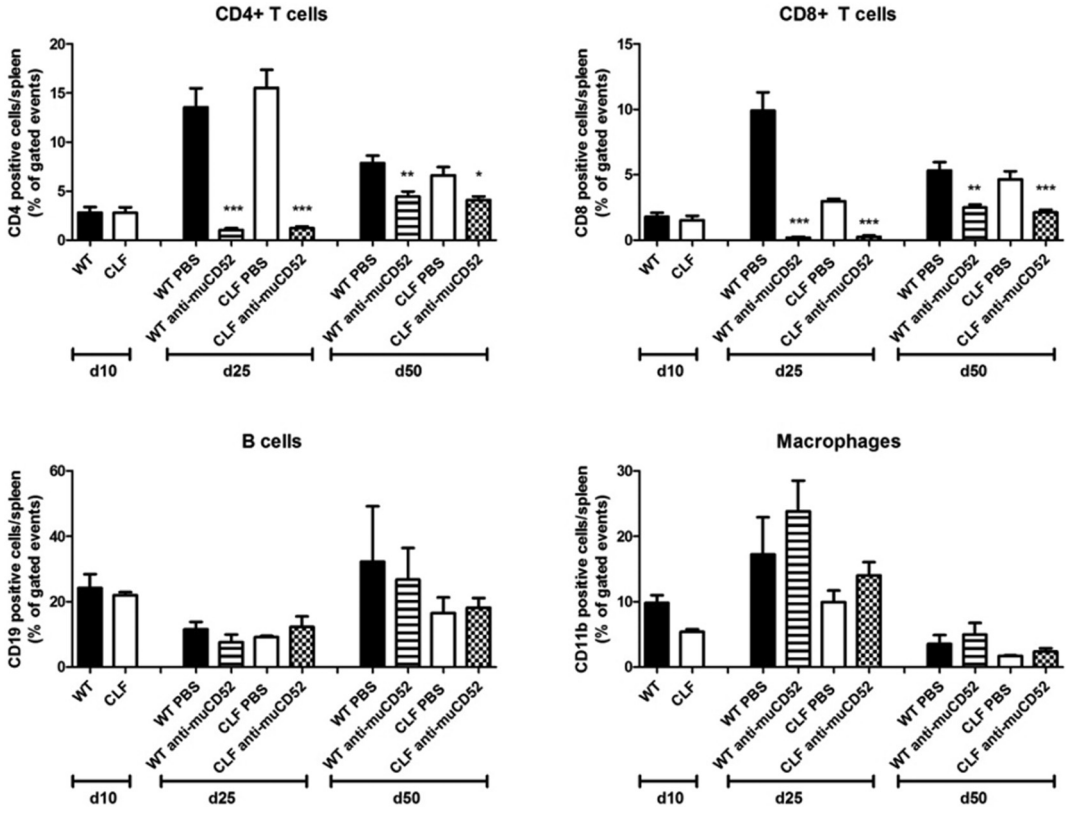

Fig. 2. Immune cell status in the spleen before and after anti-muCD52 therapy and PBS treatment. Analysis was done 10, 25 and 50 days after immunization. Directly after anti-CD52 treatment (Day 25), we could detect a significantly diminished CD4 and CD8 positive cell population in the anti-CD52 treated WT and CLF groups of about 92%. 4 independent experiments, $n = 3–10$. Data represent the mean ± SEM. P-values were considered highly significant at $^{*}P < .05$; $^{**}P < .01$ or ***P < .001.

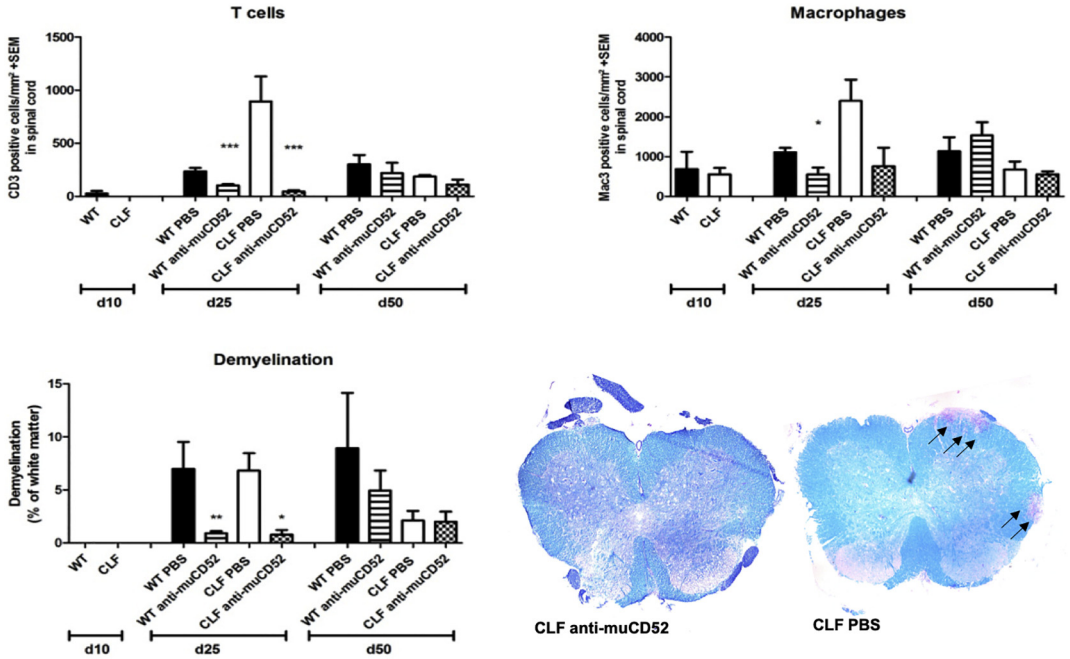

Fig. 3. Histological analysis of the spinal cord before and after anti-muCD52 therapy and PBS treatment. Analysis was done 10, 25 and 50 days after immunization. Histological analysis of spinal cord sections revealed a diminished expression of macrophages (Mac3 staining) and T cells (CD3 staining) in the anti-muCD52 treated WT and CLF groups compared to the PBS treated WT control directly after treatment (Day 25). 4 independent experiments, $n = 2–7$. Data represent the mean ± SEM. P-values were considered highly significant at $^{*}P < .05$ and $^{**}P < .01$. Representative pictures of Luxol Fast Blue myelin staining depicting more areas of demyelination (arrows) for the CLF PBS treated mice compared to anti-muCD52 treated CLF mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

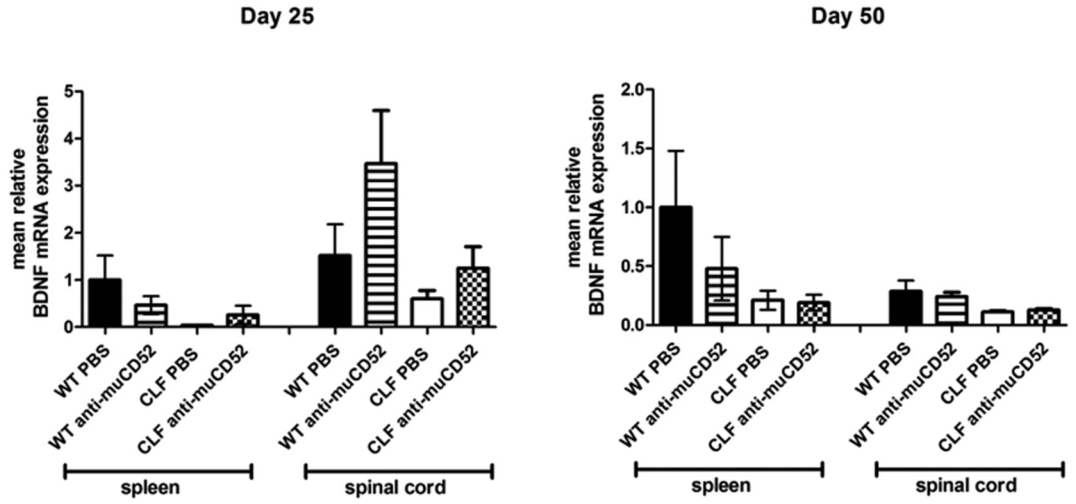

Fig. 4. Real-Time PCR analysis of BDNF mRNA expression in the spleen and spinal cord after anti-muCD52 therapy and PBS treatment. Analysis was done 25 and 50 days after immunization. While the BDNF mRNA expression in the spleen was highly reduced after anti-CD52 treatment in WT as well as CLF mice, the expression of BDNF mRNA in the spinal cord was enhanced in anti-CD52 treated WT mice. n = 3–6. Data represent the mean ± SEM.

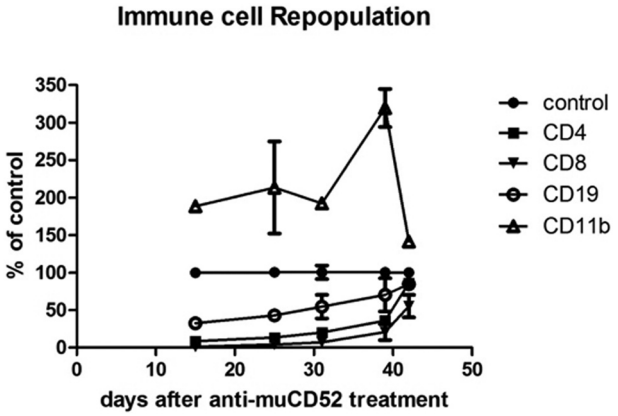

Fig. 5. Immune cell repopulation after pretreatment with anti-muCD52 compared to untreated controls. Analysis was done 15, 25, 31, 39 and 42 days after anti-muCD52 treatment. n = 1–3. Data represent the mean percentage of the control ± SEM. Flow cytometry analysis showed a gradually reconstitution of CD4, CD8 and CD19 positive cells and approached to the baseline level 42 days after anti-muCD52 treatment.

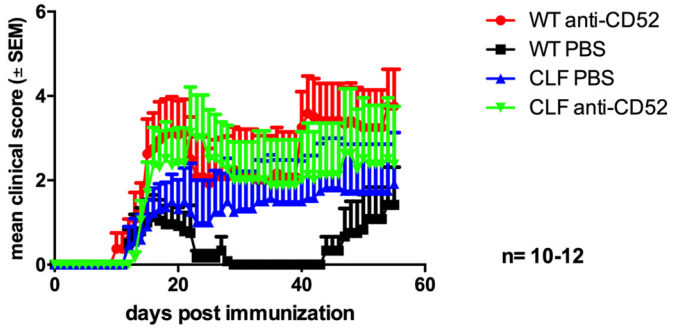

Fig. 6. Clinical disease course of WT and CLF mice pretreated with 10 mg/kg anti-muCD52 or PBS and immunized 43 days after treatment. n = 10–12 of 2 independent experiments (WT CD52: 12, WT PBS: 12, CLF CD52: 10, CLF PBS: 11). Data represent the mean ± SEM. Anti-muCD52 pretreated WT and CLF mice showed an enhanced EAE severity in the acute and chronic phase as compared to PBS treated control.

than in the CLF group (mean max. Clinical score: 2.8 vs. 2.5).

3.6. Immune cell status in the spleen after preventive treatment with anti-muCD52

To clarify if the worsened EAE disease course in anti-muCD52 treated mice is due to a maldistribution of immune cells like it is observed after anti-CD52 treatment in humans (Cox et al., 2005), a cytometer analysis of immune cells in the spleen was performed. As stated above mice were treated with anti-CD52 antibody and on day 43 (during partial immune cell reconstitution) they were immunized. The results showed no enhanced expression of CD4, CD8 or CD19 positive cells after anti-muCD52 treatment of CLF and WT mice neither in the acute (day 25 after immunization, however anti-CD25 treatment 43 days before immunization) nor in the chronic phase (day 50) (Fig. 7). Only the expression of CD11b positive cells was highly enhanced already during the repopulation phase by 2–3 times as compared to the untreated control and displayed still elevated cell numbers after immunization in the acute phase (day 25).

3.7. BDNF mRNA expression after preventive treatment with anti-muCD52

To elucidate if preventive anti-muCD52 therapy has any impact on the expression of BDNF in this model, we performed a BDNF mRNA expression analysis in the acute (day 25) and chronic phase (day 50). Interestingly, only the anti-muCD52 antibody treated WT mice showed a 4–5 times higher expression of BDNF mRNA in the spleen at both time points, while the antibody treated CLF mice displayed a significantly decreased expression of the neurotrophin in the acute phase and an increased expression in the chronic phase (Fig. 8). The expression in the spinal cord showed no differences among the treatments, but only a slightly diminished expression of BDNF mRNA in both CLF groups than compared to the WT groups.

3.8. $MOG_{35–55}$-EAE disease course after preventive treatment with anti-muCD52 and a prolonged recovery phase

Since the $MOG_{35–55}$-EAE disease course in the previous setting was increased after anti-muCD52 therapy in WT and CLF mice, we decided to prolong the repopulation phase before immunization to see, if the enhanced disease severity is due to a misregulation of immune cells and can be overcome by a longer recovery phase. Therefore, animals were pretreated with the anti-muCD52 antibody or PBS as a control and were

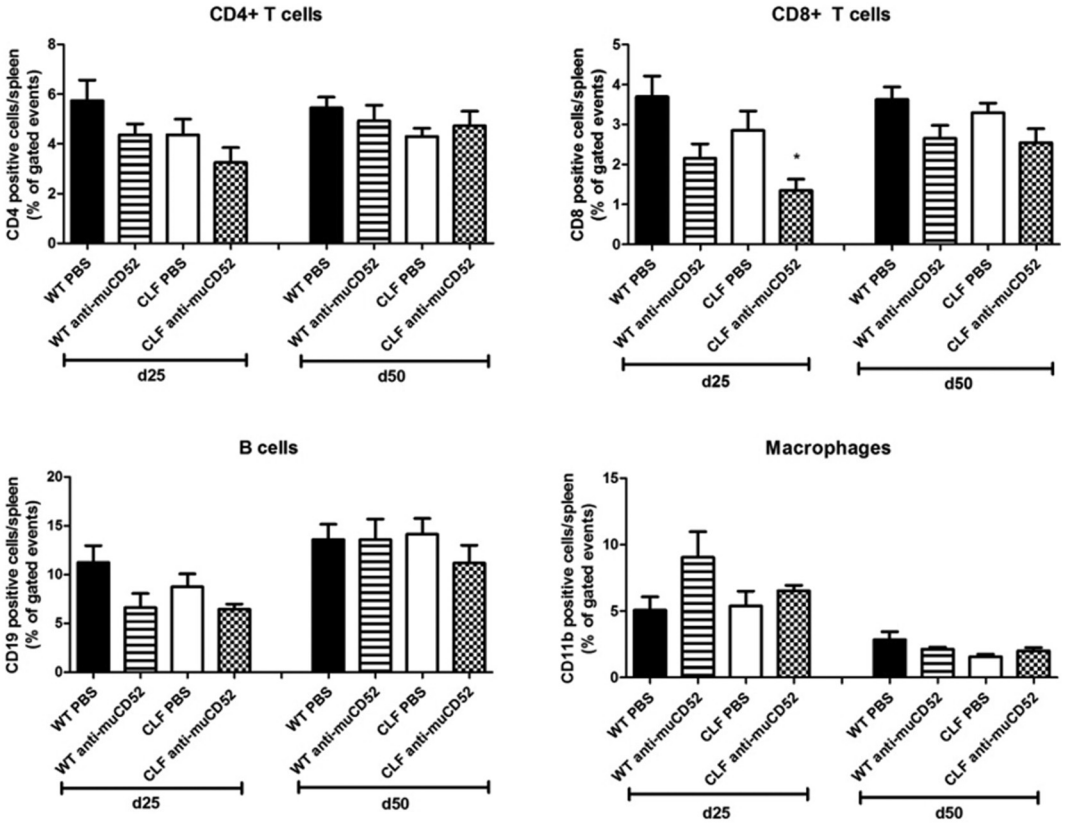

Fig. 7. Immune cell status in the spleen after preventive treatment with anti-muCD52 and PBS and a subsequent $MOG_{35–55}$ immunization after 43 days. Analysis was done 25 and 50 days after immunization. n = 3–6. Data represent the mean ± SEM. *P*-values were considered significant at $^*P < .05$. Only the expression of CD11b positive cells was highly enhanced already during the repopulation phase by 2–3 times as compared to the untreated control.

regularly monitored to check for repopulation. 85 days after pretreatment CD4, CD8 and CD19 cells were almost completely recovered and showed similar values as the untreated controls (Fig. 9). CD11b positive cells showed highly enhanced cell numbers at the beginning of the repopulation phase (approx. 2–3 times stronger expression), but were downregulated to only 50% of the untreated control expression level shortly before immunization. $MOG_{35–55}$ immunization was performed on day 86 after pretreatment and lead to approximated clinical courses of anti-muCD52 and PBS treated WT and CLF mice (Fig. 10).

4. Discussion

The humanized anti-CD52 antibody, alemtuzumab, is effectively used in the treatment of relapsing-remitting multiple sclerosis with high disease activity (Cohen et al., 2012). Beside its depleting character it was also proposed to have a neuroprotective role by inducing the expression of neurotrophins, like BDNF, in peripheral immune cells (Jones et al., 2010). That BDNF plays a pivotal role in the hypothesized concept of neuroprotective autoimmunity becomes reasonable, when its major role in preventing cell death and providing trophic support to mature neurons is combined with the observation that BDNF expressing T cells and macrophages were found in the lesions of multiple sclerosis patients (Stadelmann et al., 2002). Furthermore, studies from the animal model of multiple sclerosis, the EAE, could clearly show, that mice with a BDNF deficiency in immune cells display a more severe clinical EAE course and an increased axonal loss, while on the other hand an

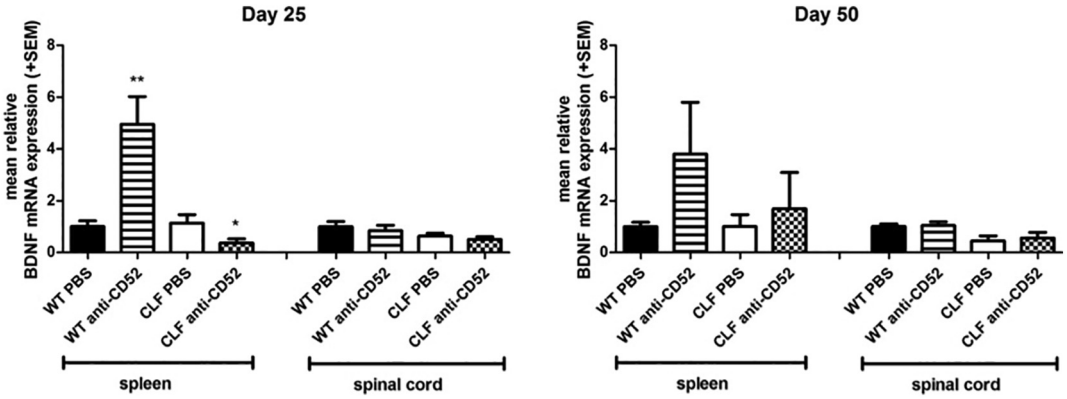

Fig. 8. Real-Time PCR analysis of BDNF mRNA expression in the spleen and spinal cord after preventive anti-muCD52 therapy and PBS treatment and a subsequent $MOG_{35–55}$ immunization after 43 days. Analysis was done 25 and 50 days after immunization. *n* = 4–6. Data represent the mean ± SEM. P-values were considered highly significant at $^*P < .05$ and $^{**}P < .01$.

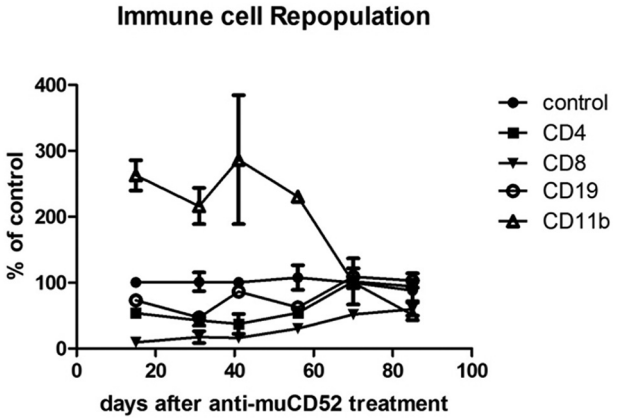

Fig. 9. Immune cell repopulation after pretreatment with anti-muCD52 compared to untreated controls. Analysis was done 15, 31, 41, 56, 70 and 85 days after anti-muCD52 treatment. n = 1–2. Data represent the mean percentage of the control ± SEM. 85 days after pretreatment CD4, CD8 and CD19 cells were almost completely recovered and showed similar values as the untreated controls.

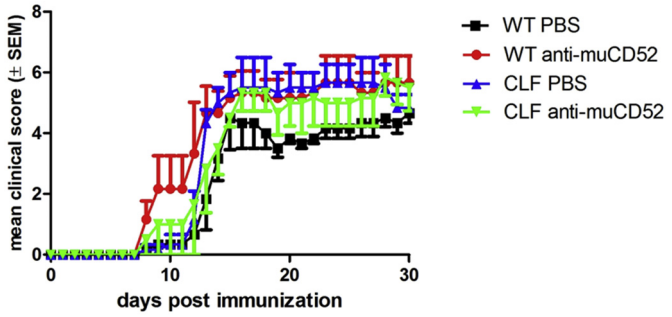

Fig. 10. Clinical disease course of WT and CLF mice pretreated with 10 mg/kg anti-muCD52 or PBS and immunized 86 days after treatment. $n = 3$. Data represent the mean ± SEM. MOG35–55 immunization was performed on day 86 after pretreatment and lead to approximated clinical courses of anti-muCD52 and PBS treated WT and CLF mice.

enhanced BDNF expression in T cells lead to axon protection (Linker et al., 2010).

To test, if immune cell derived BDNF has any impact on the clinical or subclinical outcome of anti-CD52 therapy, a murine equivalent was used in the $MOG_{35–55}$ EAE, induced in lineage specific constitutive BDNF deficient in T cells and macrophages and wildtype mice. This BDNF lineage specific constitutive knockout model was previously used in several studies. Especially in the work of Linker et al. these mice were studied in detail concerning BDNF expression in the brain. Here, no difference in the number of BDNF-reactive astrocytes or neurons was detected in the lineage specific constitutive knockout mice compared to wild-type controls (Linker et al., 2010). Therapeutic application of the anti-muCD52 antibody in the $MOG_{35–55}$ induced EAE lead to a significantly ameliorated EAE disease course in WT and lineage specific constitutive BDNF knock-out mice. Thus, both groups profit equally from the depletion of immune cells induced by anti-muCD52 treatment. Analysis of immune cell subsets in the spleen and spinal cord done after immunization and antibody treatment did not reveal a differentiated regulation of T cells, B cells and macrophages among the antibody treated WT and lineage specific constitutive BDNF knockout mice. The depletion efficiency of the murine anti-CD52 antibody concerning $CD4^+$ and $CD8^+$ positive T cells was effective as described in previous publications although in the case of B cells a slight discrepancy was there, because of the use of different surface markers: while we used the CD19 marker to detect B cells, a previous publication used the B220 marker (Turner et al., 2015).

Since therapeutic anti-muCD52 treatment did not reveal a neuroprotective character mediated by immune cell derived BDNF, we aim to study a preventive approach, where the anti-muCD52 antibody was applied before immunization. This approach will mimic more the human situation, where the human anti-CD52 antibody is applied as effective treatment to prevent a new relapse and the disability progression (phase 3 study). Furthermore, the proposed neuroprotective effect of anti-CD52 is based on the observation, that PBMCs produce increased levels of BDNF in the recovery phase after treatment (Jones et al., 2010) and are therefore possible mediators to maintain neuronal integrity. Interestingly, preventive treatment with anti-muCD52 resulted in a more severe clinical EAE course in WT and lineage specific constitutive BDNF deficient mice than compared to controls. Immune cell analysis showed a highly increased expression only of macrophages after anti-muCD52 treatment, which may cause the enhanced severity in the clinical course. Yet, after a prolonged recovery phase, where especially macrophage levels were reduced again to baseline levels, the disease courses of anti-muCD52 treated mice approached to the control ones. Hyperpopulation of B-cells after murine anti-CD52 treatment of MOG-EAE mice was not observed in the present study as well as in previous studies (Turner et al., 2015).

Since EAE is a more autoreactive T-cell driven disease (Fletcher et al., 2010), B cells play a minor role in this model. However, excessive macrophage levels after anti-CD52 was not observed before, neither in patients with multiple sclerosis after alemtuzumab therapy nor in MOG-EAE mice after the treatment with the humanized anti-CD52 antibody (Turner et al., 2015). The impact of murine anti-CD52 therapy on macrophages in the MOG-EAE was not described before. Nevertheless, macrophages are considered as potent antigen-presenting cells and important promoters of demyelination in EAE (Rawji and Wee Yong, 2013), pointing to their importance in this animal model.

Alemtuzumab is used extensively for aggressive forms of multiple sclerosis, therefore its immunomodulatory function during $CD52^+$ depletion as well as during repopulation are of great importance. In our preventive model repopulation with increased number macrophages in the spleen led to a severe disease course, however the exact mechanism leading to this deterioration remain unclear. Histological analyses of MS lesions differentiating M1 (proinflammatory) from M2 (anti-inflammatory) macrophages as well as further flow cytometric analyses and in vitro analyses of spleen macrophages are needed.

Surely, a shortcoming of our study is the fact that preventive approach does not reproduce human relapsing disease, a better model would be a spontaneous EAE model, in which anti-CD52 would be given during remission. However, in our mouse model, this was the only way to reproduce the reconstitution phase and apply the factor of autoimmune activation (relapse) during reconstitution. One of the most interesting clinical observations in patients after anti-CD52 treatment was indeed the presence of relapses, some of them very severe during the reconstitution phase. Early relapses were attributed to B cell repopulation but there are still unexplained clinical relapses during this phase. Our model introduces a new aspect through the presence of increased macrophages during clinical deterioration, which could useful for future clinical studies on alemtuzumab.

Taken together, this study showed that therapeutic as well as preventive modulation of MOG-EAE in lineage specific constitutive BDNF k.o. mice with a murine anti-CD52 antibody did not exert a neuroprotective role mediated by immune cell-derived BDNF. In this model of EAE T lymphocytes and monocytes are crucial, thus the lineage specific constitutive lack of BDNF should be an applicable model. More information may be needed to further understand how alemtuzumab contributes to neuroprotection.

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