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ORIGINAL ARTICLE

Disease Progression After Bone Marrow Transplantation in a Model of Multiple Sclerosis Is Associated With Chronic Microglial and Glial Progenitor Response

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Abstract

Multiple sclerosis (MS), the most common nontraumatic cause of neurologic disability in young adults in economically developed countries, is characterized by inflammation, gliosis, demyelination, and neuronal degeneration in the CNS. Bone marrow transplantation (BMT) can suppress inflammatory disease in a majority of patients with MS but retards clinical progression only in patients treated in the early stages of the disease. Here, we applied BMT in a mouse model of neuroinflammation, experimental autoimmune encephalomyelitis (EAE), and investigated the kinetics of reconstitution of the immune system in the periphery and in the CNS using bone marrow cells isolated from syngeneic donors constitutively expressing green fluorescent protein. This approach allowed us to dissect the contribution of donor cells to the turnover of resident microglia and to the pathogenesis of observed disease relapses after BMT. BMT effectively blocked or delayed EAE development when mice were treated early in the course of the disease but was without effect in mice with chronic disease. We found that there is minimal overall replacement of host microglia with donor cells in the CNS and that newly transplanted cells do not appear to contribute to disease progression. In contrast, EAE relapses are accompanied by the robust activation of endogenous microglial and macroglial cells, which further involves the maturation of endogenous Olig2 glial progenitor cells into reactive astrocytes through the cytoplasmic translocation of Olig2 and the expression of CD44 on the cellular membrane. The observed maturation of large numbers of reactive astrocytes from glial progenitors and the chronic activation of host microglial cells have relevance for our understanding of the resident glial response to inflammatory injury in the CNS. Our data indicate that reactivation of a local inflammatory process after BMT is sustained predominantly by endogenous microglia/macrophages.

Key Words: Autoimmune disease, Bone marrow transplantation, CD44, Glial progenitor, Microglia, Olig2, Reactive astrocyte.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory and degenerative CNS disease, which in some patients also presents with signs of increased nervous system vulnerability or compromised repair. Based on the pathology of MS lesions, on altered immune responses in patients, and on experimental animal models of disease, the inflammatory component of MS pathogenesis is believed to have a T cell-mediated autoimmune origin. Immunosuppressive and modulatory treatments have shown partial efficacy in ameliorating the course of MS. Among experimental therapies that are currently being investigated, the aim of bone marrow transplantation (BMT) or hematopoietic stem cell transplantation (HSCT) is to achieve a radical depletion of pathogenic T cells followed by reconstitution of a new and tolerant immune system from multipotent progenitor cells. Results of clinical trials in MS have suggested that BMT/HSCT is effective in suppressing inflammatory disease activity in a large proportion of patients (1, 2). In the patients in the early stage of disease, suppression of inflammation is associated with clinical stabilization (3, 4). In contrast, most patients with advanced disability continue to show disease progression clinically even in the absence of

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inflammatory flares and magnetic resonance imaging evidence of focal blood-brain barrier disruption (5, 6).

The reasons for the persistence of clinical progression in patients undergoing HSCT are unclear. Two nonmutually exclusive hypotheses can be formulated: 1) clinical progression is unrelated to new CNS inflammation but rather depends on neuronal and/or oligodendroglial degeneration occurring in later disease stages; and 2) HSCT regenerates a tolerant peripheral immune system resolving blood-brain barrier disruption and influx of inflammatory cells but does not replace and/or suppress the pathologic activation of resident CNS cells, such as microglia and reactive astrocytes.

Recent evidence indicates that brain microglial cells can arise from circulating mononuclear cells (7–10); however, it is not clear whether, how extensively, and at what rate blood-derived cells can replace resident CNS microglia under normal circumstances, in autoimmune inflammatory conditions, and after treatment with HSCT. These answers are difficult to ascertain in humans because autologous progenitor cells are used to reconstitute hematopoiesis, and current technology does not allow us to distinguish newly transplanted cells from preexisting ones in vivo without potentially risky manipulations and invasive histopathology. To address these questions in an experimental setting we used BMT in a mouse model of neuroinflammation, myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$-induced experimental autoimmune encephalomyelitis (EAE). This disease is characterized by a fast progressive clinical course, initiated by inflammatory infiltrates of antigen-specific T lymphocytes and subsequent microglial activation (11–13). Similarly to MS, this experimental disease presents reactive gliosis, demyelination, axonal loss, and paralysis.

To investigate the kinetics of reconstitution of the immune system in the periphery and in the CNS in EAE, we used syngenic bone marrow cells isolated from transgenic mice constitutively expressing green fluorescent protein (GFP) as donors. This approach allowed us to dissect the contribution of bone marrow (BM)-derived cells to the turnover of resident microglia and to the pathogenesis of observed EAE relapses after BMT. Furthermore, because prior studies have suggested that inflammation has effects on both glial scarring and remyelination, we analyzed in parallel the response of glial progenitor cells in EAE spinal cord lesions after clinical relapse. The transcription factor Olig2 is expressed by adult glial progenitors and has been identified as a crucial marker of commitment to the oligodendroglial lineage (14, 15). Surprisingly, we found that $Olig2^+$ glial progenitors differentiate into reactive astrocytes in the spinal cord lesions. The observed maturation of large numbers of reactive astrocytes from progenitors and the chronic activation of microglial cells have relevance for our understanding of the resident glial response to inflammatory injury in the CNS.

MATERIALS AND METHODS

Mouse Strains and Induction of Experimental Allergic Encephalomyelitis

All animal procedures were carried out in accordance with the Animal Care Users Committee guidelines of the National Institutes of Health. For induction of active MOG_{35-55}-induced EAE, 6- to 8-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were immunized on 4 spots on the back with 200 μl of PBS containing 200 μg of an emulsion of equal volumes of murine MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK; purity >95%; Stanford Pan Facility, Stanford, CA) supplemented with *Mycobacterium tuberculosis* H37Ra (2 mg/ml; BD/Difco, Franklin Lakes, NJ). Immunized animals were administered 200 μg of pertussis toxin (Sigma-Aldrich, St. Louis, MO) on the day of immunization and on day 2 postinduction. EAE developed after approximately 12 days and was scored on the basis of a 5-point EAE scale (0, disease-free; 0.5, partial limp tail; 1, limp tail; 1.5, uneven gait; 2, hindlimb paresis; 2.5, severe hindlimb paresis; 3, hindlimb paralysis; 3.5, severe hindlimb paralysis; 4, hindlimb paralysis and forelimb paresis; 4.5, quadriparalysis; and 5, moribund or dead). Mice that were grade 4 for more than 24 hours were killed.

Irradiation and Bone Marrow Transplantation

Mice underwent myeloablative total body irradiation (TBI) with 900 rad (2 × 450 rad, 6 hours apart) and were immediately reconstituted with 3×10^6 BM cells isolated from C57BL/6-*Tg*(ACTB-EGFP)1Osb/J transgenic donors (The Jackson Laboratory). Nonirradiated EAE and control mice were given transplants as well, whereas irradiated nontransplanted control mice died within 12 days.

Tissue Processing

Animals were killed by CO_2 inhalation and then perfused transcardially with cold PBS followed by 4% paraformaldehyde. The brains and cords were removed and postfixed for 3 hours and then transferred to a 20% sucrose solution in PBS for cryoprotection. Samples were embedded in OCT compound (Tissue-Tek; VWR Scientific, Bridgeport, NJ) and snap-frozen. Spinal cords were cut into 6 sections and embedded to allow parallel analysis at all levels of the cord, whereas brain samples were cut sagittally along the midline. Five- to 10-μm-thick sections were cut on a freezing microtome and stored at −80°C for immunofluorescent analysis. Before embedding in OCT, small amounts of tissue were removed, and paraffin sections were prepared for standard hematoxylin and eosin and Luxol fast blue histochemistry.

Immunofluorescent Analysis

Tissue sections or cell preparations were washed in PBS, blocked in 10% goat or donkey serum (Sigma-Aldrich) for 1 hour at room temperature followed by primary antibody incubation overnight at 4°C or for 3 hours at room temperature, followed by incubation with the specific secondary antibodies (Molecular Probes/Invitrogen, Carlsbad, CA) for 30 minutes. All antibodies were diluted in blocking buffer containing 5% serum diluted in 0.03% Triton X-100 PBS solution: we used combinations of antibodies against NG2 (rabbit IgG or mIgG1, both from Chemicon, Temecula, CA; 1:200), A2B5 (mIgM, 1:20; American Type Culture Collection, Manassas, VA), Olig2 (rabbit IgG, 1:100, IBL, Gunma, Japan; mIgG2b, 1:50, Invitrogen; or rabbit IgG,

1:400, Chemicon), platelet-derived growth factor receptor (PDGFR)-α (mIgG1, 1:200; BD Pharmingen, Franklin Lakes, NJ), glial fibrillary acidic protein (GFAP) (rabbit IgG, 1:4000, Dako, Carpinteria, CA; mIgG2a, 1:50, BD Pharmingen; or chicken IgY, 1:500, Chemicon), Nkx2.2 (rabbit IgG, 1:400; Chemicon), Rip (mIgG1, 1:1000; Chemicon), neurofilament 150-kDa (rabbit IgG, 1:400; Chemicon), CD44s (mIgG2b, 1:200; Chemicon), CD3 (rat IgG, 1:100; Serotec, Raleigh, NC), ionized calcium-binding adaptor molecule-1 (Iba-1) (rabbit IgG, Wako Chemical, Richmond, VA), and anti-GFP (chicken IgG, 1:500; Molecular Probes/Invitrogen), followed by specific fluorochrome-conjugated secondary antibodies (1:1000, Molecular Probes). For double stainings, we blocked with a background-reducing protein block (Dako) and used combinations of antibodies raised in different species. Negative control stainings did not show unspecific staining. Fluorescence images were acquired on an inverted microscope (Axiovert 200; Zeiss, Thornwood, NY) using the hardware and software provided (Axiovision, Zeiss) and fixed exposure times for comparisons.

Changes in tissue structure and intensity of the fluorescent signals were graded independently by 2 investigators at 3 different levels along the EAE spinal cord (cervical, thoracic, and caudal portions) by assigning a score between 0 (no change compared with control) and 4+ (severe change); sagittal and coronal sections of the brain cut along the midline were included in the analysis. The control mice (healthy BMT) were used as reference in the comparisons.

For Olig2 and Iba-1 counts, we imaged 4 to 6 representative fields (250 μm × 250 μm) in the dorsal funiculi in 2 to 3 parallel sections per animal of the cervical cord and used Volocity cell counting software (Improvision Software, Lexington, MA) for quantification.

Fluorescence Activated Cell Sorting Analysis

Mice were bled retro-orbitally, and red blood cells were lysed; immunostaining was performed on fresh peripheral blood mononuclear cells using antibodies against CD4, CD8, CD11-b, and CD19 (all from BD Biosciences) according to standard methods. Peripheral blood mononuclear cells from a GFP-transgenic donor were used for positive gating. Cellquest software (BD Biosciences) was used for acquisition and analysis of the data.

Proliferation Assay

Proliferation assays to the immunizing antigen were performed according to standard procedures. Spleen cells were isolated at T1, red blood cells were lysed, and 4×10^5 spleen cells were seeded per well and tested against a titration of MOG_{35-55} in RPMI 1640 medium supplemented with antibiotics, l-glutamine, and fetal bovine serum. Cells were incubated at 37°C for 72 hours and 1 mCi of [^{3}H]thymidine (DuPont, Wilmington, DE) was added to each well for the last 12 hours of culture. Thymidine incorporation was measured on a Wallac Microbeta Trilux (Wellesley, MA).

Statistical Analysis

To evaluate statistical differences of EAE scores between outcome groups we used an unpaired *t*-test after confirming normality of distribution and equal variance. In histopathologic assessments to perform multiple independent comparisons on cell counts between groups, we used the nonparametric Kruskal-Wallis 1-way analysis of variance by ranks and Dunn's post hoc test. For all tests the level of significance was set at $p < 0.05$.

RESULTS

Bone Marrow Transplantation Does Not Ameliorate Chronic Experimental Autoimmune Encephalomyelitis

Previous studies of EAE suggested that the timing of BMT after disease onset critically affects the clinical outcome (16–20). Because HSCT has only been investigated as a therapy for severe and long-standing MS, we first asked whether BMT could influence the clinical course when performed in the late phase of disease in our EAE model.

We immunized wild-type C57/BL6 mice to induce active EAE, which is characterized by the development of a severe ascending paralysis due to inflammatory lesions developing mainly in the dorsal funiculi of the cervical spinal cord. Forty days after disease onset, EAE mice were divided into 2 separate groups: a treatment group that received TBI followed by reconstitution with 3×10^6 BM cells intravenously (referred to as "EAE BMT" group; n = 6), and an EAE control group that did not undergo TBI, but only received an injection of BM cells ("EAE" group; n = 5). A third group consisted of healthy mice that received TBI and BMT ("BMT CTRL"; n = 4). Immunoablative radiotherapy followed by BMT did not alter the clinical course of chronic EAE, as there were no differences in clinical scores between the control group (black) and the EAE BMT group (red) (Fig. 1A). This result suggested that neurologic impairment at this chronic stage of EAE was not primarily due to ongoing inflammation. Histopathologic analysis conducted 8 months after transplantation (day 292 after disease induction) did not reveal significant differences in terms of myelin and neuronal damage and gliosis between EAE animals that had or had not been irradiated (data not shown).

Minimal Microglial Turnover After Bone Marrow Transplantation

Microglia have been shown to arise from circulating monocytes; however, it is unknown to what extent BMT replaces/renews the existing pool of CNS resident microglia in EAE. To track the long-term fate of the transplanted cells in the CNS, we reconstituted animals with BM cells isolated from transgenic animals ubiquitously expressing GFP. Immunohistochemical analysis for GFP was then performed in the spinal cords of all mice 8 months after BMT. Overall, the number of donor-derived cells in the spinal cord parenchyma was very low in all animals, despite a complete reconstitution of the peripheral blood compartment as shown by flow cytometry analysis of blood samples from the same

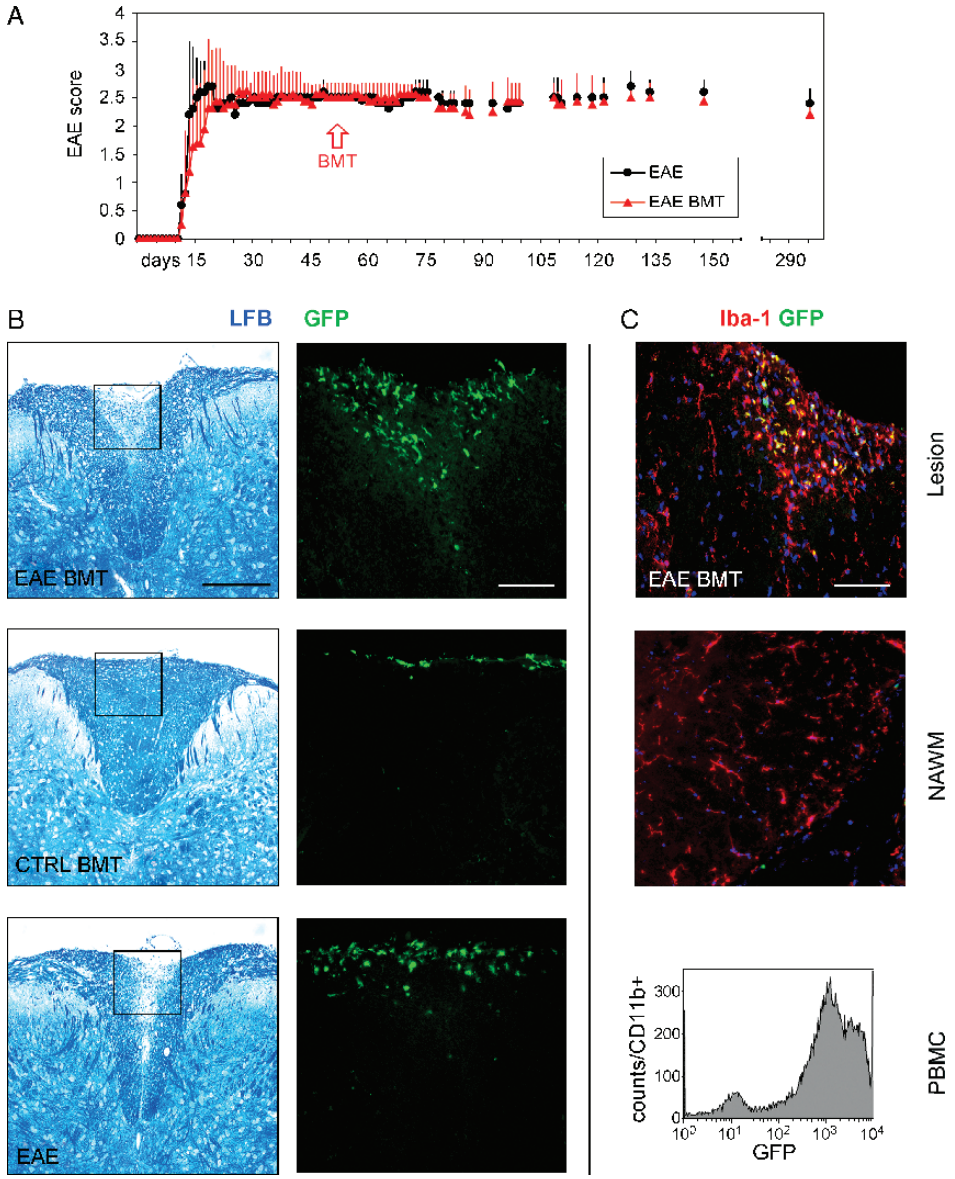


FIGURE 1. Bone marrow transplantation (BMT) in the chronic phase of experimental autoimmune encephalomyelitis (EAE). To test the effects of BMT in the late phase of MOG_{35-55}-induced EAE, 40 days after disease initiation, 1 group of EAE mice received lethal body irradiation and were reconstituted with BM cells isolated from green fluorescent protein (GFP)-expressing transgenic donors (EAE BMT, red curve; n = 6), whereas a second group received only BM cells (EAE CTRL, black curve; n = 5). A third group of healthy mice were irradiated and also received a transplant (BMT CTRL, not shown; n = 3). The graph summarizes the clinical scores for the EAE groups **(A)**, showing that BMT in the chronic stage does not ameliorate disease. To investigate the extent to which transplanted BM cells repopulate the spinal cord tissue, immunostaining for GFP was performed in EAE animals and controls 8 months after transplantation **(B)**. Representative images of the dorsal funiculi are shown for each group; Luxol fast blue (LFB) staining was used to detect myelin pathology: (top) a demyelinated lesion in EAE BMT animals shows numerous GFP^+ cells exclusively within this area (black square). In BMT CTRL animals (center), which showed no signs of demyelination, GFP^+ cells were only located within the meninges. Numerous GFP^+ cells were also detectable in lesions of nonirradiated EAE CTRL mice (bottom). **(C)** Virtually all GFP^+ cells in the lesion site coexpress Iba-1 (red), a marker of macrophage/microglial cells. In contrast, the normal appearing white matter (NAWM) in the lateral funiculi of the same section shows no signs of engraftment, despite the complete reconstitution by GFP^+ cells of the CD11-b-expressing peripheral blood mononuclear cell (PBMC) compartment in this animal at this time point. Scale bars = **(B, C)** 200 μm for LFB; 50 μm for GFP.

mice. In the spinal cord from EAE BMT animals, GFP^+ cells were detected virtually exclusively within the demyelinated lesions in the dorsal funiculi (Fig. 1B), whereas no cells were detected in the surrounding normal appearing tissue. Outside of the lesion area, the majority of GFP^+ cells were localized within the spinal cord meninges; this finding was

similar to what we observed in BMT CTRL mice, in which donor-derived cells were frequent in the meninges, but only single (1–10 cells per section) resting microglial cells were present in the tissue at this time point. Interestingly, we found GFP^+ cells in the nonirradiated EAE group, indicating that upon intravenous injection BM cells can home to the inflamed sites and then survive there for a prolonged period of time.

To characterize the cellular phenotype of GFP^+ cells in the cord, we performed double labeling with lineage-specific markers. Figure 1C shows a representative section in which the monocyte/microglial marker Iba-1 (21) was used to detect cells of this lineage. More than 95% of all GFP^+ cells inside the lesions and in the meninges were positive for this marker (top image). Less than 5% of GFP^+ cells colabeled with the T cell marker CD3 and none with glial-lineage markers such as GFAP, NG2, or Olig2 in any of the animals analyzed (data not shown). There was little donor engraftment (0%–5% of all Iba-1^+ cells/field) in healthy areas of the cord (center image), despite the complete reconstitution of the blood compartment (80%–95%) by donor-derived CD11-b^+ monocytes (histogram plot).

Together, these data demonstrated that transplanted BM-derived cells almost exclusively colonize areas of previous damage in the EAE spinal cord, where they display a monocyte/microglial phenotype. Although complete reconstitution of the peripheral immune compartment can be achieved with BMT, the exchange between blood and brain is limited and appears to be predominantly confined to lesion sites.

Greater Clinical Severity at the Time of Bone Marrow Transplantation is Associated With Experimental Autoimmune Encephalomyelitis Relapse

Next, we addressed whether BMT during in the initial phase of EAE development is clinically beneficial and affects the extent of donor cell engraftment in the CNS. We followed the same protocol of EAE induction but applied BMT before the peak of clinical symptoms. Two distinct patterns of clinical response emerged after treatment (Fig. 2A). One group of mice entered complete stable remission after BMT (hence referred to as EAE BMT remitting [REM]; open circles; n = 9). A second group showed a partial remission of the EAE symptoms, but symptoms then progressed 1 month after BMT (EAE BMT progressing [PRO]; filled circles; n = 10). These results indicated that BMT could completely arrest disease development in approximately 50% of the animals, yet the reasons for the different outcome remained unclear. Of note, the remitting versus progressing mice had significantly different mean clinical scores at the day of BMT (mean ± SEM 0.61 ± 0.07 vs 1.2 ± 0.25, respectively; $p = 0.045$; *t*-test).

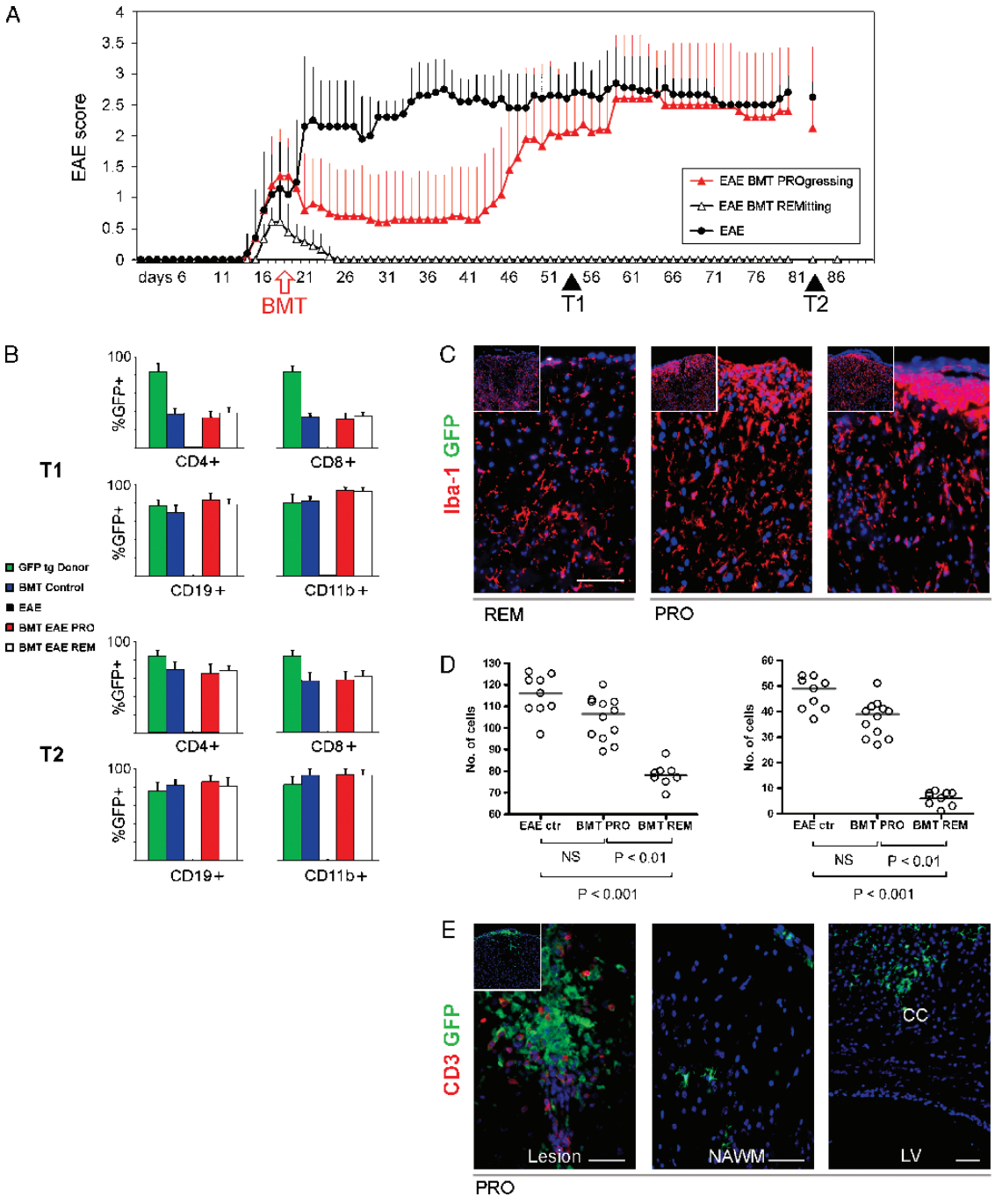
To investigate the mechanism underlying the different clinical courses, we first considered the possibility that relapses in PRO mice could be related to incomplete immune ablation and/or inefficient reconstitution by donor BM cells. We therefore analyzed the proportions of GFP^+ cells in the major subsets of peripheral blood leukocytes. In all irradiated BMT mice at 5 weeks post-transplant (T1), the proportion of GFP^+ cells in the $CD4^+$ and in the $CD8^+$ T cell subset was approximately half of that in the transgenic BM donors (Fig. 2B, T1). In contrast, the populations of $CD19^+$ cells, representing the B cell compartment, and of cells positive for CD11-b (Mac-1), a marker expressed by myeloid and natural killer cells, were entirely reconstituted by donor-derived cells. The proportions of GFP^+ cells were very similar in control mice (no EAE) that received BMT and in EAE mice. Importantly, when comparing the frequencies of GFP^+ cells in each leukocyte subset between the REM and PRO groups, there were no significant differences. The same analysis repeated at 9 weeks after BMT showed a more complete donor reconstitution of the $CD4^+$ and $CD8^+$ T subset and the persistence of complete donor chimerism of the $CD19^+$ and $CD11b^+$ populations (Fig. 2B). At both time points, analysis of phenotypic markers relevant to memory/activation and homing (CD44 and CD62L) for $CD4^+$ and $CD8^+$ T cells showed no differences between REM and PRO mice (data not shown).

Because we observed no differences between the frequencies of host versus donor-derived T cell subsets in the progressing versus remitting groups, the different clinical course could not be explained by crude differences in the efficiency of BMT nor in the kinetics of donor engraftment. To address whether EAE relapses could be mediated by the post-BMT persistence of greater numbers of memory or activated MOG-reactive host T cells (because such differences may have not been detectable in the analysis of GFP expression in T cell subsets due to the low frequency of autoreactive T cells), we compared the T cell proliferation in response to the immunizing antigen in mice that relapsed or did not relapse after BMT. Although a strong proliferation to MOG was seen in control EAE mice that had not received BMT, the response in BMT-treated EAE mice was low and not significantly different between REM and PRO mice (Supplemental Fig. 1). These results suggested that, rather than resulting from a differential reconstitution of the peripheral immune system, the different clinical outcomes may be related to events within the CNS.

To characterize the pathologic changes in the spinal cord parenchyma, we performed a qualitative histopathologic evaluation of the tissue using a blinded protocol approximately 10 days after clinical progression (T1). We graded the extent of tissue damage in representative animals from all treatment groups (n = 3/group/time point). We used hematoxylin and eosin, Luxol fast blue, and neurofilament staining to assess the degree of inflammatory infiltration, demyelination, and axonal pathology, respectively. Antibodies against CD3 and Iba-1 were used in parallel to characterize T cell infiltration and microglial activation. Anti-GFP was used to reliably identify transplanted cells. The results of this analysis are summarized in the Table and in Figure 2 and showed that, as expected from the different clinical courses, the severity of pathologic changes was lower in REM mice as opposed to PRO animals. The surprising paucity of lymphocytic infiltrates suggested that T cells were not primarily responsible for the observed progression in BMT-treated animals. Importantly, only

sparse GFP$^+$ cells were detected in some lesions or in the meninges throughout the CNS at 5 weeks post-BMT; this observation did not support the possibility that transplant-derived immune cells were pathogenetic in the CNS. In contrast, high numbers of activated Iba-1$^+$ microglia were found throughout the dorsal funiculi (Fig. 2C), emphasizing the high degree of CNS-resident immune activation in PRO mice. A quantitative comparison between PRO and REM mice demonstrated that both the total number of Iba-1$^+$ cells present in the dorsal funiculi and the number of microglial cells with an activated morphology were significantly elevated in PRO mice at T1 (Fig. 2D). The median numbers of Iba-1$^+$ cells were 106.5 cells/mm^2 (interquartile range 96–112) in the PRO group versus 78 cells/mm^2 (76–80) in the REM mice ($p < 0.01$); the median numbers of activated microglia were 39 cells/mm^2 (30.5–41.5) versus 7 cells/mm^2 (3.5–8), respectively ($p < 0.01$).

Based on the complete reconstitution of the peripheral pool of macrophage/monocytes and the robust activation of microglia in the spinal cord parenchyma, it was notable to find



a paucity of GFP^+ cells in the CNS at T1. Instead, donor cells were found in high numbers in the meninges and in the dorsal funiculi in all PRO animals 1 month later (T2, 9 weeks post-BMT; Fig. 2E). Phenotypic analysis revealed that GFP^+ cells were almost exclusively constituted by Iba-1^+ macrophages/microglia and $CD3^+$ lymphocytes constituted only a minority (<5%) of infiltrating blood cells in all lesions. A few Iba-1^+/GFP^+ cells per section were also present in the unaffected spinal cord parenchyma at this time point.

Taken together, these results demonstrated the quick and complete reconstitution of the peripheral compartment by newly transplanted cells, compared with the slow and limited turnover in the CNS and identified endogenous macrophage/microglia cells as primary effectors in disease progression.

Maturation of Glial Progenitors Into Reactive Astrocytes in White Matter Experimental Autoimmune Encephalomyelitis Lesions

Because local effects within CNS lesions appeared to have the strongest influence on disease progression, we investigated the participation of additional brain cells in this process. The expression of the glial markers NG2 and GFAP was increased in the dorsal portion of the funiculi of nonirradiated EAE mice and in PRO animals compared with REM mice (Table), consistent with the prolonged reactive gliosis that accompanies this EAE model.

To further define the intrinsic glial progenitor response occurring with relapses we analyzed the expression pattern of the transcription factor Olig2, which is a reliable marker for the identification of cells participating in the response to injury (22–24). In the healthy CNS, Olig2 is located in the nucleus of all resting glial progenitors and oligodendrocytes but is not expressed in mature astrocytes. In response to stimulation, however, glial progenitors translocate this factor to the cytoplasm at the time of differentiation into GFAP-expressing cells (25, 26). We previously found that nuclear export of Olig2 concomitant with GFAP expression is also a hallmark of glial progenitor activation in the EAE white matter (24). Therefore, we analyzed this process in PRO animals after BMT by immunofluorescent staining for GFAP and S-100 (as astrocytic markers), and Rip (an oligodendrocyte marker), in combination with Olig2, Nkx2.2, and PDGFR-α to characterize glial progenitor cells.

Figure 3A shows representative data from 1 of 2 animals that initially recovered completely from EAE symptoms after BMT (from grade 1.5 to 0), but had severely relapses 1 month later (grade 3.5). Parallel staining for Iba-1 and GFP at T1 confirmed the increased Iba-1 expression in the dorsal portion of the funiculi; however, no transplant-derived GFP^+ cells were detected in the lesion area or in the meninges. Reactive gliosis was pronounced at these sites, as shown by detection of pronounced GFAP signal. Compared with healthy control mice, the number of $Olig2^+$ (Rip^-) progenitor cells was doubled in the lesion area (Fig. 3B), although the number of oligodendrocytes ($Olig2^+$/Rip^+ cells) was not significantly affected shortly after relapse (data not shown). We analyzed the subcellular localization of Olig2 (Fig. 3B–D) and found that the majority of $Olig2^+$ progenitors in the lesion core showed both nuclear and cytoplasmic distribution of this factor, and these cells also coexpressed GFAP or S-100 (but not Rip). Moreover, we detected Olig2 translocation to the cytoplasm in conjunction with PDGFR-α or Nkx2.2 expression, further confirming that reactive $Olig2^+$ cells were glial progenitors. Of note,

FIGURE 2. Bone marrow transplantation (BMT) in the early phase of experimental autoimmune encephalomyelitis (EAE). BMT was performed in a group of EAE mice before peak of clinical symptoms (day 18). A second group of healthy mice were also transplanted, whereas a third group of nonirradiated EAE mice received only bone marrow (BM) cells. We observed 2 clearly distinct outcomes of treatment, which were related to the severity of clinical signs at the time of BMT. Mice with milder disease scores entered prolonged remission after BMT, whereas more severely affected mice only showed partial remission and their disease subsequently progressed. **(A)** Summary of the clinical scores for the different EAE groups, showing that BMT induces complete clinical remission in animals with lower disease severity (EAE remitting, open curve; n = 9); in contrast, animals with higher disease scores progressed within 1 month from BMT (BMT progressing, red curve; n = 10), despite an initial, partial disease remission, compared with untreated mice (EAE, black curve; n = 10). Mean ± SEM clinical scores at BMT in remitting versus progressing mice were 0.61 ± 0.07 versus 1.2 ± 0.25, p = 0.045. **(B)** Peripheral immune reconstitution by green fluorescent protein $(GFP)^+$ cells was analyzed by fluorescence-activated cell sorting at 5 weeks (T1) and 9 weeks (T2) after BMT. Each bar represents the percentage of GFP^+ cells among all circulating CD4 and CD8 T lymphocytes, $CD19^+$ B cells, and CD11-b^+ monocytes in each group. Peripheral blood mononuclear cells from GFP-transgenic BM donors (green; n = 2/time point) were used as positive controls for gating. At T1, among $CD4^+$ and $CD8^+$ T cells, less than 40% expressed GFP in the blood of all transplanted groups: healthy controls (BMT Control, blue; n = 3), progressing animals (EAE PRO, red; n = 7), and remitting animals (EAE REM, white; n = 6). In contrast, the engraftment among CD19 and CD11-b cells was complete. No GFP signal was detected in nonirradiated EAE mice (EAE, black; n = 5). At T2 (n = 4/group), GFP^+ cells represented about 70% of the CD4 lymphocytes and 60% of all CD8 cells, whereas all circulating CD19 and CD11-b cells were GFP^+. Staining for GFP and Iba-1 in the dorsal funiculi of EAE PRO mice at T1 shows increased expression of Iba-1 compared with REM animals **(C)**; insets show the same areas at lower magnification. No transplanted GFP^+ cells are detected within the lesions at this time. **(D)** The total numbers of Iba-1^+ cells/mm^2 (left graph) and of Iba-1^+ cells/mm^2 with an activated morphology (right graph) are significantly elevated in the cervical spinal cord in BMT PRO mice and EAE controls compared with BMT REM mice at T1 (3 consecutive sections with 3 to 4 animals per treatment group). **(E)** Staining for GFP and CD3 in a progressing mouse at T2 shows numerous donor cells within the spinal cord lesion (left image), in the normal appearing white matter (NAWM) of the lateral funiculi (center), and in the periventricular corpus callosum (CC) (right). LV, lateral ventricle. Scale bars = **(C, E)** 50 μm.

TABLE. Neuropathologic Evaluation of Brain and Spinal Cord in EAE BMT Groups

EAE Group	CNS Area	Tissue Damage (H&E)	Myelin Pathology (LFB)	Axonal Pathology (NF)	Microglial Activation (Iba-1)	T Cell Infiltrates (CD3)	Astrogliosis (GFAP)	Reactive Gliosis (NG2)	Donor Cells (GFP)
BMT PRO Mouse #1	Brain	+	−	−	+	−	−	−	−
	Cerv SC	+++	+	+	+++	++	+	+	−
	Thor SC	−	−	−	+	−	++	−	−
	Lum SC	−	−	−	−	−	−	−	−
BMT PRO Mouse #2	Brain	−	−	−	−	−	−	−	−
	Cerv SC	++	++	+	+	−	+	+	+
	Thor SC	−	++	+	+	−	++	+	−
	Lum SC	−	−	−	−	−	−	+	−
BMT PRO Mouse #3	Brain	+	−	−	+	−	+	−	−
	Cerv SC	+	+	++	++	+	++	++	−
	Thor SC	−	−	++	++	−	+	+	−
	Lum SC	+	−	−	−	−	−	−	−
BMT REM Mouse #1	Brain	−	−	−	−	−	−	−	−
	Cerv SC	−	−	−	+	−	−	−	−
	Thor SC	−	−	−	−	−	−	+	−
	Lum SC	−	−	−	−	−	−	−	−
BMT REM Mouse #2	Brain	−	−	−	−	−	−	−	+
	Cerv SC	−	−	+	−	+	−	−	−
	Thor SC	+	−	−	−	−	−	—	−
	Lum SC	−	−	−	−	−	−	−	−
BMT REM Mouse #3	Brain	−	−	−	−	−	−	−	+
	Cerv SC	−	−	−	+	−	−	−	−
	Thor SC	−	−	−	−	−	−	−	−
	Lum SC	−	−	−	−	−	−	−	−
EAE Ctrl Mouse #1	Brain	+	−	−	+	−	−	−	−
	Cerv SC	++	++	++	+	−	++	+	−
	Thor SC	++	+	++	+	−	+	+	−
	Lum SC	−	−	+	−	−	−	—	−
EAE Ctrl Mouse #2	Brain	+	−	−	−	+	−	−	−
	Cerv SC	−	+	++	+	−	++	++	−
	Thor SC	++	+	−	+	−	−	++	−
	Lum SC	−	−	−	−	−	−	−	−
EAE Ctrl Mouse #3	Brain	+	−	+	+	+	−	−	+
	Cerv SC	+++	+++	+++	++	+	+++	+	+
	Thor SC	−	+	++	+++	−	+	−	−
	Lum SC	+	−	+	+	−	−	+	−

Neuropathologic evaluation at T1 of the brain and of the cervical (Cerv), thoracic (Thor), and lumbar (Lum) spinal cord (SC) in the different experimental autoimmune encephalomyelitis (EAE) bone marrow transplantation (BMT) groups (remitting [REM], progressing [PRO], and EAE control [Ctrl]). Pathologic changes in the tissue structure and/or in the intensity of staining for the indicated markers were evaluated and compared to healthy BMT controls. The degree of change was scored from + (mild) to ++++ (severe); − indicates not changed, whereas — indicates reduced expression compared to BMT control mice. This analysis confirms that remission of EAE symptoms is accompanied with a normal histology in REM mice. In contrast, PRO mice show abundant gliosis, myelin damage, and neuronal swelling despite to a lesser degree than untreated EAE control animals.

H&E, hematoxylin and eosin; LFB, Luxol fast blue; NF, neurofilament; Iba-1, ionized calcium-binding adaptor molecule-1; GFAP, glial fibrillary acidic protein; GFP, green fluorescence protein.

Nkx2.2-expression was retained in a minority of reactive GFAP$^+$ cells, which is consistent with data showing that Nkx2.2 is expressed by a small proportion of Olig2$^+$ progenitors in the adult dorsal funiculi (27); this observation further indicates that Nkx2.2 does not hamper expression of GFAP per se.

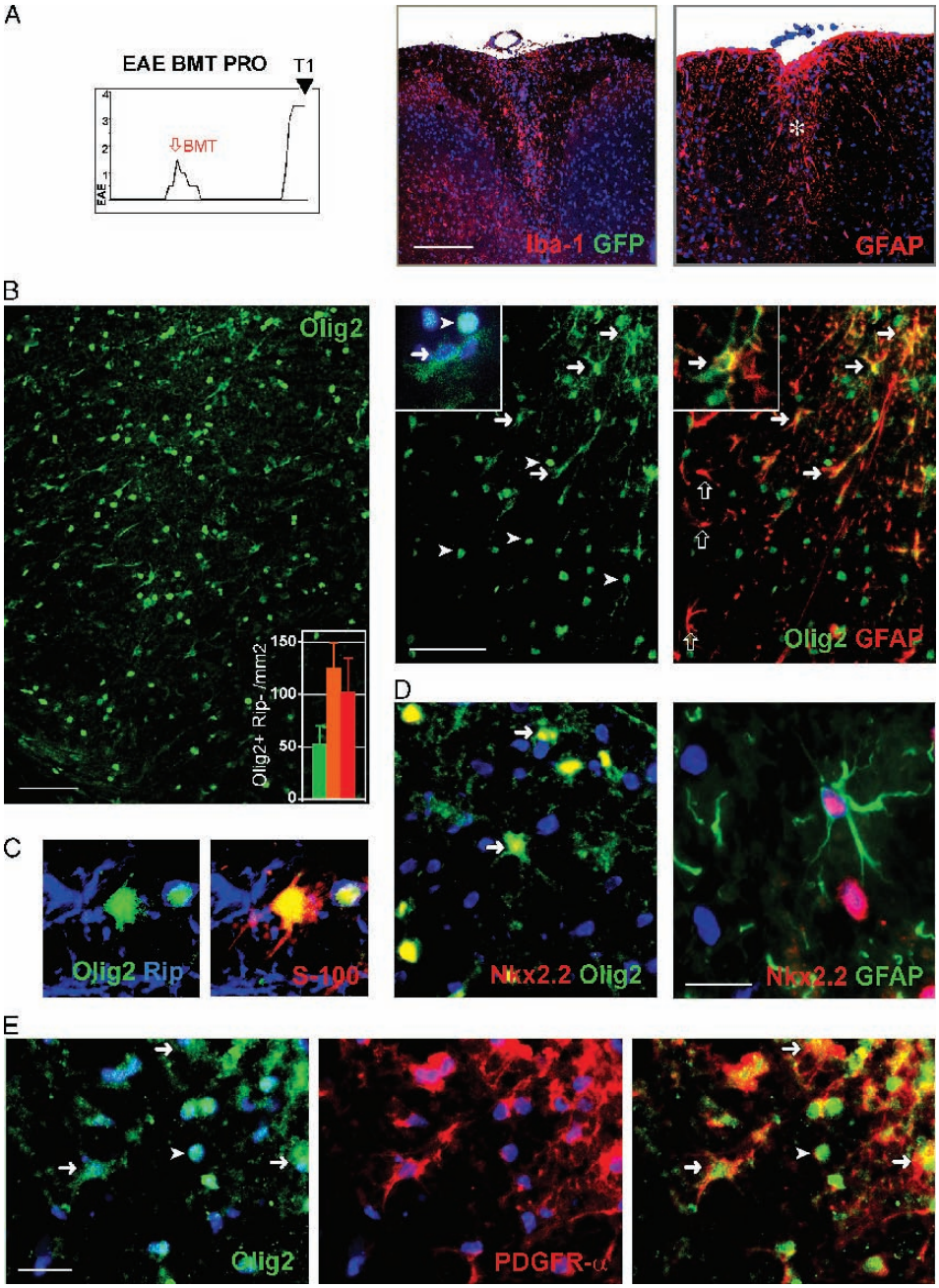


FIGURE 3. Cytoplasmic translocation of the glial transcription factor Olig2 in experimental autoimmune encephalomyelitis (EAE) lesions. **(A)** The graph depicts the clinical course of a mouse that showed complete disease remission after BMT, but had a severe relapse thereafter. Staining for Iba-1 and GFP in the dorsal funiculi at T1 shows activated microglial cells (left image), none of which are donor-derived. Reactive GFAP$^+$ cells were abundant in the dorsal white matter (right image); the asterisk indicates the area analyzed at higher magnification below **(B)**, showing the accumulation of cells expressing the glial transcription factor Olig2 (left); the inset provides cell counts of Olig2$^+$ progenitors (cells/mm^2) in the dorsal funiculi in the different treatment groups (EAE BMT REM in green, EAE BMT PRO in orange, and EAE control mice in red; n = 2/group), documenting the increase in progenitor cells that accompanies disease. At higher magnification, Olig2 labeling shows 2 different patterns of expression: cells with exclusively nuclear expression (arrowheads), next to numerous cells with both nuclear and cytoplasmic Olig2 (arrows), which also coexpress GFAP. DAPI nuclear stain is in blue. **(C)** High-magnification image showing the diffuse localization of Olig2 in a S-100$^+$ astrocyte, in contrast with the nuclear localization in a Rip$^+$ oligodendrocyte. **(D)** Olig2 translocation in cells expressing the transcription factor Nkx2.2, and, on the right, an example of Nkx2.2 and GFAP coexpression. **(E)** Nuclear and cytoplasmic Olig2 in PDGFR-α^+ progenitors at the lesion border. Scale bars = **(A)** 150 μm; **(B)** 50 μm; **(D, E)** 20 μm.

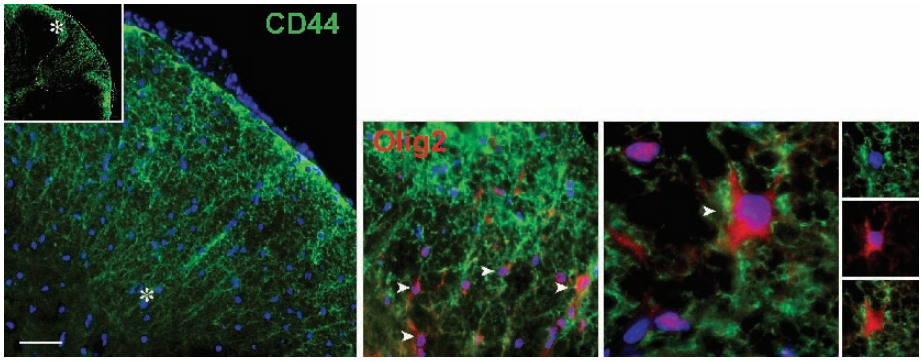


FIGURE 4. Reactive progenitors express CD44. Staining for the surface receptor CD44 shows increased expression of this marker in the white matter lesion in an EAE PRO mouse at T1; the asterisk indicates the area shown at progressively higher magnification from left to right, double labeled for CD44 and Olig2. All cells that show cytoplasmic Olig2 coexpressed CD44 (arrowheads). Scale bar = 50 μm.

We verified these findings by repeating the stainings using at least 2 different antibodies for each of the markers Nkx2.2, GFAP, and Olig2, and obtained equivalent results in parallel sections.

Reactive Cells Express CD44

Inflammatory cues released locally by activated immune cells or present in the extravasating serum, are likely to play a role in determining the glial progenitor differentiation and migration to the lesion area. Molecules such as osteopontin, certain matrix metalloproteinases, and hyaluronic acid, for example, represent important immune modulators secreted by activated macrophages and astrocytes that are known to accumulate in demyelinated lesions in MS and EAE (28–31). A major receptor for some of these factors is CD44, a transmembrane glycoprotein with multiple functions in cell adhesion and signal transduction, which participates in monitoring changes in the extracellular matrix that influence cell growth, survival, and differentiation of glial cells (32).

Consistent with previous data in this model (28, 30), we found that CD44 was highly upregulated in the inflamed EAE white matter (Fig. 4). Importantly, all reactive progenitors showing translocation of Olig2 also expressed CD44, suggesting that CD44 ligands may be involved in modulating the reactive glial progenitor response in vivo.

DISCUSSION

The broad goal of this study was to model and gain further understanding of the pathophysiologic events that follow HSCT for treatment of severe immune-mediated CNS disease. We and others have recently shown that in patients with autoimmune disorders, HSCT can regenerate a new immune system and restore immune tolerance (2, 33). However, an unsettled issue relevant to clinical efficacy is whether despite restoration of peripheral immune tolerance in treated patients, the previous extensive tissue damage may have induced reactive changes in CNS resident cells predisposing to subsequent clinical deterioration or to continued inflammatory, endogenous toxic, or dysmetabolic damage. This possibility could explain the failure of BMT/HSCT to control progression of clinical disability in a subset of patients with MS who were treated in advanced chronic stages of disease (5, 6).

It is unclear exactly how and when tissue changes in disease might predispose to recurrent inflammatory attacks or might result in chronic disturbances of homeostasis that can lead to pathogenetic glial activation. To address some of these questions, we studied disease mechanisms of EAE after BMT at different times after disease induction. The MOG-induced active EAE model has been extensively studied and well characterized. Within 7 days from immunization, antigen-specific T lymphocytes have migrated through the blood-brain barrier to initiate disease (34). Previous studies have elegantly shown that only the very initial burst of disease is T cell-mediated and that the subsequent inflammatory cascade that leads to invalidism is mediated primarily by the activation of microglial cells and is practically extinguished if this activation is impaired in transgenic models (12). Our experiments are consistent with results obtained in other EAE models showing that BMT can completely arrest disease progression and allow full clinical recovery if performed in the early stages of disease but does not significantly modify the course of disease when performed late (5, 19).

The ability to distinguish donor BMT derived from endogenous host cells through GFP expression was a novel and important component of our study and allowed us to define the contribution of pseudoautologous donor BM to the reconstitution of peripheral blood and to long-term microglial turnover in the CNS. Analysis of GFP expression in peripheral blood revealed a rapid repopulation of the B cell and myeloid compartment, whereas the replacement of the T cell pool by BM-derived cells was slower, in agreement with the kinetics of immune reconstitution in mice (35) and in humans (2, 36). We examined the contribution of transplanted BM to microglial turnover in CNS tissues and found that, despite complete donor reconstitution of peripheral circulating monocytes, the presence of Iba-1^{+} GFP-expressing microglia in the spinal cord was generally limited to demyelinating lesions and to the meninges, consistent with

the existence of a local chemoattractant gradient in response to injury.

When we evaluated the response of EAE mice to earlier BMT treatment (administered at acute clinical onset), we observed 2 clearly distinct outcomes that were related to the severity of clinical signs at the time of BMT. Mice with milder disease scores entered prolonged remission after BMT, whereas more severely affected mice improved only partially and transiently. We considered possible immunologic causes underlying the different outcomes and systematically compared in remitting versus progressing mice the overall frequency as well as the donor versus host reconstitution of major immune cell lineages and the memory/activation state of T cell subpopulations. Because there were no differences in the size of the main leukocyte populations and in the activation-related phenotype of host- or donor-derived T cell subsets, we considered the possibility that these relatively crude measures could not reveal potential subtler differences in the frequency of encephalitogenic cells, which constitute a small size of the T cell repertoire even in EAE mice. Therefore, we specifically measured the proliferative responses to the immunizing antigen. The responses in the BMT-treated groups were both suppressed compared with those in EAE control mice and did not differ between relapsing and progressing mice, suggesting that BMT treatment had eliminated pathogenic cells in the periphery to a similar extent in the 2 groups. The lack of significant differences in any of these comparisons suggested that divergent clinical courses were not related to differences in the peripheral immune system. Our results, therefore, pointed to a crucial role of the local CNS environment in determining the late course of disease. Reactive gliosis was clearly pronounced in the CNS in progressing mice, and activated macrophage/microglial cells were most abundant at the lesion sites. Lack of lymphocytic infiltration and prominent macrophage/microglial activation were also the histopathologic features observed in autopsy tissue from a small number of subjects with progressive MS at advanced stages who had received HSCT (37).

In addition, our data revealed that $Olig2^+$ glial precursors are major players in the early response to relapse and highlight how reactive gliosis should not be merely viewed as the reactivation of resident, mature, astrocytic cells around the injury site. We found that a high proportion of reactive astrocytes were derived from $Olig2^+$ progenitors and that cytoplasmic translocation of Olig2 acted as the molecular signature of this fate decision. Although lineage markers such as Olig2, NG2, CNPase, and Nkx2.2 have been commonly used for the characterization of progenitor cells committed to the oligodendrocyte lineage, several recent studies have now challenged this idea by providing evidence that cells expressing those antigens can also generate astrocytes in vivo and in vitro (24, 38–41). Thus, it is still not clear whether adult glial progenitors can generate both astrocytes and oligodendrocytes, depending on the specific type of injury and on the stage of the disease or repair process nor whether independent progenitor populations exist. Alternatively, glial progenitors could undergo a transitory expression of GFAP before generating oligodendrocytes, as previously observed in humans (42, 43) and in the murine subventricular zone (44). This interpretation would also fit our previous data showing that remyelination in the CNS is strictly dependent on the vicinity of astrocytic cells and that no remyelination occurs in areas devoid of $GFAP^+$ cell processes (45); consequently, the observed activation of $Olig2^+/GFAP^+$ cells could still reflect a process fostering remyelination. However, because glial scarring and axonal damage are irreversible in this acute EAE model and repair is poor, it is unlikely that the conspicuous activation of glial progenitors could lead to the generation of large numbers of new oligodendrocytes. Future experiments involving new transgenic animal models in which recombination of the transgene is cell-specific and inducible in the adult stage are expected to answer many of these questions.

We speculate that if glial precursors had the potential to respond in the most appropriate way, their differentiation to astrocytes in the acute phase could be a neuroprotective mechanism because astrocytes, compared with oligodendrocytes, are less susceptible to damage and can protect neurons by removing glutamate and producing neurotrophic factors (46). Consistently, next to activated microglia/macrophages, glial precursors have been shown to be among the first cells responding to disruptions in the blood-brain barrier. This reactivity indicates that progenitor cells must necessarily have the ability to sense local changes in the extracellular matrix that accompany tissue damage as well as the capacity to recognize immune-secreted factors to modulate their response accordingly. One response of the precursor cell is the expression of CD44, a transmembrane glycoprotein that participates in cell-to-matrix interactions and that functions as a major receptor for regulatory factors such as osteopontin and hyaluronan. For instance, when CD44 is forcibly expressed in glial progenitors under the CNPase-promoter, transgenic animals spontaneously develop a severe demyelinating syndrome that is accompanied by a striking increase in vivo of cells coexpressing O4 and GFAP (47, 48), indicating a disturbance in normal progenitor maturation driven by CD44 signaling. In the present study, we detected CD44 expression on all "reactive" Olig2 progenitors in the EAE white matter, further strengthening the notion that CD44 ligands are among the factors regulating the glial response during neuroinflammation and are therefore potentially important therapeutic targets.

Our demonstration that endogenous microglia and Olig2 "astrocyte progenitor cells" are conspicuously represented and activated in EAE mice, which progress clinically after BMT, provides new insight into the cellular and molecular events underlying similar pathogenetic processes that may also occur in MS and related disorders and may have significant therapeutic implications.

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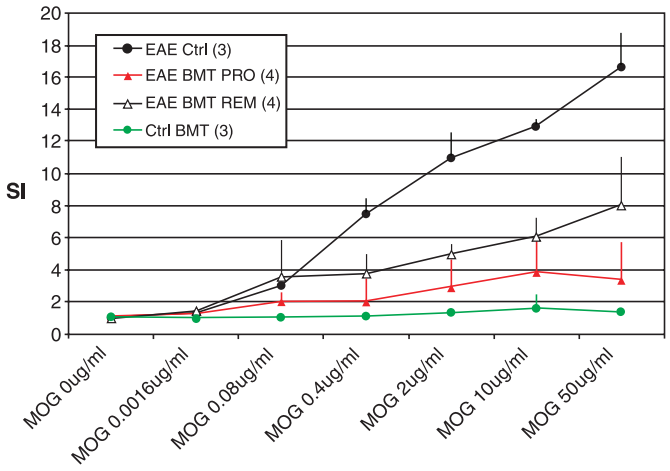
REFERENCES

1. Tyndall A, Saccardi R. Haematopoietic stem cell transplantation in the treatment of severe autoimmune disease: Results from phase I/II studies, prospective randomized trials and future directions. Clin Exp Immunol 2005;141:1–9
2. Muraro PA, Douek DC, Packer A, et al. Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. J Exp Med 2005;201:805–16
3. Fassas A, Passweg JR, Anagnostopoulos A, et al. Hematopoietic stem cell transplantation for multiple sclerosis: A retrospective multicenter study. J Neurol 2002;249:1088–97
4. Saccardi R, Mancardi GL, Solari A, et al. Autologous HSCT for severe progressive multiple sclerosis in a multicenter trial: Impact on disease activity and quality of life. Blood 2005;105:2601–7
5. Burt RK, Cohen BA, Russell E, et al. Hematopoietic stem cell transplantation for progressive multiple sclerosis: Failure of a total body irradiation-based conditioning regimen to prevent disease progression in patients with high disability scores. Blood 2003;102:2373–78
6. Nash RA, Bowen JD, McSweeney PA, et al. High-dose immunosuppressive therapy and autologous peripheral blood stem cell transplantation for severe multiple sclerosis. Blood 2003;102:2364–72
7. Bechmann I, Goldmann J, Kovac AD, et al. Circulating monocytic cells infiltrate layers of anterograde axonal degeneration where they transform into microglia. FASEB J 2005;19:647–49
8. Priller J, Flugel A, Wehner T, et al. Targeting gene-modified hematopoietic cells to the central nervous system: Use of green fluorescent protein uncovers microglial engraftment. Nat Med 2001;7:1356–61
9. Kennedy DW, Abkowitz JL. Kinetics of central nervous system microglial and macrophage engraftment: Analysis using a transgenic bone marrow transplantation model. Blood 1997;90:986–93
10. Krall WJ, Challita PM, Perlmutter LS, Skelton DC, Kohn DB. Cells expressing human glucocerebrosidase from a retroviral vector repopulate macrophages and central nervous system microglia after murine bone marrow transplantation. Blood 1994;83:2737–48
11. Gold R, Hartung HP, Toyka KV. Animal models for autoimmune demyelinating disorders of the nervous system. Mol Med Today 2000;6: 88–91
12. Heppner FL, Greter M, Marino D, et al. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. Nat Med 2005;11: 146–52
13. Benveniste EN. Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. J Mol Med 1997;75: 165–73
14. Zhou Q, Wang S, Anderson DJ. Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron 2000;25:331–43
15. Ligon KL, Kesari S, Kitada M, et al. Development of NG2 neural progenitor cells requires Olig gene function. Proc Natl Acad Sci U S A 2006;103:7853–58
16. Burt RK, Padilla J, Begolka WS, Canto MC, Miller SD. Effect of disease stage on clinical outcome after syngeneic bone marrow transplantation for relapsing experimental autoimmune encephalomyelitis. Blood 1998;91:2609–16
17. Herrmann MM, Gaertner S, Stadelmann C, et al. Tolerance induction by bone marrow transplantation in a multiple sclerosis model. Blood 2005; 106:1875–83
18. Karussis D, Slavin S. Hematopoietic stem cell transplantation in multiple sclerosis: Experimental evidence to rethink the procedures. J Neurol Sci 2004;223:59–64
19. van Bekkum DW. Stem cell transplantation in experimental models of autoimmune disease. J Clin Immunol 2000;20:10–16
20. van Gelder M, van Bekkum DW. Treatment of relapsing experimental autoimmune encephalomyelitis in rats with allogeneic bone marrow transplantation from a resistant strain. Bone Marrow Transplant 1995; 16:343–51
21. Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S. Microglia-specific localisation of a novel calcium binding protein, Iba1. Brain Res Mol Brain Res 1998;57:1–9
22. Arnett HA, Fancy SP, Alberta JA, et al. bHLH transcription factor Olig1 is required to repair demyelinated lesions in the CNS. Science 2004;306:2111–15
23. Fancy SP, Zhao C, Franklin RJ. Increased expression of Nkx2.2 and Olig2 identifies reactive oligodendrocyte progenitor cells responding to demyelination in the adult CNS. Mol Cell Neurosci 2004;27: 247–54
24. Cassiani-Ingoni R, Coksaygan T, Xue H, et al. Cytoplasmic translocation of Olig2 in adult glial progenitors marks the generation of reactive astrocytes following autoimmune inflammation. Exp Neurol 2006;201:349–58
25. Samanta J, Kessler JA. Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. Development 2004;131:4131–42
26. Setoguchi T, Kondo T. Nuclear export of OLIG2 in neural stem cells is essential for ciliary neurotrophic factor-induced astrocyte differentiation. J Cell Biol 2004;166:963–68
27. Kitada M, Rowitch DH. Transcription factor co-expression patterns indicate heterogeneity of oligodendroglial subpopulations in adult spinal cord. Glia 2006;54:35–46
28. Kim MD, Cho HJ, Shin T. Expression of osteopontin and its ligand, CD44, in the spinal cords of Lewis rats with experimental autoimmune encephalomyelitis. J Neuroimmunol 2004;151:78–84
29. Selvaraju R, Bernasconi L, Losberger C, et al. Osteopontin is upregulated during in vivo demyelination and remyelination and enhances myelin formation in vitro. Mol Cell Neurosci 2004;25:707–21
30. Back SA, Tuohy TM, Chen H, et al. Hyaluronan accumulates in demyelinated lesions and inhibits oligodendrocyte progenitor maturation. Nat Med 2005;11:966–72
31. Chabas D, Baranzini SE, Mitchell D, et al. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. Science 2001;294:1731–35
32. Ponta H, Sherman L, Herrlich PA. CD44: From adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 2003;4:33–45
33. de Kleer I, Vastert B, Klein M, et al. Autologous stem cell transplantation for autoimmunity induces immunologic self-tolerance by reprogramming autoreactive T-cells and restoring the $CD4^+CD25^+$ immune regulatory network. Blood 2006;107:1696–1702
34. Juedes AE, Hjelmstrom P, Bergman CM, Neild AL, Ruddle NH. Kinetics and cellular origin of cytokines in the central nervous system: Insight into mechanisms of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis. J Immunol 2000; 164:419–26
35. Mackall CL, Granger L, Sheard MA, Cepeda R, Gress RE. T-cell regeneration after bone marrow transplantation: Differential CD45 isoform expression on thymic-derived versus thymic-independent progeny. Blood 1993;82:2585–94
36. Douek DC, Vescio RA, Betts MR, et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. Lancet 2000;355:1875–81
37. Metz I, Lucchinetti CF, Openshaw H, et al. Autologous haematopoietic stem cell transplantation fails to stop demyelination and neurodegeneration in multiple sclerosis. Brain 2007;130:1254–62
38. Alonso G. NG2 proteoglycan-expressing cells of the adult rat brain: Possible involvement in the formation of glial scar astrocytes following stab wound. Glia 2005;49:318–38
39. Belachew S, Chittajallu R, Aguirre AA, et al. Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. J Cell Biol 2003;161:169–86
40. Masahira N, Takebayashi H, Ono K, et al. Olig2-positive progenitors in the embryonic spinal cord give rise not only to motoneurons and oligodendrocytes, but also to a subset of astrocytes and ependymal cells. Dev Biol 2006;293:358–69
41. Buffo A, Vosko MR, Erturk D, et al. Expression pattern of the transcription factor Olig2 in response to brain injuries: Implications for neuronal repair. Proc Natl Acad Sci U S A 2005;102:18183–88

42. Choi BH, Kim RC. Expression of glial fibrillary acidic protein by immature oligodendroglia and its implications. J Neuroimmunol 1985; 8:215–35
43. Choi BH, Kim RC. Expression of glial fibrillary acidic protein in immature oligodendroglia. Science 1984;223:407–9
44. Menn B, Garcia-Verdugo JM, Yaschine C, Gonzalez-Perez O, Rowitch D, Alvarez-Buylla A. Origin of oligodendrocytes in the subventricular zone of the adult brain. J Neurosci 2006;26:7907–18
45. Talbott JF, Loy DN. Liu Y, et al. Endogenous Nkx2.2$^+$/Olig2$^+$ oligodendrocyte precursor cells fail to remyelinate the demyelinated adult rat spinal cord in the absence of astrocytes. Exp Neurol 2005;192: 11–24
46. Trendelenburg G, Dirnagl U. Neuroprotective role of astrocytes in cerebral ischemia: Focus on ischemic preconditioning. Glia 2005;50: 307–20
47. Tuohy TM, Wallingford N, Liu Y, et al. CD44 overexpression by oligodendrocytes: A novel mouse model of inflammation-independent demyelination and dysmyelination. Glia 2004;47:335–45
48. Liu Y, Han SS, Wu Y, et al. CD44 expression identifies astrocyte-restricted precursor cells. Dev Biol 2004;276:31–46



Proliferation assay.
The degree of proliferation of splenocytes to different concentrations of the immunizing peptide $MOG_{35–55}$ was assessed in mice from all treatment groups (n = 3–4/group). The results demonstrate the reduced response in BMT-treated animals as opposed to untreated EAE Control mice; both the REMitting group and the PROgressing group show a comparable low degree of proliferation. SI = stimulation index.