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# Peripheral T Cells Are the Therapeutic Targets of Glucocorticoids in Experimental Autoimmune Encephalomyelitis<sup>1</sup>

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High-dose glucocorticoid (GC) therapy is widely used to treat multiple sclerosis (MS), but the underlying mechanisms remain debatable. In this study, we investigated the impact of GC administration on experimental autoimmune encephalomyelitis using different GC receptor (GR)-deficient mutants. Heterozygous GR knockout mice were less sensitive to dexamethasone therapy, indicating that the expression level of the receptor determines therapeutic efficacy. Mice reconstituted with homozygous GR knockout fetal liver cells showed an earlier onset of the disease and were largely refractory to GC treatment, indicating that the GR in hematopoietic cells is essential for the beneficial effects of endogenous GCs and dexamethasone. Using cell-type specific GR-deficient mice, we could demonstrate that GCs mainly act on T cells, while modulation of macrophage function was largely dispensable in this context. The therapeutic effects were achieved through induction of apoptosis and down-regulation of cell adhesion molecules in peripheral T<sub>H</sub>17 and bystander T cells, while similar effects were not observed within the spinal cord. In addition, dexamethasone inhibited T cell migration into the CNS, confirming that peripheral but not CNS-residing T lymphocytes are the essential targets of GCs. Collectively, our findings reveal a highly selective mechanism of GC action in experimental autoimmune encephalomyelitis and presumably multiple sclerosis. *The Journal of Immunology*, 2008, 180: 8434–8443.

Glucocorticoids (GCs)<sup>4</sup> are in widespread clinical use for the treatment of multiple sclerosis (MS), the most prevalent chronic autoimmune disease of the CNS (1). So far, there is no definitive cure available and, despite considerable progress made during the last decade, the clinical management of MS is still unsatisfactory. Although optic neuritis and other acute relapses are efficiently treated with high doses of GCs, therapy may still lead to severe complications or incomplete recovery (2). Thus, a better understanding of this commonly used therapeutic regimen is urgently needed.

In the early phases of MS, autoreactive T cells cross the blood-brain barrier (BBB) and are restimulated by local APC. This re-

sults in the release of cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , and IL-17, which initiate and perpetuate an inflammatory response by activating microglia and recruiting macrophages (3, 4). As a consequence, the BBB is breached and allows for the influx of more T lymphocytes, additional immune cells, and the deposition of humoral components (5). Subsequently, this process leads to myelin destruction, induction of oligodendrocyte death, axonal degeneration, and eventually to the development of severe functional deficits (6).

Traditionally, acute relapses of MS patients are treated with high doses of GCs, especially methylprednisolone, for a limited period of time (2). Under such conditions they exert a variety of proapoptotic and anti-inflammatory effects and thereby modulate the survival, migration, and the effector functions of multiple cell types, including leukocytes, endothelial cells, and neurons (7). Nevertheless, the relative contribution of these cell-specific effects for therapeutic efficacy remains unknown. Although most GC actions presumably require the presence of the glucocorticoid receptor (GR), additional nongenomic effects are discussed that may be exerted through nonrelated receptors or interactions with lipid membranes (8, 9). Thus, neither the cellular nor the molecular mechanisms of GCs have been fully resolved in the context of MS therapy.

GC actions can be studied in experimental autoimmune encephalomyelitis (EAE), a widely recognized rodent model of MS (10). Depending on the experimental setup, distinct features of MS are recapitulated by EAE. In the case of C57BL/6 mice, immunization with myelin oligodendrocyte glycoprotein (MOG) leads to a chronic disease, characterized by a fulminant inflammatory response, demyelinating lesions, and subsequent axonal damage. First attempts to treat EAE by administration of GCs were made almost half a century ago (11, 12). Meanwhile, a series of experiments were conducted to address the mechanism of GCs in EAE, revealing T cells, macrophages, microglia, and endothelial cells as potential targets (7). For instance, GC administration potentiates

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<sup>4</sup> Abbreviations used in this paper: GC, glucocorticoid; MS, multiple sclerosis; BBB, blood-brain barrier; GR, GC receptor; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; Dex, dexamethasone; CHS, contact hypersensitivity; GITR, glucocorticoid-induced tumor necrosis factor receptor family related gene; Treg, regulatory T cell; n.s., nonsignificant.

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lymphocyte apoptosis in Lewis rats suffering from EAE but it remains elusive whether this is indeed essential for therapeutic efficacy (13, 14). Furthermore, dexamethasone (Dex) profoundly suppresses cytokine secretion and cell-cell interactions (15). In particular, it has been found that GCs target components of the receptor-ligand pairs LFA-1/ICAM-1, VLA-4/VCAM-1, and CD44/HA that play a crucial role in the extravasation of effector T cells through the BBB (15–17). Finally, additional effects of GCs on neuronal survival and the function of the microglia have been postulated (7). However, despite the large amount of data, the relevance of these different effects, the identity of the major target cells, and the primary site of GC action, i.e., leukocytes in the periphery vs inflammatory cells in the lesion, remain largely unclear.

Studies in newborn GR knockout mice (18) as well as mice reconstituted with hematopoietic stem cells have confirmed that the GR is essential for the induction of thymocyte apoptosis (19). More recently, conditional GR knockout mice were developed that lack the GR in T cells or myeloid cells (20). In an attempt to dissect the cell type-specific actions of GCs in contact hypersensitivity (CHS), a model of allergic dermatitis, we could show that macrophages and neutrophils are the only essential targets of GCs in the treatment of this disease while control of T cell function was dispensable (20). In this study, we have used a similar approach to investigate the mechanisms of GC action in EAE. In contrast to CHS, the successful treatment of this neuroinflammatory disease by GCs depends on the presence of the GR in T lymphocytes but not myeloid cells, involves induction of apoptosis and down-regulation of cell adhesion molecules in peripheral lymphoid organs, and interferes with T cell migration to the CNS. Thus, we describe for the first time the underlying mode of GC action at the cellular and molecular level in the treatment of EAE and presumably its human counterpart MS.

## Materials and Methods

### Mice

C57BL/6 mice used for EAE induction were purchased from Charles River. GRN<sup>+/-</sup> mice were reported elsewhere (21); GR<sup>lox</sup> mice (22) were bred to mice expressing Cre either as transgene under the control of the proximal Ick promoter (IckCre) (23) or having it knocked into the LysM locus (LysMCre) (24). The different mouse lines were backcrossed to C57BL/6 mice for at least six generations. All animal experiments were approved by the responsible authorities in Lower Saxonia and Bavaria.

### Generation of HSC chimeric mice

GRN<sup>+/-</sup> mice were intercrossed and pregnant females were sacrificed on embryonic day 14.5. Fetuses were dissected and stored in ice-cold PBS while genotyping by PCR as previously described (21). The livers of fetuses were removed, passed through a nylon mesh, washed, and the cell number counted. In parallel, 6-wk-old female CD45.1- congenic C57BL/6 mice (The Jackson laboratory) were gamma-irradiated at 11.5 Gy (6.5 and 5 Gy with a 4-h intercept). Subsequently,  $2 \times 10^6$  fetal liver cells in 500  $\mu$ l of PBS were injected i.v. and the reconstituted mice were kept in individually ventilated cages for 3 wk with water supplemented with penicillin (Sigma-Aldrich). At an age of 12 wk, the mice were subjected to the EAE experiments.

### EAE induction and therapy

Mice were immunized with 50  $\mu$ g of MOG<sub>35–55</sub> peptide in PBS, emulsified in an equal volume of CFA containing *Mycobacterium tuberculosis* H37RA (Difco) at a final concentration of 1 mg/ml, and given s.c. into the flanks as previously described (25). Two injections of pertussis toxin (List Biological Laboratories; 400 ng/mouse in total) were given, one immediately after immunization and the second 2 days after immunization. Animals were weighed and scored daily for clinical signs of disease on a scale from 0 to 10 depending on severity; scores were as followed: 0 = normal; 1 = reduced tone of tail; 2 = limp tail, impaired righting; 3 = absent righting; 4 = gait ataxia; 5 = mild paraparesis of hind limbs; 6 = moderate paraparesis; 7 = severe paraparesis or paraplegia; 8 = tetraparesis; 9 =

moribund; and 10 = death. To analyze the effects of GC therapy, dexamethasone-21-dihydrogen phosphate (Dexa-ratiopharm 100 mg; Merckle or Fortecortin Inject 100 mg; Merck) was injected daily i.p. on 3 consecutive days.

### Histology and immunohistochemistry

Animals were anesthetized with ketamine/hydrochloride (Inresa) and xylazine/hydrochloride (Bayer Vital) in 0.9% NaCl and sacrificed by saline perfusion through the left ventricle. Following fixation with 4% paraformaldehyde, the spinal cord was removed, postfixed for 2 h, and embedded in paraffin. Three-micrometer cross-sections were stained with a rat anti-human CD3 Ab (1:200; Serotec) or an anti-mouse Mac-3 Ab (1:200; BD Biosciences) followed by incubation with a secondary biotinylated rabbit anti-rat Ab (1:200; Vector Laboratories). Ag unmasking was achieved by pretreating the sections in 1 mM EDTA (pH 8.0) for 30 min in a microwave oven at 850 W. The peroxidase-based ABC detection system (Dako-Cytomation) in combination with diaminobenzidine was used for visualization, and all sections were counterstained with hematoxylin. To detect the disruption of the BBB, the sections were incubated with an anti-albumin Ab (1:300; Abcam) that was detected with a biotinylated rabbit anti-sheep Ab (1:300; Southern Biotechnology Associates).

### Quantification of histological results

Enumeration of T cells and macrophages was performed in a double-blinded manner. Ten visual fields of the cervical, thoracic, and lumbar spinal cord were counted at a 400-fold magnification, two infiltrates per cross-section and three cross-sections per animal. A median was determined and the results were calculated as number of infiltrating cells per mm<sup>2</sup>.

Analysis of BBB disruption was performed by gray scale analysis. Six photographs were made of comparable histological areas of cross-sections of the cervical, thoracic, and lumbar spinal cord at a 200-fold magnification (three cross-sections per animal). The photographs were analyzed by using Scion Image software and the results of the pixel densities are depicted as arbitrary units.

### Isolation of spinal cord infiltrates

Lymphocytes were isolated from the spinal cord by density centrifugation following perfusion of the mice with NaCl. To this end, the dissected tissue was passed through a metal mesh and homogenized in PBS containing 0.1% BSA, 1% glucose, and 100  $\mu$ g/ml DNase I (Roche). After centrifugation, the spinal cord homogenate was resuspended in 6 ml of 30% Percoll, overlaid on a Percoll gradient containing 4 ml of 45% and 2 ml of 70% Percoll, and spun for 20 min (2300 rpm, 4°C). Finally, the lymphocytes were harvested at the interfaces between the layers, washed with PBS, and analyzed by flow cytometry.

### Flow cytometry

All Abs and reagents were obtained from BD Biosciences unless otherwise indicated: anti-CD3 $\epsilon$  (145-2C11), anti-CD4 (RM4-5), anti-CD44 (IM7), anti-CD11a/LFA-1 (2D7), anti-CD49d/VLA-4 (R1-2), anti-GITR (DTA-1), anti-IL-17 (TC11-18H10.1), anti-active caspase 3 (C92-605), annexin V, and anti-Foxp3 (FJK-16s; eBioscience). The Abs were directly labeled with FITC, PE, PerCP, PE-Cy7, Cy5, allophycocyanin, or allophycocyanin-Cy7. Extracellular staining was performed as previously described (26); for the intracellular staining of Foxp3 and caspase 3, we followed the manufacturer's protocols. To allow for intracellular staining of IL-17, cells were stimulated with PMA (5 ng/ml) and ionomycin (500 ng/ml) for 2 h followed by a treatment with GolgiPlug (BD Biosciences). All analyses were performed on a FACSCanto II device allowing for the detection of six fluorescent dyes (BD Biosciences).

### Tracking experiments

Splenic T cells were isolated from diseased mice by magnetic cell sorting using an AUTOMACS machine according to the manufacturer's instructions (Miltenyi Biotec). One  $\times 10^6$  cells/ml were labeled with 50 nM CFSE for 10 min at 37°C and the reaction was stopped with 2% FCS followed by extensive washings. Cells ( $5 \times 10^6$ ) were injected into each recipient mouse also suffering from EAE (grades 3 and 4), followed by daily treatment with Dex or PBS for three times, starting 1 h after adoptive transfer. Fifty-eight hours later, the leukocytes were isolated from spinal cord and spleen and the frequency of the CFSE<sup>+</sup> cells among the CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes was determined. The ratio of their relative abundance in spinal cord vs spleen was taken as a measure of specific migration.

### Statistical analysis

Analyses were routinely performed using the Mann-Whitney *U* test. When comparing more than two experimental groups, the Kruskal-Wallis test followed by the Dunn multiple comparison test was used (Microsoft Excel and GraphPad Prism version 4). Data are depicted as mean values  $\pm$  SEM; *p* values above 0.05 were considered as nonsignificant (n.s.); \*, *p* < 0.05; \*\*, *p* < 0.01; and \*\*\*, *p* < 0.001. To determine differences referring to the disease course, the whole curves rather than individual time points were compared between experimental groups. Strictly speaking, statistical analysis was performed from the onset of the disease (preventive setting) or the day after the first Dex treatment (therapeutic setting) until the end of the observation period.

## Results

### Administration of dexamethasone ameliorates MOG-induced EAE in mice

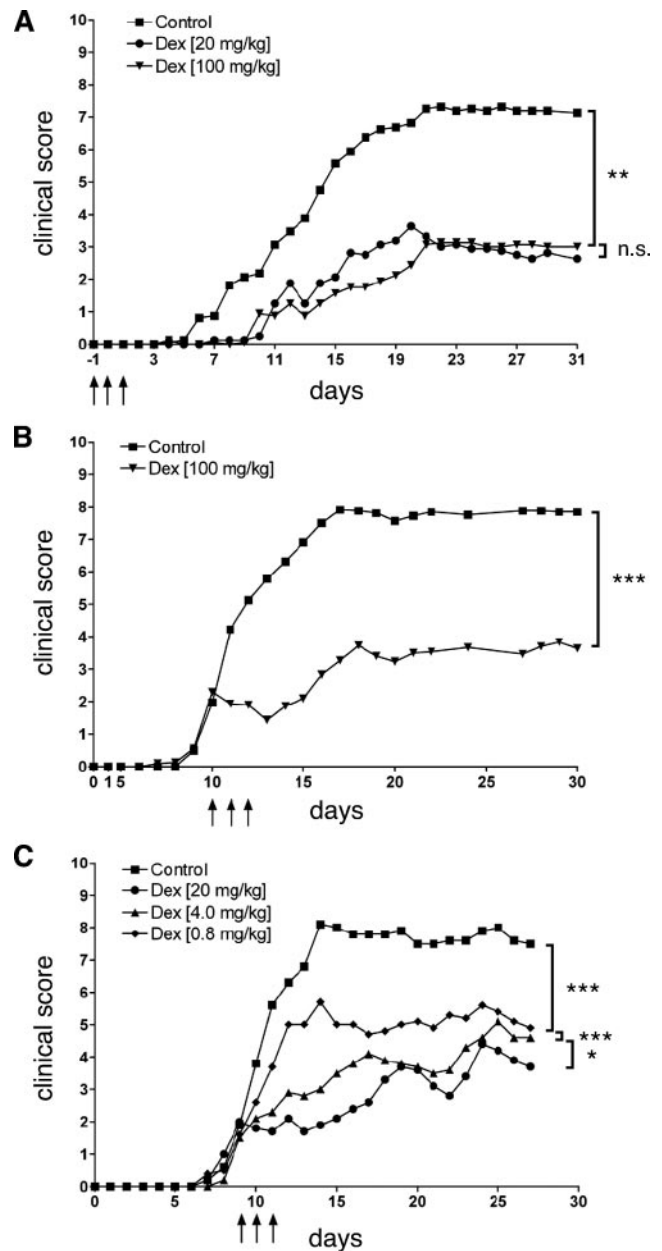
The beneficial effect of GCs is well established in the treatment of human MS and monophasic EAE in the Lewis rat, but has not yet been systematically applied to the chronic MOG-induced EAE model in C57BL/6 mice. Therefore, we first investigated whether GC application around the time of immunization impacts on the disease course. Dex was chosen instead of methylprednisolone because it is most commonly used for the treatment of EAE in rodents (7) and has a higher potency in reducing clinical symptoms in C57BL/6 mice (our unpublished data). EAE was induced by immunization with MOG and at days -1, 0, and 1 we administered 100 or 20 mg/kg Dex i.p. In this preventive setting, the onset and the severity of the disease were significantly reduced, an effect that lasted until the end of the observation period (Fig. 1A).

Next, we turned to the clinically more relevant therapeutic setting. Dex administration was started when more than half of the mice showed first signs of disease, resulting in an average score of 2–3 at the 10-point scale. When Dex was applied at an ultrahigh dose of 100 mg/kg for 3 days, therapy led to an immediate amelioration of the disease (Fig. 1B). However, a few days later, EAE exaggerated again, although its severity remained below the one of untreated mice (Fig. 1B). To establish a dose-efficiency relationship, we tested a series of different hormone concentrations (Fig. 1C). The therapeutic effect of Dex gradually decreased at lower doses but remained efficient even at 0.8 mg/kg (Fig. 1C). Taken together, GCs are an effective means to treat EAE in C57BL/6 mice, both in a preventive and a therapeutic setting, and act in a dose-dependent manner.

### GCs impact on leukocyte infiltration and the BBB

To investigate whether the clinical efficacy of GCs in EAE therapy was reflected by a reduction in leukocyte infiltration, we performed an immunohistochemical analysis of spinal cord sections from mice that had been treated with 100 mg/kg Dex either in a preventive or a therapeutic setting. T cells and macrophages that comprise the majority of the CNS infiltrate were almost absent from the spinal cord of mice that had undergone preventive Dex administration. In therapeutically treated mice, the infiltrate was less but still significantly reduced (Fig. 2, A and B). Another hallmark of EAE and MS is the disruption of the BBB, which can be studied by staining spinal cord sections for the presence of albumin. Both, in the preventive and the therapeutic setting, the BBB was partially protected by Dex administration, indicating that GCs impact on the integrity of the BBB (Fig. 2, A and B).

To determine the influence of Dex administration on the cellular composition of the spinal cord infiltrate, we isolated leukocytes by density centrifugation using a Percoll gradient and analyzed them by flow cytometry. GC treatment significantly reduced the absolute numbers of  $T_H17$  cells as well as other T lymphocytes, but only

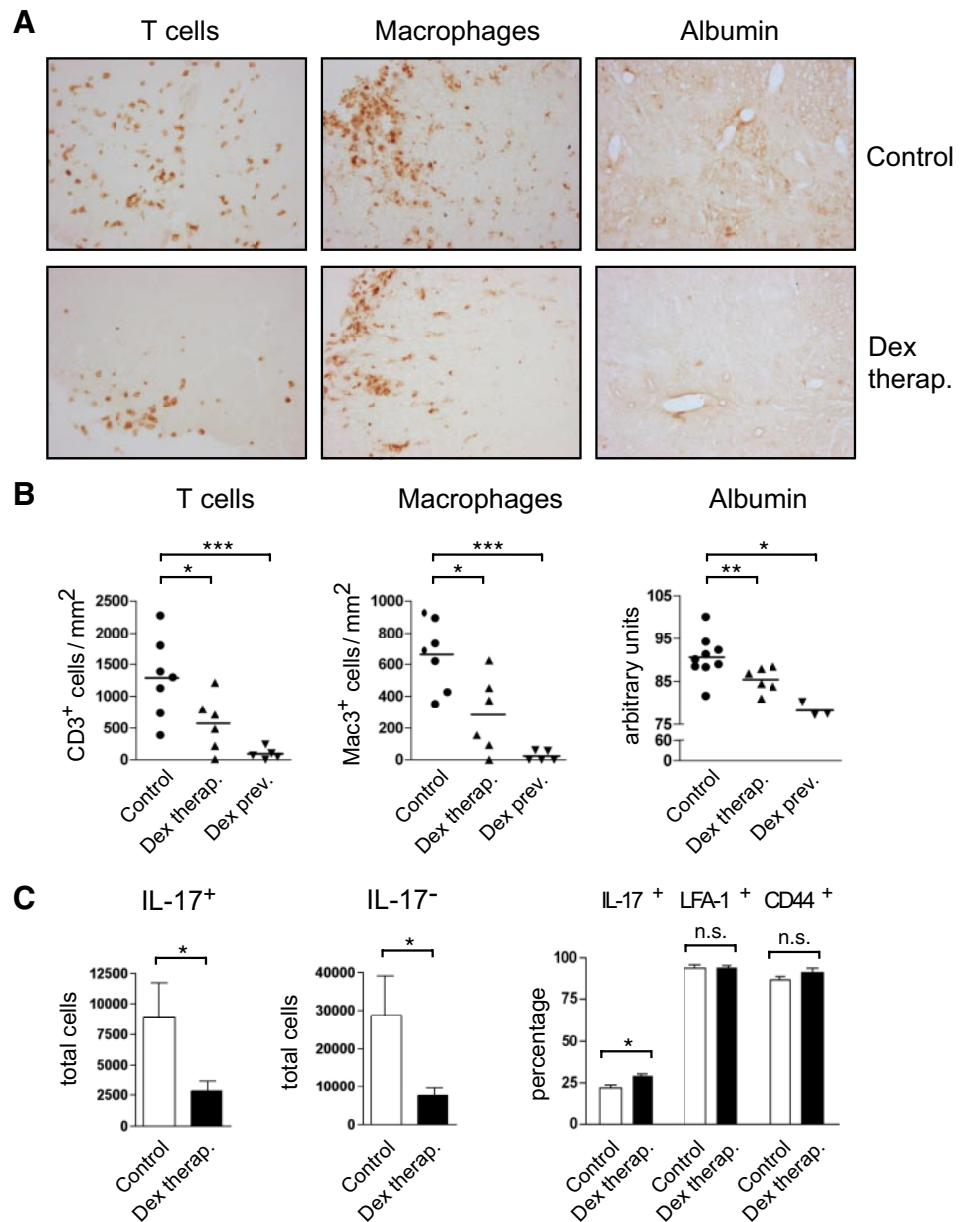


**FIGURE 1.** Dex administration interferes with EAE. *A*, In a preventive setting, EAE was induced by immunization with MOG and the mice were treated with the indicated doses of Dex for 3 days, starting 1 day before immunization (arrows); *n* = 8 for each group, statistical analysis: days 6–31. *B*, In a therapeutic setting, Dex was applied three times after the first appearance of clinical signs, starting at an average score of 2–3 (arrows); *n* = 11 for each group, statistical analysis: days 11–30. *C*, In a therapeutic setting, different doses of Dex were applied after the first appearance of clinical signs (arrows); *n* = 5 for each group, statistical analysis: days 10–27.

marginally affected their relative abundances (Fig. 2C). The frequency of  $LFA^+$  and  $CD44^+$  T cells, which represent characteristic cell populations present in CNS lesions of diseased mice, also remained unaltered (Fig. 2C). This suggests that GC administration reduces infiltration of the spinal cord largely independent of the cell type.

### Therapeutic GC effects in EAE require the presence of the GR and are receptor dosage dependent

There is a long-standing debate as to whether GC therapy of MS and EAE is mediated by the nuclear GC receptor (GR). Alternatively, it



**FIGURE 2.** Dex suppresses leukocyte infiltration and preserves BBB integrity. *A*, Immunohistochemical stainings for infiltrating T cells, macrophages, and the integrity of the BBB (albumin) in the cervical spinal cord of one control and one therapeutically Dex-treated mouse sacrificed on day 12. Original magnification,  $\times 200$ . *B*, Quantification of immunohistochemical stainings of spinal cord in control and Dex-treated mice for T cells, macrophages, and albumin as in *A*. Each symbol represents one animal.  $n = 5-7$ . *C*, Quantification of T cell subsets in the spinal cord of control and Dex-treated mice by flow cytometry following isolation by density centrifugation. IL-17 (intracellular), LFA-1, and CD44 (surface) expression was determined after gating on live CD3<sup>+</sup>CD4<sup>+</sup> T cells. Total cell numbers refer to all cells isolated from the spinal cord of a mouse and percentages refer to the relative frequency of the respective cell population among the CD4<sup>+</sup> T cells.  $n = 8$ . Control = PBS-treated mice; Dex prev. = preventive application of 100 mg/kg Dex on days -1, 0, and 1; Dex therap. = therapeutic application of 100 mg/kg Dex on days 9-11.

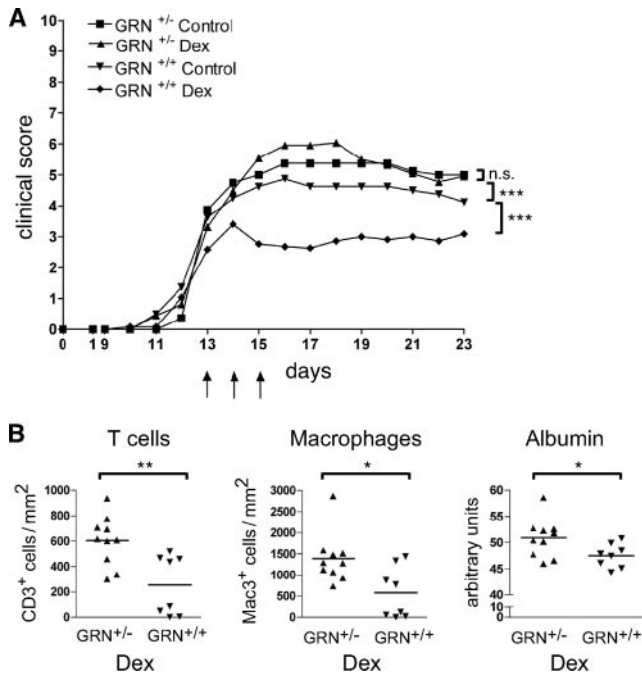
could act through a yet unknown alternative receptor or by interaction with lipid membranes (9, 27). To address this question, we first studied heterozygous GR knockout mice (GRN<sup>+/-</sup>) because homozygous mutants die around birth due to lung atelectasis (18). Importantly, GR expression in those animals is reduced by half and T cells show an increased resistance to GC-induced apoptosis (21).

We first compared the clinical course of EAE between GRN<sup>+/+</sup> and GRN<sup>+/-</sup> mice and found a minor but statistically significant difference in the disease course (Fig. 3A). This indicates that endogenous GCs impact on EAE via the GR. Next, we tested the therapeutic protocol on these mice. EAE was induced by MOG immunization and, once the mice showed the first symptoms, they were injected with Dex for 3 consecutive days. To reveal a potential gene dosage effect, we used a concentration of only 4 mg/kg, which was still effective in wild-type mice (Fig. 1C). Dex administration to GRN<sup>+/+</sup> control mice led to a halt of disease progression, while the same dose was not effective in heterozygous GRN<sup>+/-</sup> mice (Fig. 3A). To answer whether the impact of GC treatment on the disease course was also reflected by immunohis-

topathology, we analyzed spinal cord sections of Dex-treated EAE mice (Fig. 3B). Importantly, we found higher numbers of infiltrating T cells and macrophages and a reduced integrity of the BBB in GRN<sup>+/-</sup> mice. This suggests that the GR is essential for GC therapy in EAE.

*The GR in hematopoietic cells is central to the therapeutic efficacy of GCs in EAE*

A plethora of potential GC target cells is discussed in the context of EAE therapy but their individual relevance is unknown. Among them are hematopoietic cells, mainly T cells and macrophages, and nonhematopoietic cells such as endothelial and neural cells. To discriminate between these two major tissue compartments, we generated chimeric mice in which GR expression was exclusively abrogated in the hematopoietic lineage. CD45.1-congenic mice were lethally irradiated and reconstituted by transplantation of CD45.2<sup>+</sup> fetal liver cells from embryonic 14.5-day-old GRN<sup>+/+</sup> control or homozygous GRN<sup>-/-</sup> embryos. As confirmed by flow cytometry, all nonhematopoietic cell types in the resulting

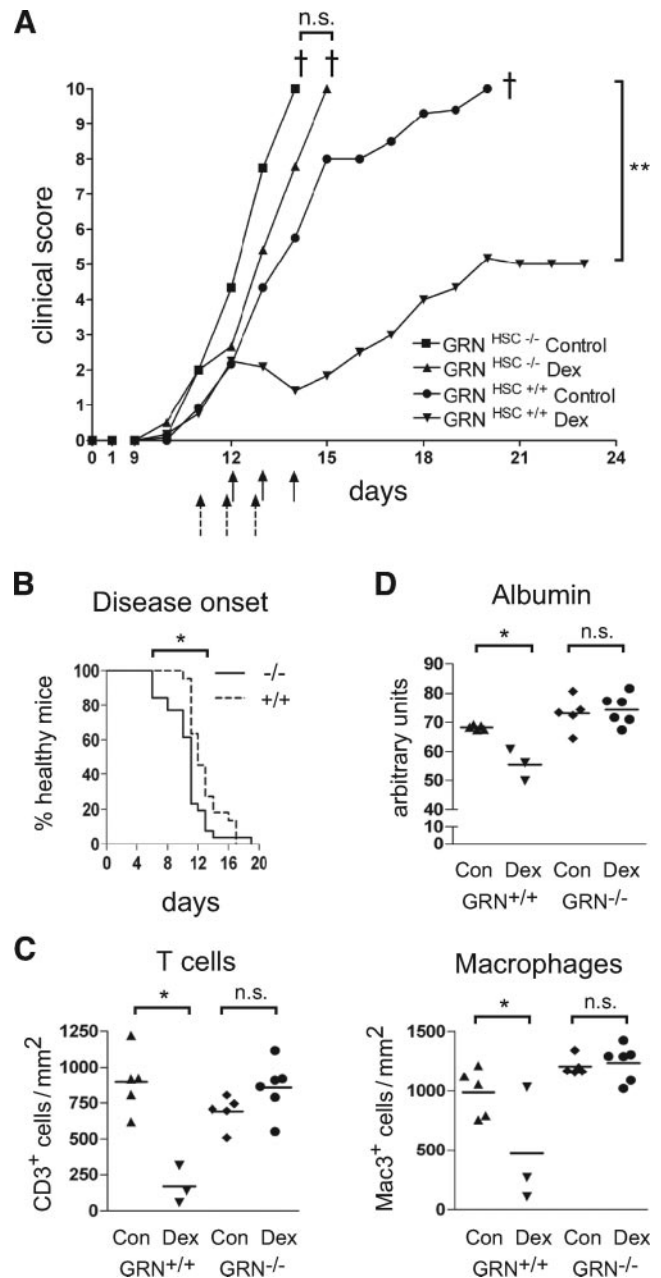


**FIGURE 3.** Haploinsufficiency of the GR attenuates the therapeutic effects of GC treatment. *A*, GRN<sup>+/+</sup> and GRN<sup>+/-</sup> mice were therapeutically treated with 4 mg/kg Dex or PBS (control), starting at an average disease score of 2–3 (arrows). *n* = 7; statistical analysis: days 14–23. *B*, Immunohistochemical analyses of T cells, macrophages, and albumin in the spinal cord of GRN<sup>+/-</sup> and GRN<sup>+/+</sup> mice treated with Dex as in *A* and sacrificed 6 h after the last injection. Each symbol represents one animal. *n* = 8–10.

GRN<sup>HSC+/+</sup> and GRN<sup>HSC-/-</sup> mice were wild type while the leukocytes either lacked or expressed the GR. The number of repopulating lymphocytes as well as their subset distribution was similar after reconstitution in both groups (data not shown), which is in accordance with previously published results (19). A functional test further showed that T cells from GRN<sup>HSC-/-</sup> mice were completely refractory to Dex-induced apoptosis, confirming the successful generation of chimeric mice (data not shown).

MOG-EAE was induced in the two types of chimeric mice followed by administration of 100 mg/kg Dex to half of each group at time points when an average disease score of 2–3 was reached. Notably, EAE was more severe in untreated GRN<sup>HSC-/-</sup> mice as compared with GRN<sup>HSC+/+</sup> controls, manifesting in a premature disease onset and rapid morbidity (Fig. 4*A*). This was confirmed by statistical analysis based on three individual experiments, revealing that GRN<sup>HSC-/-</sup> mutants showed clinical symptoms on average 4 days earlier than control mice (Fig. 4*B*). This suggests that endogenous GCs exert a suppressive effect on hematopoietic cells and thereby reduce the susceptibility to EAE in wild-type mice.

Next, we studied the effect of therapeutic GCs in the chimeric mice. As expected, GRN<sup>HSC+/+</sup> mice could be efficiently treated by Dex, resulting in an immediate and long-term amelioration of the disease, while GRN<sup>HSC-/-</sup> mice hardly responded to high-dose GC therapy (Fig. 4*A*). Although we observed a mild and transient therapeutic effect in mutants on the first day, the disease rapidly aggravated and eventually all GRN<sup>HSC-/-</sup> mice became moribund within a short period of time irrespective of Dex treatment (Fig. 4*A*). Immunohistochemical analysis of the spinal cord confirmed that Dex failed to diminish the number of T cells and macrophages in GRN<sup>HSC-/-</sup> mice while the infiltrate in GRN<sup>HSC+/+</sup> control mice was significantly reduced (Fig. 4*C*).



**FIGURE 4.** The GR in the hematopoietic system is required for the anti-inflammatory effects of Dex in EAE. *A*, MOG-EAE was induced in HSC chimeric mice followed by therapy with 100 mg/kg Dex, starting at an average score of 2–3 (GRN<sup>HSC-/-</sup> mice = dotted arrows, GRN<sup>HSC+/+</sup> mice = full arrows); *n* = 6, statistical analysis: days 13–23, one representative experiment of three is shown. The cross indicates that all animals were prematurely sacrificed for ethical reasons. *B*, Kaplan-Meier curve illustrating the day of the disease onset in GRN<sup>HSC+/+</sup> (*n* = 22; dotted line) compared with GRN<sup>HSC-/-</sup> mice (*n* = 26; full line). *C* and *D*, Immunohistochemical analyses of T cells (*C*), macrophages (*C*), and albumin (*D*) in the spinal cord of GRN<sup>HSC+/+</sup> and GRN<sup>HSC-/-</sup> mice that had been treated with Dex or PBS (control (Con)) as in *A* and sacrificed 6 h after the last injection. Each symbol represents one animal. *n* = 3–6.

Thus, the presence of the GR in hematopoietic cells is essential for the therapeutic success of Dex administration.

GC treatment partially restores the integrity of the BBB (Fig. 2, *A* and *B*), but it is unclear whether this is achieved through direct GC actions on endothelial cells or whether it is rather due to a diminished production of proinflammatory cytokines by leukocytes in

the lesion. Interestingly, GCs did not impact on the permeability of the BBB in  $GRN^{HSC-/-}$  mice despite the presence of the GR in endothelial cells (Fig. 4D). This indicates that Dex primarily reduces lymphocyte infiltration of the CNS and that the protective effect on the BBB is therefore mainly indirect.

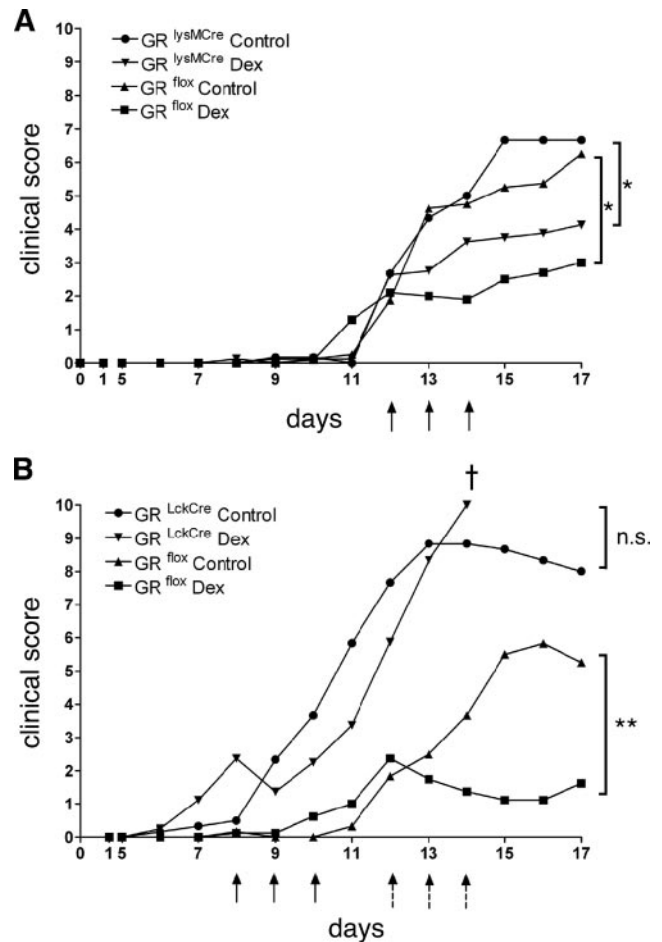
#### T cells but not macrophages are the major targets of GCs

To further dissect the therapeutic Dex effect on hematopoietic cells, we studied the individual contribution of T cells vs macrophages, which are the main CNS-infiltrating cell types in MOG-EAE. To this end, we analyzed cell type-specific GR knockout mice lacking the receptor in either of the two cellular compartments (20, 28). First, we investigated  $GR^{lysMCre}$  mice in which the GR is absent from myeloid cells. Recombination of the floxed GR allele in these animals is almost complete in macrophages and neutrophils, whereas almost no depletion is observed in T and B lymphocytes (20). The onset of the disease in  $GR^{flox}$  control and mutant  $GR^{lysMCre}$  mice was comparable. Furthermore, EAE in mice of both strains could be efficiently treated with Dex, each resulting in a three-point lower disease score as compared with untreated mice (Fig. 5A). Thus, modulation of macrophage functions does not significantly contribute to the efficacy of GCs in EAE therapy.

To reveal a potential role of the GR in T lymphocytes, we used  $GR^{lckCre}$  mice (28), which are capable of mounting normal T cell-dependent immune responses, e.g., in CHS (20). First, the onset of EAE after immunization with MOG was significantly earlier in  $GR^{lckCre}$  mice as compared with  $GR^{flox}$  controls (Fig. 5B). This is in line with the premature development of clinical signs in  $GRN^{HSC-/-}$  mice (see Fig. 4B) and demonstrates that endogenous GCs exert an inhibitory effect on the susceptibility to EAE via their action on T cells. Second, there was a clear attenuation of the therapeutic GC effect (Fig. 5B). Three daily injections of Dex, starting at an average disease score of 2–3 (day 8 for  $GR^{lckCre}$  and day 12 for  $GR^{flox}$ ), resulted in an immediate halt of the disease progression and a long-term reduced disease severity in  $GR^{flox}$  control mice (Fig. 5B). In contrast,  $GR^{lckCre}$  mice only transiently responded to the administration of Dex and already on the second day of the treatment the disease aggravated again. During the next few days, the disease worsened substantially in both PBS- and Dex- treated mice, leading to very high clinical scores, for which reason the majority of mice had to be sacrificed for ethical reasons (Fig. 5B). In conclusion, our findings demonstrate that T cells are the hematopoietic cell population that is most relevant for the therapeutic efficacy of GCs in the treatment of EAE.

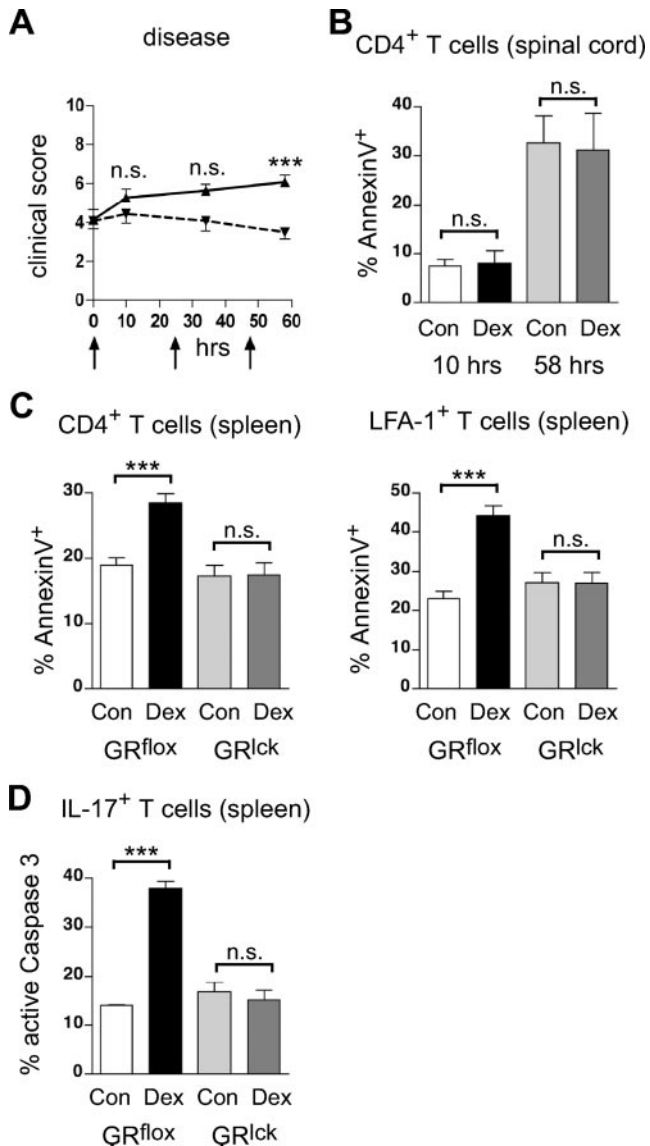
#### GCs induce T cell apoptosis in peripheral lymphoid organs but not in the CNS

Having established T lymphocytes as the major targets of GCs during EAE, we investigated the biological processes that were affected by this treatment. At first, we studied induction of T cell apoptosis in the spinal cord of wild-type mice that had been treated three times with Dex after the onset of EAE by annexin V staining and flow cytometry. Detailed analysis of the disease course revealed that GC administration gradually reduced the clinical symptoms (Fig. 6A). Concomitantly, the number of apoptotic T cells in the CNS increased in both experimental groups between 10 and 58 h after the beginning of treatment, presumably as a consequence of activation-induced cell death. Unexpectedly, however, induction of apoptosis in infiltrating T cells in the spinal cord was completely unaltered by Dex at both time points (Fig. 6B). This suggests that therapeutic efficiency of GCs in EAE is not a consequence of enhanced cell death in the CNS lesion itself.



**FIGURE 5.** T cells but not myeloid cells are essential targets of GCs in the treatment of EAE. *A*, Mice lacking the GR in macrophages ( $GR^{lysMCre}$ ) and  $GR^{flox}$  control mice were therapeutically treated with 100 mg/kg Dex (arrows);  $n = 5$ , one representative experiment of two is shown; statistical analysis: days 13–17. *B*, Mice lacking the GR in T cells ( $GR^{lckCre}$ ) and  $GR^{flox}$  control mice were therapeutically treated with 100 mg/kg Dex ( $GR^{lckCre}$  mice = marked by full arrows;  $GR^{flox}$  control mice = marked by dotted arrows);  $n = 5$ , one representative experiment of two is shown; statistical analysis: days 9–17 and 13–17, respectively. The cross indicates that all animals were prematurely sacrificed for ethical reasons; in the  $GR^{lckCre}$  group treated with PBS half of the mice had to be sacrificed.

Next, we studied splenic T cells from  $GR^{flox}$  and  $GR^{lckCre}$  mice. Interestingly, the level of apoptosis in  $CD4^+$  T cells from  $GR^{flox}$  control animals was significantly increased by Dex at 58 h. This was also true for  $LFA-1^+$  T lymphocytes known to home to the site of inflammation, as well as for T cells expressing the activation marker and cell adhesion molecule CD44 (Fig. 6C and data not shown). To assess the level of apoptosis in  $T_H17$  cells, we had to study caspase 3 activation *ex vivo* due to the incompatibility of intracellular cytokine staining with annexin V binding. Indeed, the number of apoptotic  $IL-17^+$  cells was significantly increased following Dex treatment as compared with controls, indicating that GC treatment also targets bona fide encephalitogenic effector T cells in the spleen (Fig. 6D). Most importantly, the level of apoptosis was unaltered in all cell populations of  $GR^{lckCre}$  mice after Dex administration, confirming that induction of cell death requires the presence of the GR in T cells (Fig. 6, C and D). In summary, peripheral but not infiltrating T cells undergo enhanced

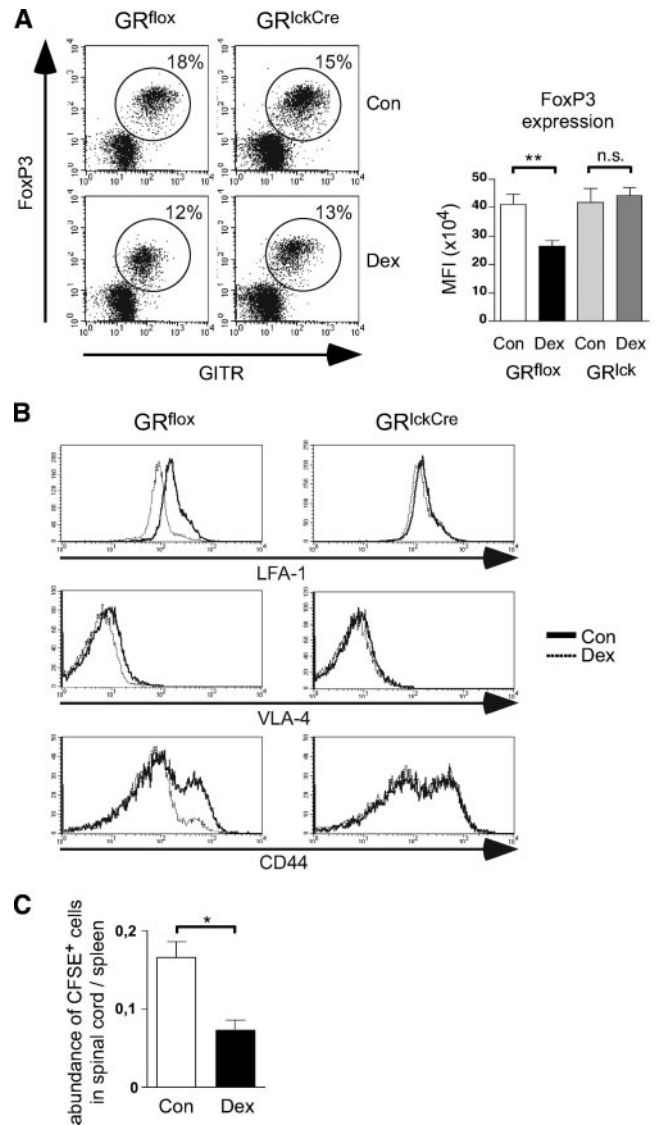


**FIGURE 6.** GCs induce apoptosis in peripheral but not CNS-infiltrating T cells. **A**, C57BL/6 mice were daily treated with Dex (dashed line) or PBS (solid line) three times after the disease onset at an average score of 4 (arrows); the clinical score was determined at the time of injection and 10, 34, and 58 h later;  $n = 14$ . **B**, Leukocytes were isolated at 10 and 58 h from the spinal cord of C57BL/6 mice treated with Dex or PBS as in **A** and apoptosis was determined in CD3<sup>+</sup>CD4<sup>+</sup> cells by flow cytometry based on the binding of annexin V.  $n = 6-13$ . **C**, Splenocytes were isolated from GR<sup>flox</sup> and GR<sup>lckCre</sup> mice at 58 h after the treatment with Dex or PBS as in **A** and apoptosis was determined in CD3<sup>+</sup>CD4<sup>+</sup> cells (*left panel*) or CD3<sup>+</sup>CD4<sup>+</sup>LFA-1<sup>+</sup> cells (*right panel*) by flow cytometry.  $n = 5$ . **D**, Splenocytes from PBS-treated mice were isolated as in **C** and cultured *ex vivo* in the absence (control (Con)) or presence of  $10^{-7}$  M Dex for 20 h. Subsequently, the degree of apoptosis was determined by intracellular staining for active caspase 3 in combination with an anti-IL-17 Ab.  $n = 2$ .

apoptosis after GC treatment, suggesting that an impaired recruitment of T cells to the site of inflammation must be central to therapeutic efficiency.

*Regulatory T cells ( $T_{reg}$ ) do not account for the beneficial effect of GCs on EAE*

Naturally occurring CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>  $T_{reg}$  play a crucial role in the control of pathogenic immune responses such as EAE and MS (29). Experimental evidence further suggested that GCs in-



**FIGURE 7.** Impact of GC treatment on  $T_{reg}$ , expression of adhesion molecules, and T cell migration. **A**, GR<sup>flox</sup> and GR<sup>lckCre</sup> mice were therapeutically treated daily for three times with Dex or PBS, starting at an average score of 2–3 and after 58 h the splenocytes were analyzed by flow cytometry.  $T_{reg}$  were identified among the CD3<sup>+</sup>CD4<sup>+</sup> cells by the concomitant expression of GITR and Foxp3 and their relative frequency is indicated in each dot blot (*left panel*). One representative analysis is depicted;  $n = 3-11$ . The relative expression level of Foxp3 on  $T_{reg}$  was determined as the mean fluorescence intensity (MFI; *right panel*). **B**, Using the same mice as in **A**, surface expression of LFA-1, VLA-4, and CD44 on CD3<sup>+</sup>CD4<sup>+</sup> splenocytes was determined by flow cytometry. One representative analysis is depicted;  $n = 3-11$ . **C**, Splenic T cells were isolated from diseased mice, labeled with CFSE, and retransferred into recipient mice also suffering from EAE. The mice were daily treated with Dex or PBS three times, starting 1 h after adoptive transfer. Fifty-eight hours later, the leukocytes were isolated from spinal cord and spleen and the percentages of CFSE<sup>+</sup> cells among the CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes were determined in both organs. The ratio of their relative frequencies in spinal cord and spleen is depicted as a measure of specific migration.  $n = 3$ .

crease the relative number of  $T_{reg}$ , which was postulated to contribute to their anti-inflammatory activity (30, 31). Therefore, we wondered whether Dex administration might have an effect on  $T_{reg}$  in our model. To address this question, we induced EAE in GR<sup>flox</sup> and GR<sup>lckCre</sup> mice and treated them three times with Dex similar to the previous experiments. Subsequently,  $T_{reg}$  were identified



among the CD4<sup>+</sup> T cells in spleen (Fig. 7A) and spinal cord (data not shown) based on the concomitant expression of FoxP3 and GITR. Against all expectations, Dex administration rather diminished than increased the relative frequency of T<sub>reg</sub> in the periphery as well as in the lesions of GR<sup>fllox</sup> mice. Noteworthy, this effect was much less pronounced in GR<sup>lckCre</sup> mice. Moreover, Dex also reduced the expression level of FoxP3 in splenic T<sub>reg</sub> of GR<sup>fllox</sup> mice while it remained unaltered in GR<sup>lckCre</sup> mice (Fig. 7A). Taken together, these data essentially rule out that T<sub>reg</sub> contribute to the anti-inflammatory activity of GCs in MOG-induced EAE in C57BL/6 mice.

#### *Cell adhesion molecules on T cells are down-regulated by GCs*

Because Dex administration reduces T cell infiltration in the spinal cord without directly inducing cell death in situ, one could assume that GCs presumably block the recruitment of new leukocytes to the site of inflammation. Although induction of peripheral T cell apoptosis is one mechanism, down-regulation of cell adhesion molecules could be another. To test this hypothesis, we determined surface expression of CD44, LFA-1, and VLA-4 on CD3<sup>+</sup>CD4<sup>+</sup> splenocytes, which all represent molecules that are involved in T cell extravasation and homing to the inflamed CNS during EAE (16, 17). Importantly, all three surface receptors were down-regulated on splenic T cells from Dex-treated GR<sup>fllox</sup> mice while their expression remained unaltered in GR<sup>lckCre</sup> mice (Fig. 7B). It is noteworthy that expression of CD44, LFA-1, and VLA-4 was only diminished on peripheral T cells after Dex administration while CNS-residing lymphocytes were completely unaffected (data not shown). Thus, GCs not only reduce the number of peripheral T cells through induction of apoptosis but also compromise their ability to replenish the pool of infiltrating T cells in the CNS by repressing cell adhesion molecules needed for homing to the site of inflammation.

#### *Dex interferes with T cell migration to the CNS*

The previous experiments suggested that GCs might interfere with T cell migration to the spinal cord. To test this hypothesis, we performed a tracking experiment in the context of an ongoing EAE. Splenic T cells were isolated from strongly diseased wild-type mice and labeled with CFSE. Subsequently,  $5 \times 10^6$  cells were adoptively transferred i.v. into syngeneic mice also suffering from EAE (grades 3–4) and allowed to equilibrate for 1 h. The mice were then injected with Dex or PBS daily for three times and 58 h after the cell transfer they were sacrificed, and the frequency of the CFSE<sup>+</sup> cells among the CD3<sup>+</sup>CD4<sup>+</sup> T cells was determined by flow cytometry. As a measure of specific cell migration to the CNS, we analyzed the abundance of CFSE-labeled cells in the spinal cord relative to their abundance in the spleen. Most importantly, our results indicate that the frequency of CFSE<sup>+</sup> infiltrating T cells in the spinal cord was significantly reduced by GC therapy (Fig. 7C). This suggests that Dex interferes with T cell migration and thereby diminishes the fresh supply of peripheral T cells to the CNS lesion.

## **Discussion**

High-dose GC therapy is the measure of choice to treat acute relapses such as optic neuritis in MS patients (2). Currently, the standard regimen is a daily injection of 0.5–2.0 g of methylprednisolone i.v. for 5 days followed by a rapid taper (32). However, adverse effects such as aseptic bone necrosis or psychosis may sometimes complicate the treatment. Although this highlights the need for improved compounds, a deep understanding of the mech-

anism underlying GC actions in neuroinflammatory disorders is still missing.

Traditionally, GCs are thought to act through their cognate receptor, the GR (8). However, the observation of rapid responses challenged the model that all GC actions were transcriptionally mediated through the GR and supported the hypothesis that so-called nongenomic effects exist (27). These could be mediated by the GR itself (33) through an unrelated protein as recently observed for the estrogen receptor (34) or even by unspecific physicochemical interactions with lipid membranes. From this it is debatable whether the GR is necessary for the therapeutic effects of GCs or not. Our experiments now unequivocally demonstrate that the GR is responsible at least for most GC actions in the treatment of EAE and presumably MS. There are three lines of evidence in favor of this notion. 1) Heterozygous GR<sup>+/-</sup> mice are significantly less susceptible to Dex treatment as compared with wild-type littermates. Thus, the level of the GR determines therapeutic success, indicating that GCs impact on the disease via the GR itself. 2) Mice reconstituted with fetal liver cells from homozygous GR<sup>-/-</sup> mice are almost fully refractory to GC therapy. The fact that the GR is completely absent from all hematopoietic cells in these animals confirms that GC effects on leukocytes in EAE intervention require the presence of the receptor itself. 3) Similarly, T cell-specific GR knockout mice hardly respond to Dex, underscoring the relevance of GR actions. We believe that this proves that the majority of GC actions in EAE depend on the presence of the GR. Consequently, the contribution of other mechanisms of GC action is, at best, of minor importance.

In the context of EAE therapy, GCs act on a plethora of cell types. T lymphocytes, macrophages, microglia, endothelial cells, and neurons are only the most important to mention (7). Therefore, we wondered whether GC actions on all these cell types were equally crucial for the efficacy of GC therapy or whether this treatment primarily impacts on a specific subset. Our studies with HSC chimeric mice and conditional knockout mouse strains now show that T cells are the most important targets of Dex in the treatment of EAE. Mice that lack the GR in all hematopoietic cells while retaining expression in endothelial and neural cells were almost fully refractory to Dex treatment and did not restore the integrity of the BBB. The same was true for T cell-specific GR mutant mice, whereas myeloid cell-specific GR mutants respond normally to GC treatment. In contrast, a small but reproducible effect of Dex on the disease course was observed in GR<sup>HSC-/-</sup> and GR<sup>lckCre</sup> mice, indicating that nonhematopoietic cell types may, to a minor extent, also contribute to the therapeutic efficacy of GCs. In the first place, this applies to endothelial cells because the adhesion molecules ICAM-1 and VCAM-1 (35) and the chemokine CXCL10/IFN- $\gamma$ -inducible protein 10 (36) are known to be down-regulated after GC administration. However, the integrity of the BBB was not restored by Dex in GR<sup>HSC-/-</sup> mice, suggesting that a direct impact on endothelial cells is at best transient. An alternative explanation for the limited effects of GC treatment in GR<sup>HSC-/-</sup> and GR<sup>lckCre</sup> mice would be nongenomic effects mediated through a GR-independent mechanism. Thus, additional experiments are required to distinguish between these two possibilities. In summary, our data suggest that GC effects on T cells are most crucial for the efficacy of Dex in the treatment of EAE.

Several mechanisms are currently discussed on how GCs impact on T cells in the treatment of MS and EAE. One is elimination of T lymphocytes by apoptosis, which was previously demonstrated for the monophasic EAE model in Lewis rats (13, 14, 37). We could now show that GCs indeed induce apoptosis in T<sub>H</sub>17 effector as well as other T cells. However, in contrast to previous reports (38), increased apoptosis after Dex administration was restricted to

peripheral T lymphocytes and not seen in infiltrating T lymphocytes. Our observation that the level of cell death in the CNS increases over the 3-day observation period (see Fig. 6B) offers a possible explanation for this discrepancy. Apparently, due to the high local levels of proinflammatory cytokines, pronounced activation-induced cell death occurs during chronic EAE in the spinal cord of C57BL/6 mice, possibly mediated through Fas or galectin-1 (39). This can be expected to selectively counteract GC-induced apoptosis in the CNS but not in peripheral T cells (28, 40). Therefore, we believe that GC-induced apoptosis in EAE and MS primarily pertains to peripheral T cells, at least under conditions of fulminant CNS inflammation.

In recent years, a crucial role of  $T_{reg}$  was established in the control of inflammatory diseases such as EAE and MS (29). Several reports indicate that GCs increase the frequency and potency of naturally occurring  $T_{reg}$  expressing Foxp3 and GITR (30, 31). Most unexpectedly, our study has revealed that in the context of EAE, the relative number of  $T_{reg}$  and the expression level of Foxp3 were even slightly reduced by Dex in a GR-dependent manner. This discrepancy could be the consequence of differences related to the strains or doses used in the EAE experiments (32). Moreover, the frequency of  $T_{reg}$  in MS studies is usually assessed after longer time intervals and not, as in our case, only 58 h after the first Dex injection. Our data therefore argue that enhanced  $T_{reg}$  function is at least no general feature underlying the anti-inflammatory activity of GCs. In contrast, we could confirm that GC treatment reduced the expression of LFA-1, VLA-4, and CD44, cell adhesion molecules that are known to be involved in the homing of effector T cells to inflammatory lesions in EAE and MS. Interestingly, similar to the induction of apoptosis, suppression of cell adhesion molecules by GCs is also restricted to peripheral T lymphocytes, suggesting again that CNS-residing T cells are not a major target of GC action in EAE. Since we nevertheless found reduced leukocyte numbers in the inflammatory lesions of Dex-treated mice, GCs must interfere with T cell migration to the CNS and thereby prevent replenishment by peripheral lymphocytes. This model is supported by our tracking experiment. Significantly less adoptively transferred T cells migrated to the spinal cord after GC administration as compared with control animals. In parallel, they were partially trapped in the spleen. We conclude that Dex impacts on peripheral T cells by inducing apoptosis and reducing cell adhesion molecules crucial for homing to the inflamed tissue. This prevents further lymphocyte influx into the CNS and thereby ameliorates the disease.

Most intriguingly, this study shows that the presence of the GR in myeloid cells is required neither for the attenuation of EAE by endogenous GCs nor for therapeutic efficacy. This contrasts with findings in other inflammatory models such as CHS (20, 28) or septic shock (41). In those models, we and others could show that exclusively GR expression in macrophages and neutrophils is essential for the anti-inflammatory activity of GCs and emphasizes that critical GC target cells differ between distinct inflammatory conditions. In summary, our data reveal for the first time the cell type that is critical for GC therapy in the treatment of EAE. In contrast to competing models, our data also show that the GR itself is a prerequisite for the beneficial effects of GCs in the treatment of EAE. Unexpectedly, peripheral but not CNS-residing T cells are targeted by such therapies. We believe these findings provide a rationale for the development of new therapeutic strategies and pave the way for an improved treatment of MS.

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## Disclosures

The authors have no financial conflict of interest.

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