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Polyclonal expansion of regulatory T cells interferes with effector cell migration in a model of multiple sclerosis

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Recruitment of naturally occurring CD4$^+$ CD25$^+$ regulatory T (T_{reg}) cells is a highly promising approach for the treatment of experimental autoimmune encephalomyelitis (EAE), a widely used model of multiple sclerosis. Here, we studied the *in vivo* interaction of T_{reg} cells, induced by the monoclonal anti-CD28 antibody JJ316, with encephalitogenic T cell lines established from eGFP-transgenic rats. By tracking these fluorescent cells using flow cytometry and confocal microscopy, we found that the activation and expansion of T_{reg} cells inhibited infiltration of the CNS by pathogenic T cells. Interference with effector cell migration occured within the secondary lymphoid organs, since the early therapeutic effects were achieved despite the absence of T_{reg} cells in the spinal cord. However, the delayed homing to the CNS seen after prophylactic JJ316 administration indicates that T_{reg} cells may play an additional role within the target tissue. In addition, the blood–brain barrier remained largely intact after JJ316 treatment, the secretion of T_H2 cytokines was augmented and the encephalitogenic T cells exhibited a reduced secretion of IFN-γ. This in turn resulted in a reduced expression of the chemokine receptor CXCR-3 on effector T cells which may interfere with their capacity to infiltrate the CNS. Importantly, these effects were not achieved by direct action of JJ316 on the encephalitogenic cells. Our data rather suggest that polyclonal activation of T_{reg} cells in the secondary lymphoid organs is instrumental in preventing the pathological transmigration of encephalitogenic T cells into the CNS. We anticipate that these results may help to better understand the role of T_{reg} cells in controlling autoimmunity in the CNS.

Keywords: experimental autoimmune encephalomyelitis; immunotherapy; regulatory T cells; cell migration; CXCR-3

Abbreviations: AT-EAE = adoptive transfer EAE; BBB = blood–brain barrier; EAE = experimental autoimmune encephalomyelitis; T_{conv} = conventional CD4$^+$ T cells; T_{reg} cells = regulatory T cells

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Introduction

The immune system is characterized by its ability to defend the organism against pathogens whilst simultaneously tolerating a variety of tissue-specific self-antigens. In the first place, this is achieved by negative selection in the thymus but some autoreactive T cells still escape this process and need to be held in check. Peripheral tolerance is achieved by several mechanisms including anergy induction, T cell ignorance and suppressive effects of regulatory T (T_{reg}) cells (Mackay, 2000). In particular, the naturally occurring CD4$^+$ CD25$^+$ T_{reg} cells have been identified as key players in this process. They arise in the thymus (Itoh *et al.*, 1999), make up 5–10% of the mature CD4$^+$ T cells and are characterized by the expression of a variety of cell surface receptors such as CTLA-4, GITR, CD134, CD103, TLR4, PD1, Nrp1, TNFRII and 4-1BB (Wing *et al.*, 2005). Whilst most of these markers are also found on other T cells, expression of the transcription factor FoxP3 is restricted to T_{reg} cells and plays an important role for their development (Hori and Sakaguchi, 2004). T_{reg} cells have been

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shown to inhibit autoimmune reactions in a number of animal models including inflammatory bowel disease (Mottet *et al.*, 2003), murine autoimmune diabetes (Lundsgaard *et al.*, 2005; Ott *et al.*, 2005) and experimental autoimmune encephalomyelitis (EAE) (Kohm *et al.*, 2002; McGeachy *et al.*, 2005). Whereas in some models T_{reg} cells were found to exert their function within the inflamed pancreas (Green *et al.*, 2002; Herman *et al.*, 2004), joint (Morgan *et al.*, 2005) or brain (Kleinewietfeld *et al.*, 2005), homing of T_{reg} cells to the place of inflammation appears to be dispensable in an experimental colitis model (Denning *et al.*, 2005). Thus, it remains uncertain whether interactions of T_{reg} cells in secondary lymphoid organs or target tissues are crucial for their suppressive function.

A plethora of potential mechanisms of how T_{reg} cells may inhibit autoimmunity are presently discussed. *In vitro*, they appear to function in a cell–cell contact dependent manner (Bluestone and Tang, 2005), but in contrast, many *in vivo* models provide evidence that suppression rather depends on circulating cytokines such as IL-10 or TGF-β (Annacker *et al.*, 2001; Chen *et al.*, 2005; von Boehmer, 2005). Importantly, also human autoimmune diseases including multiple sclerosis and type I diabetes are associated with an imbalance between T_{reg} and effector T cells, i.e. a loss of functional suppression (Viglietta *et al.*, 2004; Poussier *et al.*, 2005). Thus, T_{reg} cells play an important role in the pathogenesis of autoimmune diseases and are a potential target for immunotherapy.

EAE is a widely used animal model reproducing some important disease mechanisms of multiple sclerosis. In the Lewis rat, EAE follows a monophasic disease course and can either be induced by injecting gpMBP in CFA into the footpad (active EAE) or by adoptive transfer of encephalitogenic T cells (AT-EAE) (Ben-Nun *et al.*, 1981; Swanborg, 2001). The onset of the disease is characterized by autoreactive T lymphocytes crossing the blood–brain barrier (BBB) and secreting proinflammatory cytokines and chemokines. This attracts further autoreactive T cells, predominantly of the T_H1 type, leading to an amplification of the immune response. Subsequently, additional T cells, macrophages and granulocytes are recruited from the recipient's lymphoid organs to the inflammatory lesion, accompanied by complement deposition, antibody production and the generation of free radicals (Owens *et al.*, 2001; Gold *et al.*, 2006; Ransohoff, 2006).

It has previously been shown that the monoclonal anti-CD28 antibody JJ316 allows for the expansion of T cells without a need for TCR engagement (Tacke *et al.*, 1997; Rodriguez-Palmero *et al.*, 1999). *In vivo*, JJ316 preferentially activates and expands T_{reg} cells (Lin and Hünig, 2003) and this is believed to underlie its efficacy in the treatment of rodent models of autoimmune diseases such as EAE (Beyersdorf *et al.*, 2005), experimental autoimmune neuritis (Schmidt *et al.*, 2003*a*) and rheumatoid arthritis (Rodriguez-Palmero *et al.*, 2006). However, how and where the activated T_{reg} cells interfere with the development of the autoimmune response remains elusive. Therefore, we followed $eGFP^+$ encephalitogenic T cells and endogenous $FoxP3^+$ T_{reg} cells in AT-EAE after JJ316 treatment. We show that this leads to an inhibition of AT-EAE by expanding $FoxP3^+$ T_{reg} cells in the secondary lymphoid organs, and blocks infiltration of the spinal cord by encephalitogenic T cells. This can be assigned to the downregulation of IFN-γ and subsequently reduced expression of the chemokine receptor CXCR-3, which is required for the homing of encephalitogenic T cells to the CNS. Thus, JJ316 interferes with a pathological autoimmune reaction through inhibition of T effector cell migration.

Material and methods

Animals

Lewis rats were purchased from Charles River (Sulzfeld, Germany) and used at 6–8 weeks of age. eGFP-transgenic Lewis rats (UGC) were bred in our own animal facility (van den Brandt *et al.*, 2004*b*; Herrmann *et al.*, 2005). All experiments were conducted according to Bavarian state regulations for animal experimentation and approved by the responsible authorities.

T cell culture

The encephalitogenic MBP-specific T cell line MBP-V1 and the $eGFP^+$ line GFP–MBP-V were established either from wild-type or eGFP-transgenic Lewis rats by four rounds of antigenic restimulation as described earlier (Gold *et al.*, 1995; Jung *et al.*, 1995). Both cell lines were cultured in RPMI 1640 medium supplemented with 10% normal rat serum, 2 mM glutamine and standard antibiotics. Prior to disease induction, 3×10^6 MBP-specific T cell blasts were freshly thawed and activated for 3 days using 1.5×10^8 irradiated (3000 rad) thymocytes and 10 μg/ml gpMBP. At the end of the culture period, the encephalitogenic cells were purified by gradient centrifugation, resuspended in phosphate-buffered saline (PBS) and used for injection. T cell specificity was tested *in vitro* in 96-well microtitre plates using 1.5×10^4 responder T cells, 7.5×10^5 irradiated thymocytes and graded doses of gpMBP (Eylar *et al.*, 1974).

To investigate the influence of JJ316 on the encephalitogenic T cells *in vitro*, 5×10^5 cells/well were cultured in 12-well plates for 48 h in the presence of 5 μg/ml JJ316 or in RPMI complete medium only.

Proliferation assay

Encephalitogenic T cells were cultured in 96-well plates at a density of 2×10^5 cells/well in RPMI complete medium in the presence or absence of 5 μg/ml JJ316. Proliferation was assessed on the basis of [^{3}H]thymidine incorporation, added during the last 18 h of a 72 h culturing period. The DNA of the [^{3}H]thymidine-pulsed cells was harvested onto fibreglass filters and the amount of incorporated radioacitivity was quantified using a β-plate reader.

Purification of $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells

Single-cell suspensions were prepared from the lymph nodes of rats that had been injected with 1 mg of JJ316 3 days before. Subsequently, T cell subsets were purified by magnetic cell sorting using an AutoMACS machine according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). At first, $CD4^+$ T cells were enriched by negative depletion of $CD8^+$ T cells and B cells using anti-CD8α and anti-CD45RA microbeads. Afterwards, $CD4^+$

cells were stained with a biotinylated mAb against CD25 (clone OX39, BD Pharmingen, Heidelberg, Germany) followed by positive selection using streptavidin microbeads. The purity of the $CD4^+$ $CD25^+$ and the $CD4^+$ $CD25^-$ T cells was 95% on average.

Induction of AT-EAE and experimental design

Typically AT-EAE was induced by injecting 8×10^6 MBP-specific $eGFP^+CD4^+$ T cells (see above) into the tail vein of wild-type Lewis rats. One milligram of JJ316 or PBS as a control was administered i.v. on Day 1 (prophylactic treatment) or on Day 3 after adoptive transfer of the encephalitogenic T cells, just at disease onset (therapeutic approach). Animals were weighed daily and inspected for signs of EAE. The severity of EAE was assessed employing a 6-grade disease score as follows: 0 = healthy; 1 = weight loss, limp tip of tail; 2 = limp tail, mild paresis; 3 = moderate paraparesis, ataxia; 4 = tetraparesis; 5 = moribund; 6 = dead (Schmidt *et al.*, 2003*b*). To follow the clinical score and the change in body weight over time, some animals from each group were left alive during the whole course of the experiment until they had fully recovered from the disease. In addition, animals were sacrificed on Days 2, 4, 6, 8, 10 and 14 (prophylactic group) or Days 3, 5, 7 and 10 (therapeutic group), and analysed by histology and flow cytometry.

To investigate the migration pattern of co-transferred indicator cells in AT-EAE after JJ316 treatment, 1×10^7 $eGFP^+$ T_{reg} cells or 1×10^7 $eGFP^+$ conventional $CD4^+$ T cells (T_{conv}) cells were purified from antibody-treated eGFP-transgenic Lewis rats and injected into the tail vein on Day 0 in parallel with 8×10^6 $eGFP^-$ encephalitogenic T cells. In a control experiment $eGFP^+$ T_{reg} or $eGFP^+$ T_{conv} were transferred without inducing the disease. In both settings, 1 mg JJ316 was administered on the day following adoptive transfer. The rats were sacrificed on Day 6 and the abundance of $eGFP^+$ cells was determined by flow cytometry and cell counting.

Flow cytometry

All antibodies used during FACS analysis were obtained from BD Biosciences unless otherwise indicated: Ox35 (CD4), Ox38 (CD4), Ox39 (CD25), Ox40 (CD134), R73 (αβTCR), DB-1 (IFN-γ), OX-81 (IL-4), A5-4 (IL-10) and FJK-16s (FoxP3, eBioscience, San Diego, USA). Extracellular staining was performed as previously described (van den Brandt *et al.*, 2004*a*), for the intracellular staining of FoxP3, IFN-γ, IL-4 and IL-10 we followed the protocol provided by eBioscience. To allow for intracellular staining of cytokines in cultured encephalitogenic T cells, they were stimulated with phorbol 12-myristate 13-acetate (PMA) (5 ng/ml) and ionomycin (500 ng/ml) for 2 h followed by treatment with GolgiPlug according to the manufacturer's instructions (BD Biosciences, Heidelberg, Germany).

Cytokine assay

In vitro production of IL-4, IL-10 and IFN-γ by freshly isolated splenocytes or cultured encephalitogenic T cells (see above) was determined using the cytometric bead array (CBA) according to the manufacturer's instructions (BD Pharmingen, Heidelberg, Germany). Splenocytes were isolated on Day 4 after disease induction followed by erythrocyte lysis. A total of 2×10^6 cells/well were cultured in duplicate in 48-well plates for 24 h using X-vivo15 medium (Bio Whittaker, Rockland, USA). The cells were either stimulated with 10 μg/ml gpMBP (Eylar *et al.*, 1974), 2 μg/ml Con A (Sigma-Aldrich, Taufkirchen, Germany) or left untreated. The total amount of cytokines was determined using the FCAP Array 1.0 software (Soft Flow).

Histological analysis and immunocytochemistry

Samples from cervical spinal cord were embedded in paraffin, 5 μm sections mounted on poly-L-lysine coated slides and processed as described (Zettl *et al.*, 1995). Pretreatment with hydroxylamine (0.9%, Sigma-Aldrich, Taufkirchen, Germany) was required for the albumin staining. Immunohistochemistry was performed as detailed previously (Zettl *et al.*, 1995). For detection of pan-T cells, serial sections were stained with the monoclonal antibody B115-1 (Holland Biotechnology, Lelden, Netherlands) and for the analysis of macrophages with ED 1 (Serotec, Düsseldorf, Germany). An anti-albumin antibody (Nordic, Tilburg, Netherlands) was used to study the integrity of the BBB. Stainings were visualized using the ABC-system (Dako, Hamburg, Germany) with New fuchsin as alkaline phosphatase substrate, or 3,3′-diaminobenzidine as peroxidase substrate. Coded sections from spleens, cervical and mesenteric lymph nodes and spinal cords were examined by masked observers.

Confocal microscopy

$CD4^+$ T cells were attached to poly-L-lysine coated chamber slides (Lab-Tek II, Nunc, Wiesbaden, Germany), fixed with 3.7% PFA and permeabilized with 0.1% Triton. After blocking with 5% BSA, samples were incubated with anti-GFP (Abcam, Cambridge, UK) and anti-CXCR-3 (clone 17, Santa Cruz, Heidelberg, Germany) antibodies overnight at 4°C. Subsequently, the cells were stained with secondary antibodies for 45 min at room temperature (Alexa594-conjugated anti-goat for the detection of CXCR-3 and Alexa488-conjugated anti-rabbit for the detection eGFP, both from Molecular Probes, Karlsruhe, Germany). Imaging was performed using a Zeiss LSM510 confocal microscope and a 63× oil objective (NA 1.4). Laser lines at 488 and 543 nm were used for excitation and 150–200 $eGFP^+$ cells were analysed per sample. Image acquisition and quantification of the fluorescence intensity were performed with the Zeiss LSM510 Software 3.2 SP2. In brief, the total fluorescence intensity measured by the program was added up within the borders of individual cells, resulting in the depicted absolute values. Spleen cells from eGFP-transgenic rats cultured with or without 100 U/ml IFN-γ for 24 h and cultured encephalitogenic cells served as a control for the expression of CXCR-3.

Statistical analysis

The data were analysed by Mann–Whitney rank sum test or one-way ANOVA and Tukey HSD as *post hoc* test as indicated in the text (Statistica 6.0, Statsoft GmbH). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ were considered as significant *P*-values; n.s. = non-significant.

Results

Prophylactic administration of the monoclonal antibody JJ316 prevents leucocyte infiltration into the spinal cord

Previously, we have shown that administration of the monoclonal anti-CD28 antibody JJ316 ameliorates the disease

course in a number of different EAE models by preferentially activating and expanding T_{reg} cells (Beyersdorf *et al.*, 2005). To further investigate the underlying mechanism we induced AT-EAE by transferring eGFP$^+$ encephalitogenic T cells into female Lewis rats. Subsequently, we studied their migration pattern and abundance in the secondary lymphoid organs and the spinal cord. The encephalitogenic T cell lines used in this experiment have been generated in eGFP-transgenic Lewis rats (van den Brandt *et al.*, 2004*b*), thus excluding any skewing of their functional properties by retroviral transduction (Flügel *et al.*, 1999). Rats treated with JJ316 on the day following disease induction remained healthy over the whole experimental period. In contrast, the PBS-treated animals showed the typical disease course (Schmidt *et al.*, 2003*b*) characterized by paraparesis (Fig. 1A) and weight loss (data not shown). By Day 14 all animals had fully recovered without residual signs of paralysis (Fig. 1A). Importantly, no relapses occurred in either of the two groups up to Day 18 after disease induction, indicating that JJ316 confers long-term protection against AT-EAE (data not shown).

To determine the effect of JJ316 on the infiltration of the CNS by eGFP$^+$ encephalitogenic T cells, we studied their abundance by flow cytometry in spinal cord homogenates. In accord with previous reports (Flügel *et al.*, 2001) the pathogenic cells started to infiltrate the spinal cord of control rats around Day 3 after disease induction and reached a maximum between Day 4 and Day 5. Subsequently, their number declined and from Day 8 on they were almost undetectable (Fig. 1B). In JJ316-treated animals the situation was completely different. Although in general eGFP$^+$ encephalitogenic T cells were even more abundant in the secondary lymphoid organs (Fig. 1B), they only scarcely infiltrated the CNS. Up to Day 4 after disease induction no eGFP$^+$ encephalitogenic T cells could be detected in the spinal cord of JJ316-treated rats and even at later time points their numbers remained small (Fig. 1B). In summary, administration of JJ316 prevents EAE by interfering with the infiltration of encephalitogenic T cells into the CNS.

To analyse whether JJ316 treatment reduced the activation of the eGFP$^+$ encephalitogenic T cells we stained spleen and lymph node cells with monoclonal antibodies against CD25 and CD134, two markers indicating reactivation of encephalitogenic T cells during EAE (Flügel *et al.*, 2001). Despite their compromised ability to migrate into the spinal cord, expression of CD25 and CD134 on the eGFP$^+$ encephalitogenic T cells was even higher in animals injected with JJ316 as compared with controls (Fig. 1C). Thus, although activation and expansion of the pathogenic T cells was boosted after antibody administration they still failed to infiltrate the spinal cord.

Finally, we assessed leucocyte infiltration of the CNS by immunohistochemistry. On Day 4 after disease induction T cells and macrophages were highly abundant, both in the white and the grey matter of the spinal cord of control animals (Fig. 2). In contrast, after administration of JJ316 they were only detectable in small numbers (Fig. 2). Notably,

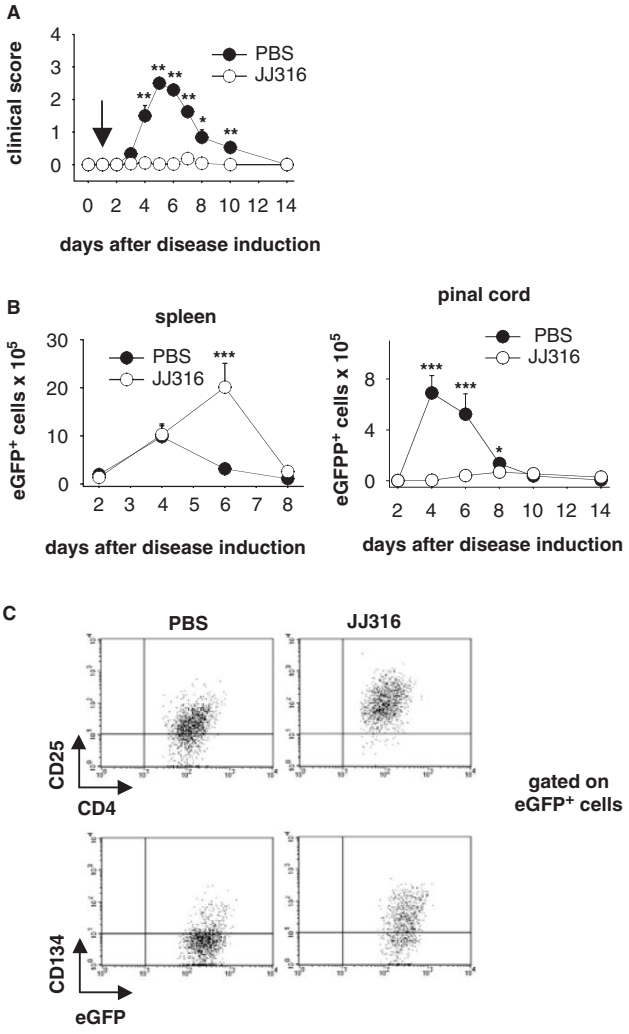

Fig. 1 Prophylactic administration of JJ316 prevents infiltration of the spinal cord. (**A**) AT-EAE was induced in female Lewis rats using eGFP$^+$ encephalitogenic T cells. JJ316 or PBS was injected 1 day later (marked by an arrow). Animals were scored for clinical signs of EAE as described in Material and methods. $n = 10–12$ per group; mean ± SEM; data were pooled from two individual experiments. (**B**) The absolute cell number of eGFP$^+$ encephalitogenic T cells in spleen and spinal cord was calculated from their percentages among all live cells (as determined by flow cytometry) and the total cell count of the respective tissue preparations (as determined by microscopy). This was performed at different time points during AT-EAE after administration of PBS or JJ316. $n = 6–11$ per group; mean ± SEM; data were pooled from three experiments. (**C**) Expression of activation markers by eGFP$^+$ encephalitogenic T cells. Spleen cells from animals treated with PBS or JJ316 were isolated on Day 4 after disease induction and stained either for CD4/CD25 or CD134. From a group of six animals, the result for one representative rat is shown. Statistical analysis was performed using the Mann–Whitney *U*-test.

eGFP$^+$ encephalitogenic T cells in the spleen were present at a similar frequency in both groups at that time (Fig. 1B). This supports our notion that JJ316 interferes with the pathological transmigration of T cells and macrophages into the CNS.

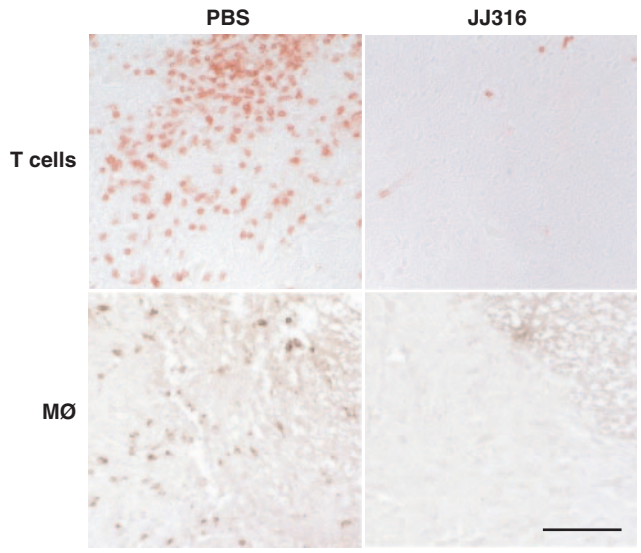

Fig. 2 Leucocyte infiltration into the spinal cord. On Day 4 after disease induction the cervical part of the spinal cord was analysed by immunohistochemistry for the prevalence of T cells and macrophages (MØ) in the white and in the grey matter. The result for 1 representative animal out of 11 is depicted. Bar = 100 μm.

T_{reg} cells infiltrate the spinal cord late after JJ316 administration

JJ316 has been shown to inhibit EAE by preferentially activating and expanding T_{reg} cells but their site of action has remained elusive. Therefore, we investigated the effect of prophylactic JJ316 treatment on the frequency of T_{reg} cells in spleen, lymph nodes and spinal cord in the context of an ongoing AT-EAE by flow cytometry using monoclonal antibodies against CD4 and FoxP3. As expected, administration of the monoclonal antibody JJ316 on the day following AT-EAE induction led to a massive increase in the number of T_{reg} cells in spleen as compared with the control group (Fig. 3A). The maximum of expansion was observed on Day 6 and remained significantly elevated throughout the experiment. In contrast, increased numbers of T_{reg} cells were never observed in animals injected with PBS. Similar results for the expansion of T_{reg} cells were obtained in the cervical and mesenteric lymph nodes (data not shown). Importantly, we could not detect T_{reg} cells in the spinal cord up to Day 4 after disease induction in either experimental group (Fig. 3A). At that time the disease in control rats had almost reached its peak, whereas the JJ316-treated animals were still healthy. This strongly suggests that T_{reg} cell actions at the site of inflammation are dispensable for the preventive effect of JJ316 in EAE. However, we observed increasing infiltration of the spinal cord by T_{reg} cells in antibody-treated animals from Day 6 on, reaching a maximum at Day 8 (Fig. 3A). Subsequently their number declined, albeit remaining significantly elevated until Day 14. In contrast, the number of T_{reg} cells in the spinal cord of PBS-treated rats did not significantly increase at any time point during the course of the analysis.

Besides their absolute number, also the percentage of T_{reg} cells among the $CD4^+$ T cells was strongly elevated on Days 6 and 8 in JJ316-injected animals, both in spleen and spinal cord (Fig. 3B and data not shown). In contrast, T_{reg} cells were never detected in significant numbers in the spinal cord of healthy rats, irrespective of whether they had been treated with JJ316 or not (Fig. 3B). Thus, T_{reg} cells in JJ316-treated rats start to home to the site of inflammation once a small number of pathogenic T cells have infiltrated the CNS (see Fig. 1B). This appears to be a specific characteristic of T_{reg} cells since such behaviour was never observed for conventional non-encephalitogenic T cells (data not shown). Also this effect was only observed after JJ316 treatment, i.e. JJ316 does not only preferentially expand but also activate T_{reg} cells. In summary, specific migration of T_{reg} cells into the CNS is observed only after the presumed peak of disease, which is too late to explain the prophylactic effect of JJ316 on EAE. However, it might still prevent a delayed onset of EAE caused by the few infiltrating encephalitogenic T cells.

Finally, we wished to confirm that administration of JJ316 indeed induced preferential migration of T_{reg} cells to the spinal cord after induction of EAE. Therefore, we isolated both T_{reg} and T_{conv} cells from eGFP-transgenic rats and used them as indicator cells in the adoptive transfer model. Either $eGFP^+$ T_{reg} or T_{conv} cells were transferred together with $eGFP^-$ pathogenic cells. JJ316 was administered on the day following AT-EAE induction and the fate of the $eGFP^+$ indicator cells was studied. On Day 6, a considerable number of $eGFP^+$ T_{reg} cells had infiltrated the spinal cord, whereas only a few $eGFP^+$ T_{conv} cells could be detected (Fig. 3C). In contrast, minute numbers of transferred $eGFP^+$ cells were present in the spinal cord of healthy control animals, i.e. without co-injection of encephalitogenic T cells and irrespective of whether the rats had been treated with JJ316 or not (Fig. 3C). This indicates that the T_{reg} cells are specifically recruited to the CNS during the late phase of the ongoing AT-EAE.

Therapeutic administration of JJ316 ameliorates EAE without driving T_{reg} cells into the spinal cord

Next, we wondered whether JJ316 was also able to ameliorate the disease when administered in a therapeutic setting, i.e. at a time point when the spinal cord had already been infiltrated by pathogenic T cells. AT-EAE rats were treated with JJ316 or PBS on Day 3 after cell transfer. After administration of the antibody there was an immediate halt of clinical progression, whereas the disease further progressed in control animals. Two days after JJ316 treatment the rats had already fully recovered from the disease whilst the controls were still severely affected (Fig. 4A). Interestingly, JJ316 treatment caused an expansion of the $eGFP^+$ encephalitogenic T cell population in the spleen. Yet they stopped to further infiltrate into the spinal cord and subsequently their numbers declined

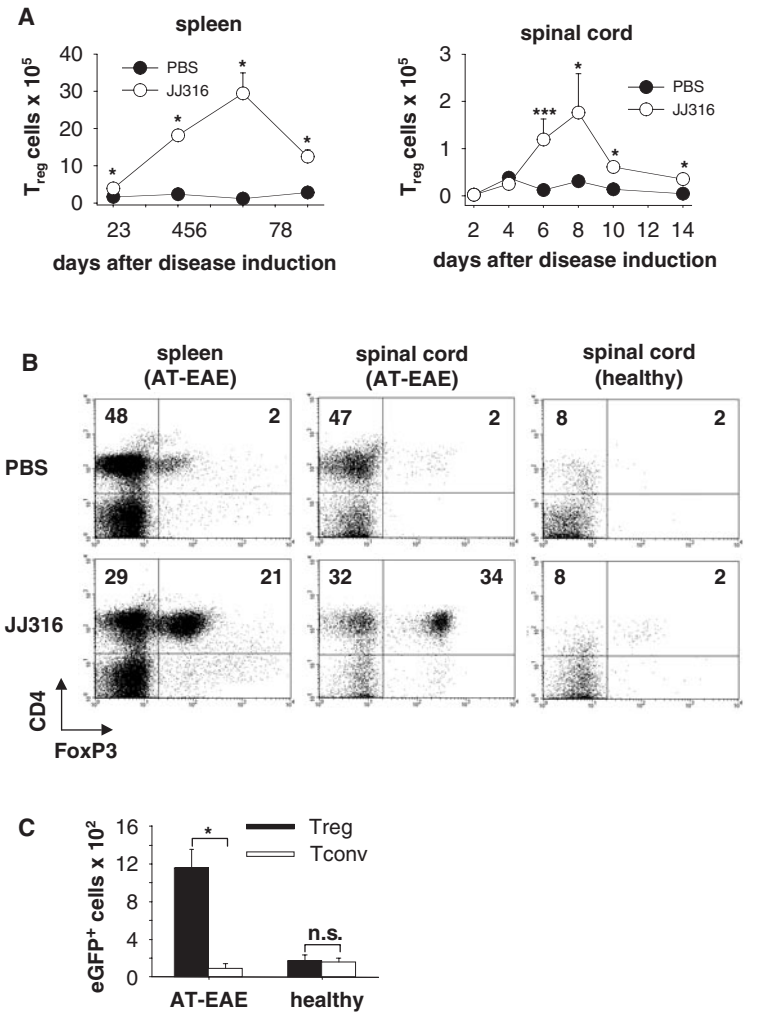

Fig. 3 Expansion and migration of T_{reg} cells after JJ316 treatment. (**A**) The absolute number of T_{reg} cells isolated from animals treated with PBS or JJ316 was determined at different time points after disease induction in spleen and spinal cord as described for Fig. 1 using monoclonal antibodies against CD4 and FoxP3. $n = 3$ for spleen and $n = 11$ for the spinal cord; mean ± SEM; data were pooled from two experiments. (**B**) AT-EAE was induced by transfer of encephalitogenic T cells or the animals were left untouched (referred to as 'healthy'). On the following day either PBS or JJ316 was administered. The percentage of $CD4^+FoxP3^+$ T_{reg} cells was then determined by flow cytometry in the spleen on Day 6 and in the spinal cord on Day 8 (corresponding to the maximum of expansion in each tissue). (**C**) Either $eGFP^+$ T_{reg} or T_{conv} cells were injected as indicator cells along with $eGFP^-$ encephalitogenic cells that were used to induce AT-EAE. Control animals received $eGFP^+$ indicator cells in the absence of encephalitogenic T cells (referred to as 'healthy'). One day later the animals were injected with 1 mg JJ316 and on Day 6 the total number of $eGFP^+$ T_{reg} or T_{conv} cells in the spinal cord was determined. $n = 3$ per group; mean ± SEM; one out of two representative experiments is shown. Statistical analysis in all experiments was performed by the Mann–Whitney *U*-test.

progressively. This is in sharp contrast to the PBS-treated animals, where the number of pathogenic T cells was maximal on Day 5 and still elevated on Day 7 (Fig. 4B). Finally, on Day 10 after disease induction, the number of encephalitogenic T cells was almost undetectable in both groups. As expected, administration of JJ316 also induced a strong expansion of the T_{reg} cells in the spleen. However, T_{reg} cells were never detected in the spinal cord, neither in JJ316-treated nor in control rats (Fig. 4C). This strongly supports our conclusion that inhibition of encephalitogenic T cells by T_{reg} cells occurs in the secondary lymphoid organs.

Administration of JJ316 strongly diminishes disruption of the BBB

To search for possible mechanisms by which JJ316 prevents infiltration of encephalitogenic T cells into the spinal cord, we first analysed the effect of the antibody treatment on the integrity of the BBB. This was achieved by an established approach using anti-albumin immunohistochemistry, considering the staining intensity as a semiquantitative measure for the permeability of the BBB (Schmidt *et al.*, 2003*b*). Again we used prophylactic treatment with JJ316 or PBS in AT-EAE as our model. Disruption of the BBB was more pronounced in PBS-treated animals as compared with rats having

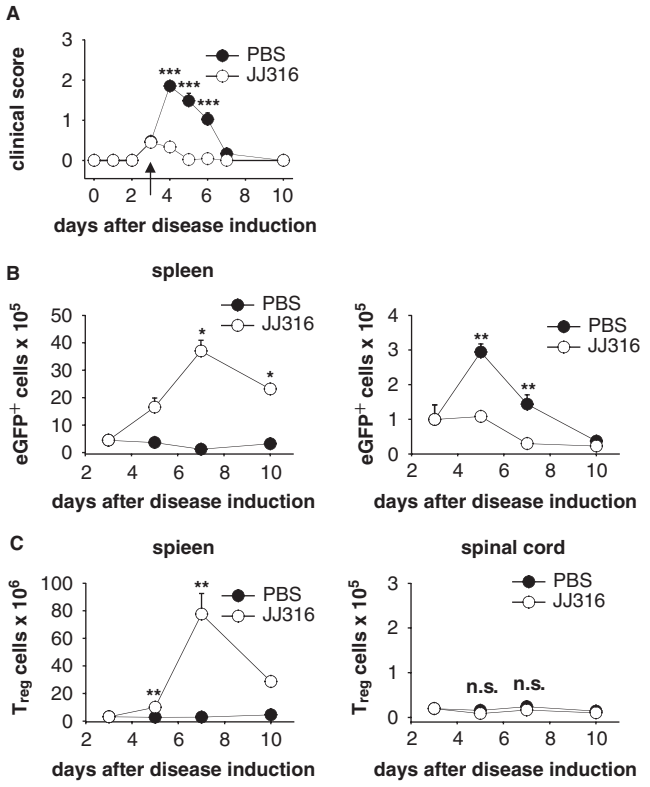

Fig. 4 Therapeutic administration of JJ316. (**A**) PBS or JJ316 was administered on Day 3 after disease induction to female Lewis rats with first signs of AT-EAE and the animals were scored for clinical signs of EAE as described in Material and methods. $n = 6$–18 per group; mean ± SEM; the data were pooled from two experiments because the groups were very similar; the arrow indicates the time point when JJ316/PBS was injected. (**B**) The total cell number of eGFP$^+$ encephalitogenic T cells in the spleen and the spinal cord was determined as described for Fig. 1. $n = 6$ per group; mean ± SEM; the data were pooled from two experiments. (**C**) The total cell number of T_{reg} cells in the spleen and the spinal cord was determined as described for Fig. 1. $n = 6$ per group; mean ± SEM; the data were pooled from two experiments. Statistical analysis was performed using the Mann–Whitney *U*-test.

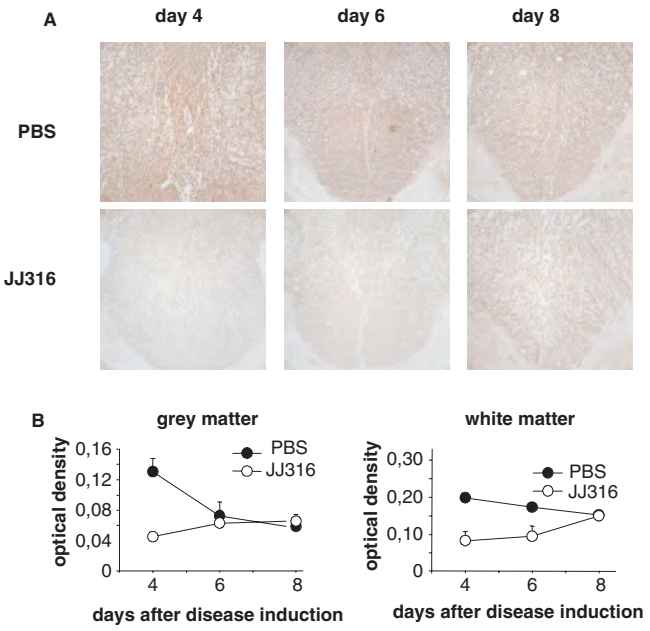

Fig. 5 Integrity of the BBB. (**A**) AT-EAE was induced followed by treatment with PBS or JJ316 on Day 1; sections from the cervical part of the spinal cord were obtained at various time points. Subsequently, they were stained with an anti-albumin antibody and visualized with immunoperoxidase to determine the degree of BBB disruption. Histological sections from the grey matter are depicted to exemplify the observed differences. (**B**) The albumin-staining intensities in the grey and white matter of the spinal cord were quantified by densitometry ($n = 3$ per group, mean ± SEM), using the MetaVue program, version 6.3r2 (Visitron Systems, Munich, Germany).

received JJ316, both in the white and in the grey matter (Fig. 5A). On Day 4, the disease-associated permeability of the BBB was maximal in the control group and its integrity was only slowly restored. In contrast, almost no albumin was detected in the spinal cord of JJ316-treated rats on Days 4 and 6 after disease induction and only on Day 8 a weak staining was observed. These findings were confirmed by quantifying the albumin stainings in the grey as well as in the white matter of the spinal cord by densitometry (Fig. 5B). Importantly, the weak but increasing leakage of the BBB in JJ316-treated rats is presumably due to the few encephalitogenic T cells found in the CNS from Day 6 on and may also explain the homing of T_{reg} cells to the spinal cord seen around that time. Taken together, the disruption of the BBB, both in the white and the grey matter, correlates with the degree of infiltration of the spinal cord by both pathogenic and T_{reg} cells (Figs 2 and 5).

Administration of JJ316 induces a T_H2 shift and inhibits IFN-γ production

After having identified the secondary lymphoid organs as the primary site of T_{reg} cell action in our setting, we aimed at characterizing the phenotypic changes induced in encephalitogenic T cells upon JJ316 administration. To this end, we analysed the cytokine profile of splenocytes isolated from AT-EAE rats after prophylactic administration of either JJ316 or PBS. Spleen cells were obtained on Day 4 after disease induction and activated for 24 h using Con A or gpMBP, the cognate antigen of the encephalitogenic T cells. Subsequently, the amount of IL-4, IL-10 and IFN-γ in the supernatants was analysed by cytokine bead array. Whereas IL-4 and IL-10 are the characteristic cytokines of a T_H2 dominated immune response, IL-10 is also produced by T_{reg} cells (Lin and Hünig, 2003). In contrast, IFN-γ is typically made by T_H1 cells and represents the major effector cytokine of the pathogenic T cells (Renno *et al.*, 1998). After activation with Con A, splenocytes from JJ316-treated rats secreted significantly elevated amounts of IL-4 and IL-10 as compared with PBS-treated animals (Fig. 6). In the presence of gpMBP, the cultured cells failed to produce any T_H2 cytokines in both experimental groups (Fig. 6). This suggests that T cells other than the encephalitogenic cells produce IL-4 and IL-10 after

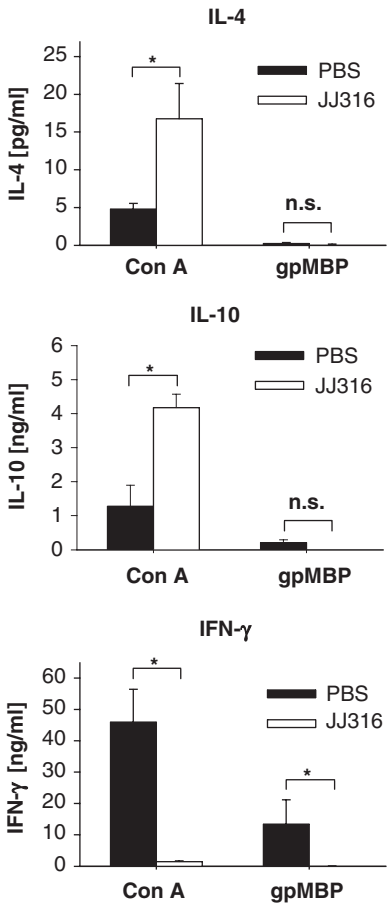

Fig. 6 Cytokine profile of spleen cells during AT-EAE. Spleen cells were isolated on Day 4 after disease induction from animals that had been prophylactically treated with PBS or JJ316, and these were stimulated with Con A or gpMBP for 24 h. The amounts of IL-4, IL-10 and IFN-γ in the supernatants were analysed as described in Material and methods (n = 3 per group; statistical analysis by the Mann–Whitney U-test).

administration of JJ316 and indicates a generalized T_H2 shift. More importantly, the production of the T_H1 cytokine IFN-γ, both after restimulation with Con A and gpMBP, was completely abrogated in splenocytes isolated from JJ316-injected rats (Fig. 6). Thus, JJ316 inhibits the production of IFN-γ by encephalitogenic T cells, most likely through activated T_{reg} cells.

JJ316 downregulates CXCR-3 expression on encephalitogenic T cells

CXCR-3 expression is induced by IFN-γ on pathogenic T cells, allowing them to home to sites of inflammation (Engelhardt and Ransohoff, 2005). Moreover, T cells expressing this chemokine receptor are enriched in the cerebrospinal fluid of multiple sclerosis patients as well as rats and mice undergoing EAE (Sorensen *et al.*, 1999; Xie *et al.*, 2003). Therefore, we asked whether reduced CXCR-3 expression may explain the impaired effector T cell migration seen in our model.

Confocal microscopy revealed specific membrane staining for CXCR-3 on $eGFP^+$ spleen cells from a naïve rat cultured in the presence of IFN-γ but not in controls (Fig. 7A). In addition, the encephalitogenic cells used for disease induction also stained positive for CXCR-3 (Fig. 7A and D). Notably, the eGFP fluorescence was always observed as a ring-like structure owing to the fact that the cytosol in lymphocytes forms only a thin rim around the prominent nucleus (Fig. 7A).

Firstly, we determined the expression level of CXCR-3 on Day 4 after disease induction on encephalitogenic T cells isolated from spleen and spinal cord of animals that had prophylactically received PBS or JJ316. Whilst almost all encephalitogenic T cells express high levels of CXCR-3 before transfer, the vast majority of them continued to express CXCR-3 in PBS-treated animals, both in spleen and spinal cord (Fig. 7B and D). In contrast, the percentage of CXCR-3 positive encephalitogenic T cells was significantly reduced in the spleen of JJ316-treated rats (Fig. 7B and D). Furthermore, also the fluorescence intensity of CXCR-3 on $eGFP^+$ encephalitogenic T cells in the spleen was strongly diminished after JJ316 treatment (Fig. 7B and C). Expression of CXCR-3 on encephalitogenic T cells in the spinal cord of JJ316-treated rats could not be assessed since the cells were not present in the CNS under these conditions (see above).

Next, we investigated whether CXCR-3 was also downregulated on non-encephalitogenic T cells. To this end, T cells were purified from spleen and spinal cord using magnetic bead separation and analysed by confocal microscopy. Only a minor fraction of the splenic T cells from a naïve rat express CXCR-3 (Fig. 7E). However, on Day 4 after disease induction, the percentage of $CXCR\text{-}3^+$ $eGFP^-$ T cells was increased in PBS-treated rats as compared with healthy animals (Fig. 7E). This suggests that CXCR-3 is induced also on bystander cells during AT-EAE. Interestingly, administration of JJ316 prevented an increase in the percentage of $CXCR\text{-}3^+$ cells among the $eGFP^-$ splenic T cells as compared with the PBS control (Fig. 7E). Thus, T_{reg} cell mediated inhibition of IFN-γ production is associated with impaired expression of CXCR-3, both on encephalitogenic and bystander T cells, which presumably is the key event in preventing leucocyte infiltration of the CNS after JJ316 treatment.

JJ316 activates encephalitogenic T cells *in vitro*

Finally, we wanted to exclude that JJ316 confers protection from AT-EAE by directly repressing the pathogenic cells. To this end, the encephalitogenic T cells used for disease induction were cultured in the presence or absence of JJ316 and analysed by flow cytometry and confocal microscopy. Firstly, we found that the pathogenic cells contained <0.2% of T_{reg} cells (Fig. 8A). Moreover, culture in the presence of JJ316 did neither result in an induction nor an accumulation of T_{reg} cells although the antibody clearly induced proliferation (Fig. 8A and B). This indicates that

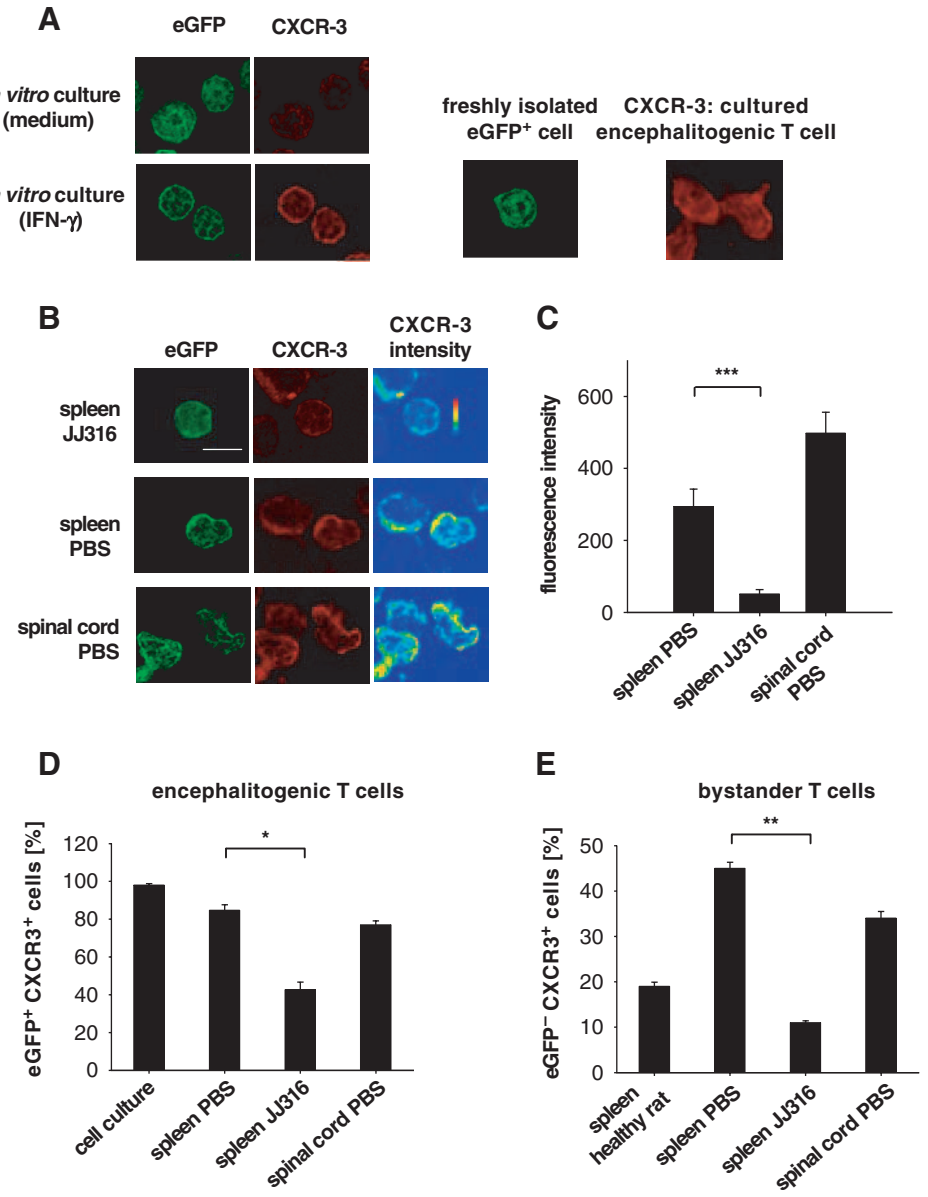

Fig. 7 CXCR-3 expression by encephalitogenic and bystander T cells. (**A**) eGFP$^+$ spleen cells were cultured for 24 h in the presence or absence of 100 U/ml IFN-γ. Subsequently they were analysed for CXCR-3 expression by confocal microscopy using antibodies against eGFP and CXCR-3. In parallel, the fluorescence of freshly isolated eGFP$^+$ cells and the CXCR-3 immunostaining of cultured encephalitogenic T cells were visualized by confocal microscopy. (**B**) AT-EAE was induced in female Lewis rats followed by prophylactic administration of PBS or JJ316. On Day 4, T cells were isolated from spleen and spinal cord and stained for the expression of eGFP and CXCR-3. For better illustration, CXCR-3 expression is also depicted in false-colour mode to quantitatively visualize its intensity based on the code display in the upper panel (the bar corresponds to 5 μm). (**C**) Quantification of the fluorescence intensity of CXCR-3 on positively stained encephalitogenic cells as shown in panel B. (**D**) The percentage of eGFP$^+$CXCR-3$^+$ encephalitogenic T cells among all eGFP$^+$ cells is depicted for cultured encephalitogenic T cells before transfer, for the reisolated T cells from spleen and spinal cord of PBS-treated animals and for the spleen of JJ316-injected rats (n = 3; mean ± SEM). (**E**) The percentage of eGFP$^-$CXCR-3$^+$ bystander T cells among all eGFP$^-$ T cells is depicted for the spleen of a healthy rat, the spleen and the spinal cord of PBS-treated animals and for the spleen of JJ316-injected animals (n = 3; mean ± SEM). Statistics: one-way ANOVA, *post hoc*: Tukey HSD test.

the eGFP$^+$ cells analysed *ex vivo* during AT-EAE represent a pure population of encephalitogenic cells and do not contain any T_{reg} cells.

To investigate whether JJ316 represses the function of the encephalitogenic cells, we studied CXCR-3 expression and cytokine production. CXCR-3 expression remained unaffected by the antibody treatment (Fig. 8C). Furthermore, all cells stained positive for IFN-γ while being negative for IL-4 and IL-10, irrespective of whether JJ316 was present or not (Fig. 8D). When measuring cytokine production in the supernatant of the encephalitogenic T cell cultures we found that JJ316 even induced IFN-γ synthesis rather than

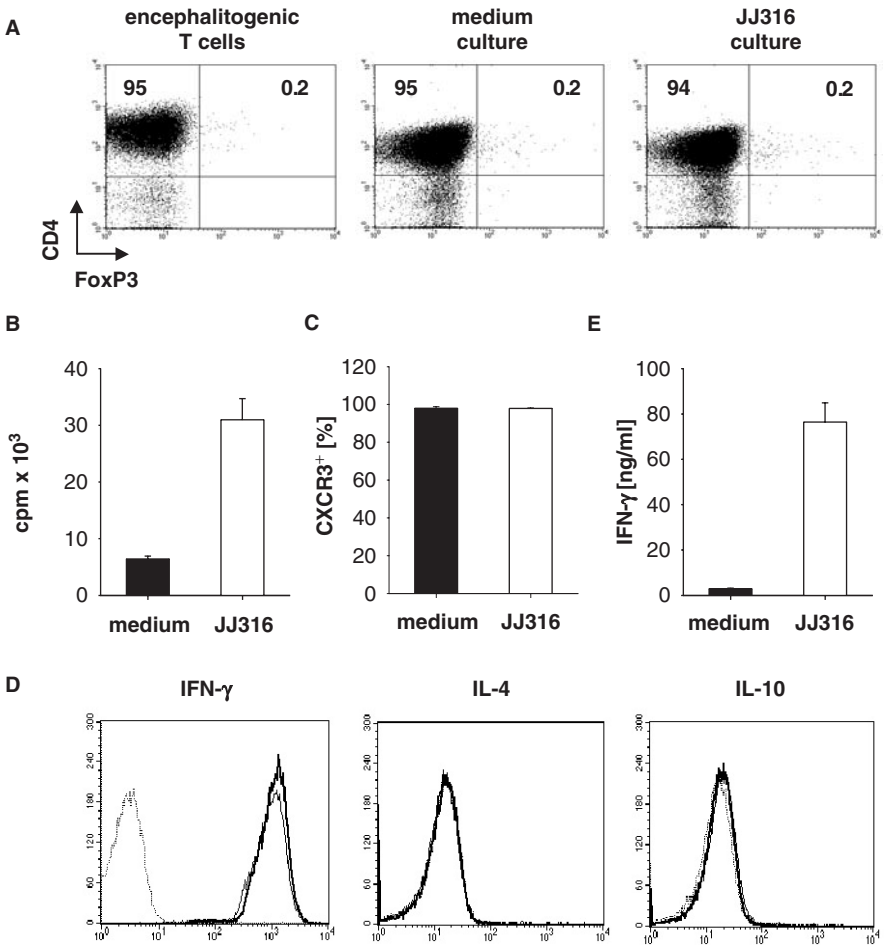

Fig. 8 Effect of JJ316 on encephalitogenic T cells in culture. (**A**) Encephalitogenic T cells used for disease induction were either directly analysed by flow cytometry or cultured in the presence or absence of 5 μg/ml JJ316 for 48 h prior to the analysis. The percentages of $CD4^+FoxP3^-$ and $CD4^+FoxP3^+$ are depicted. (**B**) Proliferation of the encephalitogenic T cells after culture in medium alone or after stimulation with JJ316. (**C**) The encephalitogenic T cells were cultured in the presence or absence of JJ316 as described above. Subsequently, the percentage of $CXCR\text{-}3^+$ cells was assessed by confocal microscopy. (**D**) After culturing the encephalitogenic T cells in the presence or absence of JJ316, they were stimulated with PMA and ionomycin for 2 h. The histogram plots of the intracellular stainings for IFN-γ, IL-4 and IL-10 gated on $\alpha\beta TCR^+CD4^+$ T cells are depicted. The dotted line represents the isotype control; the thin line the medium culture and the bold line stimulation with JJ316. (**E**) The amount of IFN-γ was analysed in the supernatant by CBA 24 h after culturing the encephalitogenic T cells in the presence or absence of JJ316. Each experiment was repeated at least twice with similar results.

suppressing it (Fig. 8E). In summary, JJ316 stimulation directly represses neither chemokine receptor nor cytokine expression by encephalitogenic T cells. Therefore, we conclude that the activation and expansion of pre-existing T_{reg} cells must be responsible for the downregulation of IFN-γ and CXCR-3 observed during AT-EAE rather than direct effects of JJ316 on the encephalitogenic T cells.

Discussion

Administration of the monoclonal antibody JJ316 ameliorates EAE by activating and expanding T_{reg} cells but the underlying mechanisms have remained elusive (Beyersdorf *et al.*, 2005). Here, we have shown that infiltration of the spinal cord by encephalitogenic T cells can be prevented without a need for T_{reg} cells to home to the CNS. T_{reg} cells rather impact on the effector cells in the secondary lymphoid organs where they suppress IFN-γ secretion by encephalitogenic T cells. In turn, expression of the chemokine receptor CXCR-3 on the pathogenic cells is reduced which presumably explains their impaired migratory ability. We propose that this low expression of CXCR-3 is at least one crucial mechanism contributing to the beneficial effect of T_{reg} cell activation in the treatment of AT-EAE and other model autoimmune diseases (Schmidt *et al.*, 2003*a*; Beyersdorf *et al.*, 2005; Rodriguez-Palmero *et al.*, 2006).

It is widely accepted that T_{reg} cells are important to control autoimmunity and thus any approach to enhance their activity may be useful as a therapeutic concept for autoimmune diseases. However, both their site of action as well as their mechanism appears to vary under different conditions. T_{reg} cells were described to act either in the inflamed tissue (Kohm

et al., 2002; Mottet *et al.*, 2003; Lundsgaard *et al.*, 2005; McGeachy *et al.*, 2005; Ott *et al.*, 2005) or in the secondary lymphoid organs (Sarween *et al.*, 2004; Viglietta *et al.*, 2004). For the JJ316-mediated treatment of AT-EAE we now show that T_{reg} cell action on effector cells is mainly confined to the secondary lymphoid organs. Several observations support this notion. (i) Encephalitogenic T cells are co-expanded after a single prophylactic treatment with JJ316 yet they do not readily infiltrate the spinal cord: neither T cells nor macrophages are detected in the spinal cord, and the integrity of the BBB also remains largely intact. We argue that the potential for transmigration of the pathogenic T cells into the CNS is inhibited in peripheral tissues. (ii) T_{reg} cells are preferentially activated and expanded in the spleen after prophylactic administration of JJ316 but until Day 4 they do not home to the spinal cord. However, at this time point, the control animals have almost reached the peak of EAE whilst the antibody-treated rats remained healthy. Therefore, it is unlikely that T_{reg} cells prevent AT-EAE by acting at the site of inflammation. (iii) Therapeutic administration of JJ316 was highly effective in ameliorating EAE although the CNS had already been infiltrated by encephalitogenic T cells. This beneficial effect was observed very rapidly although T_{reg} cells were not detected in the spinal cord under these conditions. This also strongly supports our view that JJ316 activates T_{reg} cells in the secondary lymphoid organs, possibly with an immediate impact on the effector capacity of the pathogenic T cells *in situ*.

Although T_{reg} cells were not seen in the CNS early on, they did migrate to the spinal cord on Day 6 after disease induction. This was observed in the prophylactic treatment protocol and confirmed by co-transfer experiments using $eGFP^+$ T_{reg} cells. Presumably, the small number of pathogenic T cells found in the CNS even after administration of JJ316 may facilitate a limited breakdown of the BBB. This homing to the spinal cord may represent a specific characteristic of T_{reg} cells, suggesting that they play an additional role at the site of inflammation, e.g. in the resolution of ongoing autoimmune diseases or in preventing relapses. This is of fundamental interest and requires further investigations.

The second major finding of this study is the identification of the potential mechanism underlying the inhibitory effect of JJ316 activated T_{reg} cells on the infiltration of the CNS by encephalitogenic T cells. In the past, a plethora of different mechanisms have been discussed how T_{reg} cells may ameliorate or prevent autoimmunity. Suppression could be achieved by cell–cell contact or by secreted factors such as anti-inflammatory cytokines (von Boehmer, 2005). We have shown that administration of JJ316 induces a general T_H2 shift, characterized by increased levels of IL-4 and IL-10. While IL-10 is directly produced by T_{reg} cells, IL-4 is capable of indirectly augmenting their suppressive efficacy (Maerten *et al.*, 2005). Therefore, the shift in the cytokine profile presumably contributes to the potency of T_{reg} cell action. More importantly, administration of JJ316 completely abrogated the production of the proinflammatory cytokine IFN-γ by the encephalitogenic T cells. T_H1 cells are known to play an important role in the induction and development of EAE in mice (Yura *et al.*, 2001) as well as multiple sclerosis in humans (Moldovan *et al.*, 2003). Furthermore, a similar effect of JJ316 treatment on IFN-γ production has been described for EAN (Schmidt *et al.*, 2003*a*). IFN-γ is the major effector cytokine of the pathogenic T cells and responsible for many of their effector functions. One important property of IFN-γ is its ability to induce the chemokine receptor CXCR-3 on pathogenic T cells, a receptor that is necessary for the migration of effector cells to their target tissue in autoimmune disease such as type 1 diabetes and multiple sclerosis (Sorensen *et al.*, 2002; Sarween *et al.*, 2004; Engelhardt and Ransohoff, 2005). Accordingly, we have now observed that JJ316 treatment not only abrogates IFN-γ production but also reduces the frequency and intensity of CXCR-3 expression on the encephalitogenic T cells. Thus, CXCR-3 provides a plausible link between T_{reg} cell action and impaired effector cell migration to the CNS. Importantly, our analyses have clearly shown that the antibody does not directly target the function of the pathogenic cells. Indeed, in the presence of JJ316 but in the absence of T_{reg} cells encephalitogenic cells proliferate, remain $CXCR\text{-}3^+$ and start to produce IFN-γ. This underlines again the great potency of JJ316 to interfere with a pathological immune response by preferentially addressing T_{reg} cells.

While CD28-specific antibodies such as JJ316 are highly efficient in preventing autoimmune diseases in animal models, a recent clinical trial using a humanized antibody with comparable specificity was accompanied by devastating side-effects (see http://www.tegenero.com). Thus, antibodies from this class may not be applicable to patients in their current form. However, our studies still indicate that polyclonal activation of T_{reg} cells represents a promising strategy to treat multiple sclerosis and other autoimmune diseases. It will therefore be a challenge for the future to develop alternative reagents with similar efficacy but less harmful side-effects.

In summary, our findings indicate that both prophylactic and therapeutic administration of the monoclonal antibody JJ316 activates and expands T_{reg} cells, which prevents encephalitogenic T cells from infiltrating the CNS. This is achieved within the secondary lymphoid organs by compromising the migratory ability of the pathogenic cells by impacting on the cytokine profile and the expression of the chemokine receptor CXCR-3. We believe that this explains the beneficial effect of polyclonal T_{reg} cell activation by therapeutic antibodies in a model autoimmune disorder.

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