Tolerance induction by bone marrow transplantation in a multiple sclerosis model

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Abbreviations used in this paper: BBB, blood brain barrier; BM, bone marrow; BMT, bone marrow transplantation; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; HSCT, hematopoetic stem cell transplantation; LN, lymph node; MOG, myelin-oligodendrocyte-glycoprotein; MS, multiple sclerosis; RT1, MHC of rat; MNC, mononuclear cell; p.i., post immunization; SPF, specific pathogen free; TBI, total body irradiation.
Abstract

Experimental autoimmune encephalomyelitis (EAE) in rats is a highly valuable model of multiple sclerosis (MS) since it mimics major hallmarks of the human disease. EAE induced with myelin-oligodendrocyte-glycoprotein (MOG) in DA rats is relapsing-remitting and lesions in the central nervous system show inflammation, demyelination, axonal and neuronal loss. Recently, bone marrow transplantation (BMT) was introduced as a novel strategy to treat MS but its efficiency and the underlying mechanism are debatable. In MOG induced EAE we found that BMT at the peak of EAE but not in the chronic phase leads to disease attenuation. In both settings bone marrow (BM) transplanted rats were protected from subsequently induced relapses. These findings could be confirmed by histopathology in which BM transplanted rats did not have lesions compared to non-transplanted controls. Importantly, the protective effect was achieved by allogeneic, syngeneic and grafts from diseased rats. BMT resulted in increased numbers of CD4+CD25bright regulatory T cells, increased Foxp3 expression, a shift in T cell epitope recognition and a strong reduction of autoantibodies even after re-challenge with MOG. Thus, our results indicate potential mechanisms of how BMT may contribute to the improvement of MS and provide a rationale for its application in patients suffering from various autoimmune diseases.
Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) which is characterized by demyelinating plaques and axonal loss. Although MS is highly prevalent in northern Europe and the USA there is presently no cure available and the clinical management of the disease is unsatisfactory. The etiology of MS is unknown. However, it is generally believed that both genetic and environmental factors contribute to the development of its pathology. The onset of MS is presumably characterized by autoreactive T cells crossing the blood brain barrier (BBB) and initiating an inflammatory response which results in an opening of the BBB. This allows influx of additional immune cells such as granulocytes, macrophages, NK cells and B cells, as well as antibodies and complement. Subsequently, this process leads to myelin destruction, induction of oligodendrocyte death, axonal degeneration and ultimately to the functional deficits seen in MS patients. Experimental autoimmune encephalomyelitis (EAE) is a frequently used animal model for MS which can be induced in rodents and monkeys. In particular, myelin-oligodendrocyte-glycoprotein (MOG) induced EAE in DA rats mimics major hallmarks of the human disease. These include the relapsing/remitting type of disease course, the occurrence of demyelinated plaques in brain and spinal cord, axonal loss, and the involvement of both antibodies and complement in the pathogenesis.

Autologous bone marrow transplantation (BMT) and hematopoietic stem cell transplantation (HSCT) are presently discussed as novel options for the treatment of patients with MS with fast progressive disease courses that do not respond to conventional treatment. Clinical trials were to some extent inconclusive since they arrived at divergent results. Preclinical studies were performed in EAE that
assessed certain aspects of BMT like timing and source of grafts to some extent in rats and mice\textsuperscript{17-24}. In order to lay further ground for clinical studies we conducted BMT using MOG induced EAE in the rat. We performed all possible combinations of BMT in susceptible DA and resistant ACI rats that share the RT1\textsuperscript{av1} haplotype (RT1 is MHC in rats)\textsuperscript{8}. Also, we analyzed the effect of BMT in acute versus chronic disease and the specificity of the protection after BMT in this model. Importantly, we were able to investigate the immunological mechanisms operative during BMT regarding the T cell recognition, autoantibodies and T regulatory cells in the treatment of EAE. This allows us to suggest a strategy of how BMT could be most efficiently employed in the therapy of MS and how clinical markers could be defined to assess treatment success.
Materials and Methods

Rats
Female rats, 10-14 weeks of age, were used in all experiments. DA rats were obtained from Harlan Winkelmann (Borchen, Germany) and ACI rats from Harlan USA (Indiana, IN). Rats were kept under specific pathogen free (SPF) conditions and obtained food and water ad libitum. Generation and characterization of GFP-transgenic Lewis rats\textsuperscript{25} (line UGC) have been described elsewhere. All animal experiments were approved by the regional boards in Tübingen and Würzburg, Germany.

Synthetic peptides and antigens
MOG 73-90 (KESIGEGKVALRIQNVRF), MOG 91-108 (SDEGGYTCFFRDHSYQEE) and MBP 63-88 (HTRTTHYGSLPQKSQRTQDENPVVHF) were prepared by Karl-Heinz Wiesmüller, (EMC microcollections, Tübingen, Germany) by solid phase peptide synthesis using F-moc/tBu chemistry. Peptides were purified by preparative HPLC (Abimed, Langenfeld, Germany). The identity of the purified peptides was confirmed by electrospray mass spectrometry. The purity of peptides was >95% as determined by analytical HPLC (Abimed). Recombinant rat MOG, corresponding to the N-terminal sequence of MOG (amino acids 1–125, MOG 1-125) was expressed in \textit{Escherichia coli} and purified. The purified protein in 6 M urea was dialyzed against PBS and stored at -20°C.

BMT
For TBI 6 MV photons at a dose rate of approximately 1 Gy/min delivered from a linear accelerator (Elekta, Hamburg, Germany) were used. Rats were irradiated with a lethal
dose of 10 Gy. BM was flushed out of the bones of naïve or diseased DA or ACI rats and resuspended in PBS. 2x10^7 viable cells were injected one day after irradiation i.v. into recipient rats. BM from GFP transgenic LEW^25 rats was harvested and transplanted into LEW rats as described above.

**Determination of chimerism**

DNA was isolated from blood using a Quiagen DNeasy kit. Primers for the microsatellite marker 21RHAP115FF5\(^26\) (forward: 6-FAM AGGAGAGCTCGTGAGCTGAG and reverse: ACAAGGAATGACACCCAAGG) were used in a PCR reaction which give a product lengths for DA (214 bp) and ACI (192 bp) alleles. Products were separated and quantified with a polyacrylamide gel on an ABI prism 377 DNA sequencer running with genescan software (Applied Biosystems). Results were set in relation to a standard obtained by mixing known amounts of ACI and DA blood.

**Blood cell enumeration**

To assess reconstitution of cell lineages in transplanted rats blood samples from the tail vein were analyzed in an automated blood analyzer (VetABC, Scil, Viernheim, Germany).

**Induction and scoring of EAE**

EAE was induced by administration of 200 µl inoculum intradermally at the base of the tail. The inoculum consisted of 50 µg MOG 1-125 in PBS emulsified with CFA (Sigma) (1:1) containing 200 µg heat inactivated *Mycobacterium tuberculosis* (strain H 37 RA; Difco, Detroit, MI). Clinical signs were scored as follows: grade 1, tail weakness or tail
paralysis; grade 2, hind leg paraparesis or hemiparesis; grade 3, hind leg paralysis or hemiparalysis; grade 4, complete paralysis tetraplegia, moribund state, or death. Rats which died after the transplantation procedure were excluded from the experiment and rats that died of EAE after reimmunization were scored with a score of 4.

Isolation of mononuclear cells (MNC) from lymph nodes (LN) and spleens

Draining inguinal LN and spleens were dissected out under deep anesthesia. LN were disrupted and MNC washed twice in Dulbecco’s modified eagle medium (DMEM, Life Technologies, Paisley, U.K.), resuspended in complete medium (CM) containing DMEM supplemented with 5% fetal calf serum (PAA Laboratories Linz, Austria), 1% penicillin/streptomycin (Life Technologies), 1% glutamine (Life Technologies), and 50 µM 2-mercaptoethanol (Life Technologies) and flushed through a 70-µm plastic strainer (Falcon; BD Biosciences, Franklin Lakes, NJ). MNC from spleen were prepared in the same way as from LN with the difference that red blood cells (RBC) were lysed with lysis buffer consisting of 0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM Na₂ EDTA adjusted to pH 7.4.

Elispot

Nitrocellulose bottomed 96 well plates (MAHA; Millipore, Molsheim, France) were coated with anti IFN-γ mouse mAb DB1 (a generous gift of Peter van der Meide, TNO Primate Center, Rijswijk, The Netherlands). Following washing with PBS, plates were blocked with 5% FCS in DMEM (Life Technologies). MNC (4 x 10⁵ per well) in 200 µl CM and antigen was added to the plates and incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO₂. For each Ag, triplicate determinations were performed.
MOG 1-125 was used at a concentration of 3 µg/ml and peptide at a concentration of 10 µg/ml. ConA was purchased from Sigma (St. Louis, MO) and used at a concentration of 1 µg/ml. Cells were discarded, and plates were washed four times with PBS. Secreted and bound IFN-γ was visualized with biotinylated DB12 mAb against rat IFN-γ (also a generous gift of Peter van der Meide), avidin-biotin peroxidase (Vector Laboratories, Burlingame, CA), and subsequently by staining with carbazole (Sigma).

**ELISA**

Blood samples for antibody measurements were taken at the end of experiments. ELISA plates (96 well; Nunc, Roskilde, Denmark) were coated with 2.5 µg/ml (100 µl/well) MOG 1-125 overnight at 4°C. Plates were washed with PBS/0.05% Tween 20 and blocked with milk powder for 1 h at room temperature. After washing, diluted serum samples were added and plates were incubated for 1 h at room temperature. Then, plates were washed and rabbit anti rat antiserum (IgG, IgG1, IgG2a, IgG2b, IgG2c, Nordic, Tilburg, The Netherlands) was added and incubated for 1 h at room temperature. Plates were washed prior to the addition of peroxidase conjugated goat anti rabbit antiserum (Nordic) diluted in PBS/0.05% Tween 20. After 30 min incubation, plates were washed and bound Abs were visualized by addition of ABTS (Roche Diagnostics Mannheim, Germany). After 15 min of incubation optical density was read at 405 nm.

**Quantitative real time RT PCR**

Total RNA was extracted from splenocytes using RNeasy Mini Kit (Qiagen, Hilden, Germany). To avoid amplification/detection of contaminating genomic DNA, extracted RNA was treated with Rnase free DNase (Promega, Madison, WI). Subsequently, cDNA
was synthesized by reverse transcription with Moloney murine leukemia virus reverse transcriptase and random pdN6 primers in the presence of RNase inhibitor (Promega). Amplification was performed on an Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using a SYBR green protocol. The primer sequences 5’ to 3’ were as follows: GAPDH: forward: GGTGTCTCCTGTGACTTCAA, reverse: CATACCAGAAATGAGCTTCAC; Foxp3: forward: CCACACCTCCTTTCTTCCTT, reverse: TGACTAGGGGCACTGTAGGC. Results were expressed as 2-ΔΔCT values.

**Antibodies and flow cytometry**

FITC conjugated mAb against CD45RA (OX-33), CD25 (OX-39) and TCRAB (R73) and PE conjugated mAb against CD4 (OX-35) and CD8 (OX-8) and appropriate isotype controls were purchased from Becton Dickins on (Heidelberg, Germany). Flow cytometry was performed on a FACScalibur running with Cellquest software (Becton Dickinson). Cells were gated on the lymphocyte population in the FSC-SSC dot plot.

**Enrichment of CD4+ CD25+ T cells.**

A single cell suspension of MNC from spleens was obtained as described above. The cell suspension was incubated with OX42-FITC (Becton Dickinson) recognizing the complement type 3 receptor. After washing cells were incubated with OX33 (anti-CD45RA), OX8 (anti-CD8), and anti-FITC magnetic bead labeled antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). The cell suspension was then applied to a LD column (Miltenyi Biotec) to deplete granulocytes/macrophages, B cells, and CD8+ cells respectively. The remaining CD4+ T cells (purity >94% determined by flow cytometry)
were then incubated with OX39 FITC (anti CD25), incubated with anti-FITC magnetic bead labeled antibodies, and finally enriched on a LS column (Miltenyi Biotec). Success of the enrichment procedure was determined by double staining with OX35-PE and OX39-FITC. 1 x 10^6 of these cells were injected i.v. into recipient rats.

**Histopathological evaluation**

Histological evaluation was performed on paraformaldehyde fixed, paraffin embedded sections of brains and spinal cords. Paraffin sections were stained with hematoxylin and eosin, Luxol fast blue, and Bielschowsky silver impregnation to assess inflammation, demyelination, and axonal pathology, respectively as described 8-10. Immunohistochemistry was performed using a modified avidin-biotin-based technique using diaminobenzidine as chromogen 27. After microwave pretreatment, sections were incubated over night with antibodies against ED1 (Serotec), CD3 (Serotec), rat immunoglobulin (Amersham), rat C9 (kindly provided by BP Morgan, University of Cardiff, UK) and APP (Chemicon) followed by appropriate biotinylated secondary antibodies (Amersham). Sections incubated without primary antibody, with iso-type control antibodies and with pre-immune sera did not show any immunoreactivity. Sections were counterstained with haemalaun.

**Data analysis**

Statistical significance was tested by students t-test or ANOVA as indicated. For posthoc analysis we used multiple t-test with an $\alpha$ level of 0.05. All statistical analysis were performed with JMP for Windows (release 5.0). Error bars in the graphs represent standard error of mean (SEM).
Results

Chimerism and engraftment

We chose DA and ACI rats as a source for the BM grafts since these rat strains are MHC matched (RT1\(^{av1}\)) while differing in their non-MHC genome and their susceptibility to MOG 1-125 induced EAE: DA rats are EAE susceptible while ACI rats are resistant to MOG 1-125 induced EAE: DA rats are EAE susceptible while ACI rats are resistant \(^8,28\). We determined the degree of engraftment of donor BM after BMT on day 107 after transplantation. In order to differentiate between cells of ACI or DA rat origin we analyzed the relative intensity of a microsatellite marker in a quantitative PCR reaction of transplanted rats. DA rats with ACI BM (n=6) had a high percentage of cells with the ACI allele in their blood (96.9% +/-0.55), while the ACI allele in the blood of ACI rats with DA BM (n=8) was low (5.1% +/-1.19) (Figure 1A). We determined the number of leukocytes, platelets and erythrocytes as well as hematocrit and hemoglobin concentration in BM transplanted DA rats on day 107 post transplantation. We did not detect any difference compared to non transplanted animals except for the leukocyte counts (\(P=0.0014\), ANOVA) confirming complete reconstitution of the recipient rats (Table 1).

To further verify the quality of the transplantation procedure we transplanted BM from eGFP transgenic LEW rats \(^25\) into syngeneic LEW rats (n=16). Susceptibility to MBP 63-88 induced EAE was unchanged in LEW rats reconstituted with GFP transgenic BM (n=5) compared to naïve LEW rats (n=5) (Figure 1B). We confirmed the successful reconstitution of host lymphatic tissues (lymph nodes, thymus) and BM using our grafting protocol (Figure 1C). LN cells in LEW rats transplanted with BM from eGFP transgenic LEW rats were comparable in GFP expression compared to naïve eGFP transgenic LEW rats (pool of n=5 naïve eGFP transgenic LEW rats, LEW rats transplanted with BM from eGFP transgenic LEW rats n=5). Cells eluted from the CNS...
in the course of EAE were also highly GFP-positive (Figure 1D).

**BMT studies in naïve rats**

DA rats develop a relapsing/remitting disease course after immunization with MOG 1-125 (n=5). To address the question whether BMT by itself alters the susceptibility to EAE we performed total body irradiation (TBI) with 10 Gy in naïve DA rats and transplanted them with DA BM (n=4). On day 60 post BMT after BM reconstitution, rats were immunized with MOG 1-125. Similar to rats which had not received BMT, the disease course in these transplanted rats was of the relapsing/remitting disease type (Figure 2A). Confirmatory experiments gave the same results (naïve DA transplanted with DA BM [n=5], data not shown). In contrast, DA rats transplanted with BM from the EAE resistant ACI rat strain developed a progressive type of EAE (n=7).

In a second set of experiments ACI rats which are resistant to EAE induced with MOG 1-125 were used as recipients of the BM graft (n=4). When ACI rats were treated with DA BM and subsequently EAE was induced on day 60 post BMT they developed a mild relapsing/remitting disease course (n=8). Unexpectedly, EAE induction after engraftment of ACI BM into ACI rats (n=4) resulted in severe EAE (Figure 2B). Repeated experiments gave the similar results (naïve ACI transplanted with ACI BM [n=11] and naïve ACI transplanted with DA BM [n=4], data not shown).

**Effects of BMT on established disease and induced relapses**

To evaluate the effect of TBI and subsequent BMT on EAE we divided DA rats in three groups: DA rats grafted with DA BM, DA rats grafted with ACI BM and not irradiated ungrafted DA rats. At the peak of acute disease on day 17 post immunization (p.i.), rats
were treated with 10 Gy of TBI and received BM of either DA (n=6) or ACI (n=6) origin. Importantly, this treatment led to a significant clinical improvement as compared to DA rats which had not been transplanted (n=10) (cumulative score day 19-87 p.i., \( P=0.036 \), ANOVA) (Figure 3A). On day 90 p.i. rats were reimmunized with MOG 1-125. While the non-transplanted rats relapsed, rats treated with BM were largely protected from induced relapses (cumulative score day 91-115 p.i., \( P<0.0001 \), ANOVA). It is further noteworthy that the difference between the groups treated with syngeneic DA and allogeneic ACI BM was not significant (NS, ANOVA) (Figure 3A). To confirm our results we conducted a second experiment under similar conditions (Figure not shown). This time DA rats were irradiated on day 16 after immunization and received BM of either DA (n=8) or ACI (n=6) origin on the following day. Controls were left untreated (n=9). Again, there was a significant effect of the treatment on the disease course (cumulative score day 17-131 p.i., \( P=0.027 \), ANOVA). Furthermore, rats were also strongly protected from induced relapse when reimmunized on day 131 p.i. (cumulative score day 140-154 p.i., \( P=0.0007 \), ANOVA). Again, there was no difference in the efficiency of BMT in its ability to protect from EAE with regard to the source of BM (DA versus ACI, NS, ANOVA).

In order to investigate whether BMT also had a positive effect on the disease course when performed in the chronic phase of EAE, we chose a later time point (day 140 p.i.) for BMT. Interestingly, we did not observe any improvement of EAE after transplantation with DA BM (n=9) in this setting. To further evaluate whether rats were protected from following relapses under these conditions we reimmunized with MOG 1-125 them after reconstitution (day 181 p.i.). While we were able to demonstrate partial improvement of EAE using this protocol (n=4), protection induced by BMT in the chronic phase was slightly weaker as compared to the situation when BMT was performed at the peak of...
EAE (cumulative score day 188-234 p.i., \( P=0.021 \), t-test) (Figure 3B).

**Histopathology**

We performed a histopathological examination of brains and spinal cords at the end of the experiments (Figure 3A and repetition of experiment). 13 out of 15 DA rats without BMT had strong inflammation and widespread demyelination in the CNS (Figure 4A-H). In contrast only three out of 14 DA rats treated with DA BM and none of the eleven DA rats treated with ACI BM showed inflammation or/and demyelination in the CNS (Figure 4I-O). Lesion pathology in untreated animals was characterized by large, confluent demyelination (Figure 4A), macrophage infiltration (Figure 4B) and deposition of immunoglobulin and C9 indicative of antibody mediated demyelination (Fig. 4C, D). CD3-positive T cells were found scattered in the lesions (Figure 4E). Inflammatory infiltrates were accompanied by acute axonal damage (Figure 4F, G) and a reduction in axonal density. No pathology was observed in a representative DA rat treated with DA BM (Figure 4I-O).

**Syngeneic BMT with BM from diseased rats**

To mimic the clinical setting more closely we conducted an experiment in which rats were treated in the acute phase of EAE with syngeneic BM of rats with EAE. This transplantation regimen led to an amelioration of the disease course (n=9) as compared to those which had not been transplanted (n=9) (Figure 5A). Syngeneic BM from rats with EAE also protected from induced relapses with MOG 1-125 (Figure 5B).
Antigen specificity of the protection from EAE mediated by BMT

All our experiments described so far suggested that protection from EAE is due to induction of tolerance to MOG induced EAE relapses. In order to assess whether protection is antigen specific, we rechallenged rats that had received a DA BM transplant from DA rats with EAE at the peak of MOG-EAE with the immunodominant MBP 63-88 peptide \((n=4)\) (Figure 5C). As controls, rats that were BM transplanted as naïve rats were challenged with MBP 63-88 \((n=4)\). Both groups were not protected against MBP 63-88 induced EAE (Figure 5C). These results indicate that the tolerance achieved by BMT is specific for the antigen used to induce disease.

Autoantibodies

Antibodies against MOG play an important role in mediating widespread demyelination in DA rats \(^{11}\). Therefore we assessed total IgG levels against MOG 1-125. Upon immunization the antibody levels increased to MOG \((n=6)\) compared to naïve controls \((n=4)\) \((P<0.002)\). On day 123 p.i., 107 day after BMT, DA rats transplanted with DA BM \((n=8)\) and DA rats transplanted with ACI BM \((n=6)\) showed a strong reduction of autoantibody titers compared to the non transplanted control group with EAE \((n=8)\) \((P=0.0001)\). This effect was even more pronounced on day 154 p.i. post re-immunization \((\text{no BMT } n=6; \text{ DA BM } n=6, \text{ ACI BM } n=5)\) \((P<0.0001)\) (Figure 6A).

To test whether BMT by itself leads to a reduced antibody response we measured serum antibody levels in rats which were transplanted as naïve rats and immunized with MOG 1-125. These rats developed an antibody response comparable to rats without BMT and significantly higher than the rats transplanted during acute EAE \((P<0.0001)\) (Figure 6A).
Moreover we determined IgG isotype distribution against MOG 1-125 (IgG1, IgG2a, IgG2b and IgG2c) on day 115 p.i. or day 98 post transplantation. Total IgG against MOG 1-125 as well as all IgG subtypes (IgG1, IgG2a, IgG2b, IgG2c) were strongly reduced in the BM transplanted groups (DA BM graft [n=6], ACI BM graft [n=6]) compared to the non transplanted group (n=8) (IgG1 $P < 0.004$; IgG2a, IgG2b, IgG2c each $P < 0.0001$, ANOVA) (Figure 6B). Based on the isotype distribution, we did not find evidence for a Th2 shift.

**Lymphocyte subset distribution**

To evaluate whether differences in the lymphocyte subset distribution between EAE protected transplanted rats (DA BM graft [n=6], ACI BM graft [n=6]) and controls (no BMT [n=6]) were present, cell surface expression of CD4, CD8, and CD45RA (B cells) were analyzed in the blood before reimmunization and in lymph nodes after reimmunization. In the blood, the percentage of B cells was increased whilst the percentage of CD4+ and CD8+ T cells was reduced when comparing transplanted rats to controls. Significant changes were seen for B cells ($P < 0.001$, ANOVA), CD4+ cells ($P < 0.0001$, ANOVA) and CD8+ cells ($P = 0.04$, ANOVA) between transplanted and non transplanted rats (Figure 6C).

Similar changes were detected in the lymphocyte subpopulation distribution after BMT in lymph nodes (DA BM graft [n=5], ACI BM graft [n=6]) and controls (no BMT [n=6] and naïve BMT [n=5]). Here the group of naïve rats transplanted with BM showed a pattern comparable to the BMT groups (DA BM graft and ACI BM graft). This indicates that the observed changes are linked to the BMT. The differences between the group that had not received a BMT and the transplanted groups were significant in both the T cell
(CD4+ and CD8+) and the B cell compartment (for all \( P<0.001 \), ANOVA) (Figure 6D).

**T cell responses towards MOG peptides**

We used the elispot method for quantification of MOG specific IFN-\( \gamma \) producing cells. The numbers of MOG 73-90 or MOG 91-108 (dominant determinants) \(^{29}\) specific IFN-\( \gamma \) secreting cells in relation to those specific for the MOG 1-125 were determined. The two groups which had received BMT (DA BM graft or ACI BM graft) showed an increase of MOG 73-90 specific IFN-\( \gamma \) secreting cells but no changes in numbers of MOG 91-108 specific IFN-\( \gamma \) secreting cells in relation to MOG 1-125 specific cells (IFN-\( \gamma \) secreting cells of DA and ACI BM transplanted rats treated on the peak of disease were different from IFN-\( \gamma \) secreting cells of naïve rats treated with BMT and IFN-\( \gamma \) secreting cells of rats without BMT, \( P=0.0029 \), ANOVA). In contrast naïve control rats and MOG 1-125 immunized rats that had not received a BM graft (no BMT) had about equal numbers of MOG 73-90 and MOG 91-108 specific cells in relation to MOG 1-125 specific cells (NS, ANOVA) (DA BM graft [n=15], ACI BM graft [n=10], no BMT [n=16], naïve DA BMT [n=5]). These results indicate that the relative increase of IFN-\( \gamma \) secreting cells in response to MOG 73-90 is directly related to BMT in context of ongoing MOG-EAE (Figure 6E).

**Encephalitogenicity of MOG 73-90 and MOG 91-108 peptides**

Next, we tested the encephalitogenicity of MOG 73-90 by immunizing groups of DA rats (each n=5) with MOG 73-90 or MOG 91-108 in CFA. Only DA rats immunized with MOG 91-108 developed disease while DA rats immunized with MOG 73-90 did not develop any signs of EAE (cumulative score day 0-18 p.i., \( P=0.0009 \), t-test, Figure 6F).
**Induction of T cells with a regulatory phenotype by BMT**

Finally, cytofluorometric analysis of CD25 expression of CD4+ T cells in LN revealed that CD4+CD25bright cells were increased in DA rats transplanted with either DA BM (n=6) or ACI BM (n=6) compared to not transplanted rats (n=8) (*P*<0.01, ANOVA) (Figure 7A,B). Also the mRNA of transcription factor *Foxp3* was increased in lymphocytes from spleen of BM transplanted rats (n=7) compared to controls (n=7) measured by quantitative real time PCR (*P*=0.05, t-test) (Figure 7C). Transfer experiments with enriched CD4+CD25+ T cells and CD4+ T cells depleted of CD25+ cells from naïve DA (n=10) into DA recipients (n=7 each) resulted in protection from EAE (*P*<0.001, t-test) (Figure 7D).

In order to find out if CD4+CD25+ regulatory T cells are also responsible for the EAE resistance of ACI rats we stained LN cells from naïve DA and ACI rats for CD4+CD25+ T cells (each n=5). DA rats had higher numbers of CD4+CD25+ T cells (5.1%±0.2%) compared to ACI rats (2.9%±0.1%, *P*<0.001, t-test). This data indicates that CD4+CD25+ regulatory T cells are not the reason for protection in naive ACI rats.
Discussion

Autologous BMT is a novel option for treatment of fast progressive patients with MS but initial clinical trials have proven inconclusive \textsuperscript{14-16}. Even though there have been a number of studies regarding BMT in EAE \textsuperscript{17-24}, our studies on BMT therapy in a well characterized animal model for MS represent a comprehensive analysis of this innovative therapy and provide a mean to investigate its characteristics in detail with regard to the timing of BMT, the source of the graft and, the mechanisms of its action. MOG induced EAE in rats is currently one of the best models for MS since it largely reproduces the immunological complexity of MS which is characterized by a combination of T cell mediated effector mechanisms, the action of autoantibodies, macrophages and NK cells in lesion development \textsuperscript{11}. In this sense MOG induced EAE in DA rats used in our studies contrasts purely T cell mediated models, e.g. T cell transfer studies, widely used for investigations on mechanisms operating in MS and BMT \textsuperscript{24,30,31}. The similarity of our model to MS is shown in histopathology where widespread demyelination and axonal loss are found \textsuperscript{8-10}. In this study we also show by histopathology large demyelinated plaques and axonal pathology with the presence of T cells, macrophages, antibodies and complement factors. Therefore the model described here can be considered to be highly relevant for the preclinical testing of novel strategies for the treatment of MS. Furthermore it might contribute to a better understanding of the pathogenesis of MS \textsuperscript{29,32-34}.

In agreement with earlier studies we demonstrate that BMT leads to disease attenuation and protects from further induced relapses with the disease inducing antigen, strongly suggesting that this therapy is a promising option for the treatment of MS in humans \textsuperscript{17-24}. For the first time we demonstrate in direct comparisons that protection can be
achieved by MHC matched allogeneic, syngeneic and BM grafts from EAE diseased DA rats. The protective effect was strongest when BM was transplanted in the acute phase of EAE. BMT was not effective in attenuating the disease course and severity when performed during the chronic phase of EAE. Similar observations have been done in a transfer EAE model and in humans\textsuperscript{16,24}. Importantly, in the acute and chronic phase of the disease BMT prevented subsequently induced relapses. For BMT in the acute phase of EAE this has been shown before\textsuperscript{18,23}. BMT in the chronic phase and subsequent demonstration of protection from relapses has not been shown before. We conclude that BMT in clinical trials should be preferentially performed at peak of disease while it is still worth considering also in later stages of disease progression as long as inflammatory activity is present.

As to the mode of action of BMT, three novel interconnected mechanisms for protection could be found: i) induction of CD4+CD25\textsuperscript{bright} regulatory T cells; and ii) an increased reactivity of T cells responsive to a non-encephalitogenic determinant, namely MOG 73-90; and iii) reduced autoantibody levels.

T cell tolerance is achieved by positive and negative selection in the thymus, peripheral induction of anergy and by the action of regulatory T cells\textsuperscript{35}. Our data using MBP 63-88 for rechallenge in MOG induced EAE suggests that the protection from EAE conferred by BMT is specific for the disease inducing antigen and not a general immunosuppressive effect. CD4+CD25+ regulatory T cells play an important role in the maintenance of immune tolerance to self\textsuperscript{36}. CD4+CD25+ cells have an antigen specific regulatory function \textit{in vivo} in EAE\textsuperscript{37}. By transfer studies we show that CD4+CD25+ cells are also protective when transferred in naïve rats. Further studies regarding the role of regulatory T cells in EAE and BMT are ongoing in our laboratory. In the present study
we demonstrate increased numbers of CD4+CD25bright T cells in rats after BMT as compared to non transplanted control rats. *Foxp3* is a transcription factor which is necessary for the function of these regulatory T cells \(^{38}\). Male scurfy mice with a mutation in *Foxp3* and *Foxp3* knockout mice develop a lethal lymphoproliferative disorder stressing the importance of this gene in immune homeostasis \(^{38,39}\). *Foxp3* acts as a T regulatory cell lineage specification factor and mediator of the genetic mechanisms of dominant tolerance \(^{40}\). We observed an increased expression of *Foxp3* in splenocytes of BM transplanted rats compared to controls. This data strongly indicates that regulatory T cells are suppressing subsequent relapses in BM transplanted rats. The potential of purified CD4+CD25+ T regulatory cells should be evaluated in multiple sclerosis in humans.

B cell tolerance is predominantly mediated by negative selection in the BM \(^{41}\). An altered homing behavior and a reduced life span of autoreactive B cells contributes to peripheral B cell tolerance. We show that BMT leads to a reduction of autoantibody levels while the relative size of the B cell compartment increases. Moreover and most interestingly also rechallenge with the autoantigen does not lead an increase in autoantibody titers in BM transplanted rats. This data underscores that long lasting B cell tolerance is induced by BMT. These findings are novel and could be very important in regard to the clinical effects of BMT. Such analysis has not been performed in EAE and in patients with MS up to now. There are strong indications that anti-MOG antibodies are of clinical importance and can be used as novel biomarkers for treatment effects \(^{42}\). Analysis of IgG isotypes did not indicate a BMT procedure mediated shift in Th1/Th2 balance \(^{43}\).

Naïve irradiated and DA rats transplanted with BM from resistant ACI rats were not protected from disease induction and also BM from resistant rats transplanted in
resistant rats did not protect from EAE. This data in connection with BMT studies in established EAE clearly shows that specific protection is only achieved in the context of ongoing inflammation and suggests that availability of the autoantigen during reconstitution of the immune system is important for subsequent protection. MOG-EAE resistant ACI rats become susceptible when irradiated and BM either ACI or DA rat origin is transplanted in a naïve state. These data reinforces the concept that autoantigen or mimicking antigens need to be present during the establishment of tolerance. Furthermore impaired homeostasis could be of great importance and lead to increased disease susceptibility in non-diseased transplanted rats. Reduction of numbers of regulatory cells could be a further factor affecting susceptibility in naïve BM transplanted rats. MOG is sequestered behind the BBB in the CNS as immunoprivileged site and not easily accessible to the immune system. The fetal and postnatal period during which orchestrated gene expression of most self antigens takes place is necessary for long lasting tolerance induction to various autoantigens. Obviously the same quality of tolerance can not be achieved after BMT in adult life. Therefore, we propose that the eradication of lymphocytes after irradiation leads to an altered thymic output in the adult rat in context with inflammation and the exposure to autoantigen which leads to the induction and production of regulatory T cells with the ability to suppress EAE. Importantly, it has to be considered in the clinical therapy that BMT, even though effective in the context of a specific autoimmune disease, could therefore result in increased susceptibility to other autoimmune diseases.

We found a reduction of the CD4 compartment in transplanted rats. This was an effect of the transplantation procedure since we measured the same reduction in rats that had been transplanted in the naïve state as compared to rats that had been transplanted in
context of EAE. It has been claimed that the reduction of the CD4 compartment could be of importance in the effect of BMT in autoimmune conditions. Our data would challenge this hypothesis, since we observed in MOG and MBP induced EAE in naïve BMT rats unaltered or increased susceptibility.

Others have analyzed the influence of BMT on the T cell repertoire and on autoantigen specific T cell responses. Recently it was shown that BMT in humans can change the TCR repertoire. In another study a transient reduction and change in MBP specific T cell responses to different MBP determinants after BMT could be demonstrated. We define for the first time that BMT leads to a change in the functional outcome of autoantigen recognition: although the response to the encephalitogenic determinant MOG 91-108 remained similar an increased response was observed to the non-encephalitogenic determinant MOG 73-90. This was substantiated by immunization with the encephalitogenic and non-encephalitogenic determinants. Our data could be of relevance for the effects of BMT in humans and warrants further studies in analyzing responses to different autoantigens.

Taken together our data clearly indicate that BMT arrests the progression of neuroinflammatory autoimmune diseases which is best achieved in an early phase of disease. Given the relevance and good comparability of the MOG induced EAE model for human MS, these data are highly promising and argue for a continuation of clinical trials using autologous BMT considering our findings on the timing of BMT therapy and the source of the BM grafts. Nevertheless, despite these optimistic results BMT should be used with caution since it may result in increased susceptibility to other autoimmune diseases. The increased size of the CD4+CD25bright population and the upregulation of Foxp3 after BMT as a treatment for ongoing EAE give important evidence for the
involvement of regulatory T cells in the process of the observed tolerance after induced relapses. The efficacy of BMT is not only due to a transient immunosuppression but as we demonstrate due to multiple interconnected mechanisms of immunomodulation.

Acknowledgements

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Figure legends

Figure 1. Chimerism and engraftment. (A) Chimerism was determined in DA rats grafted with ACI BM (n=6) and ACI rats grafted with DA BM (n=8) on day 107 post BMT from blood by a microsatellite as described in Materials and Methods. (B) EAE was induced with MBP 63-85 in the LEW/eGFP-LEW bone marrow chimeras on day 54 post BMT. The LEW/eGFP-LEW chimeric rats with EAE (n=5) showed a similar disease course compared to a not transplanted LEW rat control group (n=5). (C) GFP expressing cells in LN in naïve eGFP expressing LEW rats (pool of n=5) and LEW rats transplanted with BM from eGFP transgenic rats and subsequently induced with EAE (n=5). (D) example of the numbers of GFP expressing CNS infiltrating cells eluted from the CNS of a LEW rat with MBP induced EAE that had been BM transplanted with BM from a eGFP transgenic LEW rat.

Figure 2. BMT of naïve rats and subsequent EAE induction with MOG. (A) DA rats (n=5) without BMT develop a relapsing/remitting type of disease course after immunization with MOG 1-125. Naïve DA rats transplanted with DA BM graft (n=4) or with ACI BM graft (n=7) were immunized with MOG 1-125 on day 60 post irradiation. MOG-EAE was induced in all groups. (B) ACI rats without BMT do not develop EAE (n=4). ACI rats with DA BM graft (n=8) develop a mild relapsing/remitting disease course if induced 68 days after BMT. Engraftment of ACI BM into ACI rats (n=4) equally resulted in susceptibility to EAE.

Figure 3. BMT as a treatment for EAE. (A) DA rats were irradiated on day 17 after immunization and received BMT of either DA (n=6) or ACI (n=6) origin. Controls were
not irradiated and did not receive BMT (n=10). Differences between the BM transplanted groups compared to the control were significant (cumulative score day 19-87 p.i. \( P=0.036, \) ANOVA). Rats were reimmunized for EAE on day 90. BM transplanted rats had significantly lower disease scores upon reimmunization compared to non transplanted controls \( (P<0.0001, \) ANOVA). A second confirmatory experiment gave similar results (data not shown). (B) Rats were immunized on day 0 with MOG 1-125, transplanted on day 140 with BM of DA origin (n=9) or did not receive BMT (n=4). There was no effect of BMT at a late time point. Rats were then reimmunized on day 181 with MOG 1-125. After reimmunization only a slight exacerbation of disease was observed in the transplanted group compared to the no BMT group (cumulative score day 188-234 p.i., \( P=0.021, \) t-test).

**Figure 4. Histopathology of the transplanted rats from Figure 3A and repeated experiment.** (A-H) Characteristics of MOG induced EAE in a non BM transplanted DA rat. Large, confluent areas of demyelination and macrophage infiltration in the dorsal funiculus of a representative untreated DA rat. Macrophages containing LFB-positive myelin degradation products indicative of ongoing demyelination are shown in the inset (A; LFB-PAS staining). Infiltration by foamy macrophages/activated microglia cells shown by immunohistochemistry for ED1 (B; brown reaction product). Dense deposits of immunoglobulin (C) and C9 (D) in the lesions (plaque edge indicated by arrows). CD3-positive T cells scattered in the lesional area (E). APP-positive axonal profiles indicative of acute axonal damage (F; plaque edge indicated by arrows). Acutely damaged axons indicated by arrows (G; enlarged from (F); brown reaction product). Reduction in axonal density as demonstrated by Bielschowsky silver impregnation, plaque edge indicated by
arrows (H). I-O: DA rat immunized with MOG and transplanted with DA BM. No evidence for demyelination (I), macrophage infiltration (ED1) (K), immunoglobulin (L) and C9 deposition, (M) acute axonal damage (APP) or axonal loss in a representative immunized and treated DA rat. A, B, I-O: bar: 100µm; C, D, F, H: bar: 50µm; E, G: bar: 20µm.

Figure 5. BMT with BM from diseased rats and specificity of protection. (A) DA rats were immunized with MOG 1-125 in CFA on day 0 and boosted with MOG 1-125 in IFA on day 18. Subsequently, they received a BMT on day 36 from DA rats with EAE (n=9). Control rats received no BMT (n=9). (B) A subgroup of rats after transplantation of BM from diseased rats (n=4) and naïve rats (naïve BMT n=5) were immunized with MOG 1-125. Naïve rats developed EAE while rats that had received BM from diseased rats did not relapse. (C) DA rats after BMT (DA BM from diseased rats n=4) and rats that were naïve at the time of transplantation (naïve BMT n=4) were immunized with MBP 63-88 on day 79. Both groups developed EAE after immunization with MBP 63-88 indicating specificity of BMT induced tolerance.

Figure 6. Immunological changes in BM transplanted rats. (A) Anti-MOG IgG titers in different immunization and transplantation settings. BMT leads to strong reduction of autoantibody titers which persist also after secondary challenge (time point day 0: naïve DA rats n=4; time point day 15: EAE without BMT n=6; time point day 123: DA BM n=8, ACI BM n=6, no BM n=8; time point day 154: DA BM n=6, ACI BM n=5, no BM n=6). EAE lead to an increase in anti-MOG antibody titers (P<0.002). On day 123 and day 154 BM transplanted groups should strongly reduced autoantibody titers compared to non
transplanted rats (each $P<0.0001$). Rats that had been transplanted as naïve animals (day 23 p.i.) and been subsequently induced with EAE did not show a reduction of autoantibodies compared to not transplanted controls (day 15 p.i.) (NS). (B) Reduction of anti-MOG 1-125 IgG, IgG1, IgG2a and IgG2c levels in both transplanted groups at day 115 p.i. (day 98 post BMT, DA BM graft [n=6], ACI BM graft [n=5]) compared to the non transplanted group (n=6) (each $P<0.0001$, ANOVA). No significant differences for IgG2b between the DA/ACI BM treatment groups were observed. Only the non transplanted group had higher levels of IgG2b and IgG2c antibodies ($P<0.0001$, ANOVA). (C) Lymphocytes isolated from draining lymph nodes of rats were analysed by FACS (DA BM graft [n=5], ACI BM graft [n=6], naïve BMT [n=5], no BMT [n=6]). The relative size of the CD4+ T cell compartment was reduced in BM transplanted rats if compared to controls, while the relative size of the B cell compartment was enlarged compared to controls. The differences between the non transplanted group and the transplanted groups were significant in the T cell (CD4 and CD8) and B cell compartment (ANOVA, for all $P<0.001$). (D) DA BM and ACI BM grafted rats at the height of EAE had an increase in numbers of MOG 73-90 specific IFN-γ secreting cells related to numbers of MOG 1-125 specific IFN-γ secreting cells compared to the other investigated groups ($P=0.0029$, ANOVA) (DA BM [n=15], ACI BM [n=10], no BMT [n=16], naïve DA BMT [n=5]). In contrast the numbers of MOG 91-108 specific IFN-γ secreting cells in relation to MOG 1-125 specific IFN-γ secreting cells did not differ between the groups (NS, ANOVA). (E) Only DA rats immunized with MOG 91-108 (n=5) developed EAE but not DA rats immunized with MOG 73-90 (n=5) (cumulative score day 0-18 p.i., $P=0.0009$, t-test).
Figure 7. Induction of CD4+CD25bright regulatory T cells in BM transplanted rats.

(A) Representative FACS blots of DA rats without BMT and with BMT with DA or ACI BM. (B) CD4+CD25bright cells in LN of DA rats without BMT and with BMT (DA rats without BMT [n=8], DA rats after BMT with BM from DA rats [n=6] or ACI [n=6] rats). There were differences between the non transplanted and transplanted groups (P<0.01, ANOVA). (C) Foxp3 expression in spleen cells from DA rats without BMT (n=7) and with BMT with syngeneic BM (n=7). There were differences between the non transplanted and the transplanted group (P=0.05, t-test). (D) Transfer of CD4+CD25+ T cells results in protection from MOG induced EAE in DA rats as compared to rats transferred with CD4+ T cells depleted of CD25+ cells (n=7 each group, P<0.001, t-test). Repeated experiments showed similar results (n=3 each group). * indicates significance.
Table 1. Blood cell parameters in BM transplanted and non-transplanted rats

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<td>DA: DA BM (n=8)</td>
<td>8.9±0.3</td>
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<td>8.2±0.1</td>
<td>14.2±0.1</td>
<td>40.5±0.3</td>
<td>775.9±17.8</td>
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Figure 1
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Tolerance induction by bone marrow transplantation in a multiple sclerosis model

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