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The “window of susceptibility” for inflammation in the immature central nervous system is characterized by a leaky blood brain barrier and the local expression of inflammatory chemokines

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Abstract

Early in postnatal development, the immature central nervous system (CNS) is more susceptible to inflammation than its adult counterpart. We show here that this “window of susceptibility” is characterized by the presence of leaky vessels in the CNS, and by a global chemokine expression profile which is clearly distinct from the one observed in the adult CNS and has three important characteristics. First, it contains chemokines with known roles in the differentiation and maturation of glia and neurons. Secondly, these chemokines have been described before in inflammatory lesions of the CNS, where they are important for the recruitment of monocytes and T cells. And last, the chemokine profile is shaped by pathological changes like oligodendrocyte stress and attempts of myelin repair. Changes in the chemokine expression profile along with a leaky blood brain barrier pave the ground for an accelerated development of CNS inflammation.

Keywords

immature CNS; inflammation; chemokines

Introduction

Several different observations suggested that the immature brain has an enhanced susceptibility towards inflammation (Galea et al., 2007). First, experimental autoimmune encephalomyelitis (EAE), an inflammatory disease of the CNS caused by the injection of myelin basic protein (MBP)-specific T cells took a hyperacute course in 4-8 day-old rats, where it was characterized by very severe changes with edema formation selectively in the white matter, and by cellular infiltrates dominated by polymorphonuclear cells and macrophages. In adult rats, however, clinical disease was less severe, characterized by gray and white matter involvement, and by the formation of lesions dominated by perivascular mononuclear cells (Umehara et al., 1990). Secondly, injection of 100 units of IL-1 β into the striatum of juvenile rats led to an increase in blood-brain barrier permeability and recruitment of polymorphonuclear neutrophils into the tissue around the injection site, while even 50 000 units of IL-1 β injected into the adult striatum failed to do so: In these animals, vascular permeability remained essentially normal, and leukocyte recruitment at the injection site was undetectable (Anthony et al., 1997). We reasoned that this enhanced susceptibility of the immature CNS might have two different causes: First, a chemokine

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expression pattern that is different from the one observed in the mature CNS, and secondly, distinct blood brain barrier properties that allow the access of brain borne chemokines to the luminal side of vessels and facilitate the entry of immune cells into the CNS.

To test this hypothesis, we characterized age-related chemokine gene expression patterns and blood-brain barrier properties of wildtype Lewis rats, and of proteolipid protein (PLP) overexpressing transgenic Lewis rats with pathological changes characterized by oligodendrocyte stress and attempts of myelin repair (Bradl et al., 1999) (Bauer et al., 2002). We then related our findings to the recruitment of immune cells into and within the immature CNS during postnatal development and in the course of inflammation.

Materials and methods

Animals

Wildtype (wt) Lewis and hemizygous PLP transgenic Lewis rats were obtained from the Decentral Facilities of the Institute for Biomedical Research, Medical University Vienna. The genotype of the transgenic animals was determined by PCR analysis as described (Bradl et al., 1999). They were used within 24 hours after birth ("neonates"), 21-25 days after birth ("juveniles") or 2-9 months after birth ("adults").

Tissue sampling

The animals were sacrificed by inhalation of an overdose of CO₂. For molecular analysis of total spinal cords, the tissue was carefully dissected and snap-frozen at -80°C, or transferred to DMEM/HEPES medium.

RNA isolation

The RNA isolation was done according to standard protocols, using the RNeasy kit and QIAshredder (both from Qiagen, Vienna, Austria) according to the instructions of the manufacturer.

cRNA synthesis and microarray analysis

All steps of the cRNA synthesis and labeling were performed with materials from the TrueLabeling-AMP Kit (SuperArray Bioscience Corp., Frederick, USA), and biotin-16-UTP (10mM, Roche Diagnostics), using the protocol provided by SuperArray Bioscience Corp. Briefly, ~ 3µg of total RNA in a volume of 9µl were mixed with 1µl of the TrueLabeling Primer. The contents were mixed well, briefly centrifuged and incubated for 10min at 70°C. The sample was briefly centrifuged and placed on ice. The cDNA synthesis master mix (consisting of 4µl RNase-free H₂O, 4µl 5x cDNA Synthesis Buffer, 1µl RNase Inhibitor and 1µl cDNA Synthesis Enzyme Mix) was added. The cDNA synthesis was performed during an 50 min incubation of the samples at 42°C. Then, the amplification master mix (consisting of 16µl 2.5x RNA Polymerase Buffer, 2µl Biotin-16-UTP (10mM), and 2µl RNA Polymerase Enzyme) was added, and labelled cRNA was synthesized during a 4-6hrs incubation at 37°C. 8-20µg labeled cRNA was used to probe the Oligo GEArray® Rat Chemokines & Receptors Microarray (SuperArray Bioscience Corp., Frederick, USA) according to the protocols of the manufacturer. Hybridization was carried out over night (60°C/5rpm). After several washing steps, bound probes were identified using the Chemoluminescence detection kit (SuperArray Bioscience Corp., Frederick, USA), and the array images were recorded using X-ray films with exposure times between 2min and 1h. Recorded images were calibrated according to the signal intensities of two different house keeping genes (peptidylprolyl isomerase A (PPiA) and ribosomal protein L32 (Rpl32)). The absence of signals on these images could indicate either the complete lack of expression of a

given gene, or gene expression levels below the threshold of detection under the calibration conditions used.

The signal intensities of the identified gene probes were determined with the program quantity one version 4.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

Confirmation of identified products with polymerase chain reactions (PCR)

For PCR analysis, RNA was first converted to cDNA. For cDNA synthesis, 2µl random hexamer primer (10pMol/µl) and up to 12µl RNA (max. 1µg RNA in a max. volume of 12µl) were supplemented with DEPC-H₂O to obtain 14µl total volume. The mixture was first incubated for 5min at 70°C and then for 5min on wet ice. After a brief centrifugation 5µl 5x M-MLV RT Buffer (Promega, Mannheim, Germany), 1.25µl dNTP-Mix (10mM each, Fermentas, St.Leon-Rot, Germany), 1.25µl RNase Inhibitor (Eppendorf, Wien, Austria) and 2.5µl DEPC H₂O were added and the mixture incubated for 2min at room temperature. 1µl M-MLV Reverse Transkriptase, RNase H(-), Point Mutant (Promega) was added. After incubation for 10min at room temperature, cDNA synthesis was then performed for 70min at 46°C. The reaction was stopped afterwards by incubation for 15min at 70°C.

For PCR analysis, 5µl 10x PCR buffer (150 mM Tris-HCl pH 8.0 at 25°C, 500 mM KCl, 0.1% (v/v) Tween 20.), 6µl 25mM MgCl₂, 1µl dNTP mix (10mM each), 1.5µl forward primer (100pmol/µl), 1µl reverse primer (100pmol/µl), 0.2µl HotGoldstar Polymerase (Eurogentec, Seraing, Belgium), 2µl cDNA and 32.8µl DEPC H₂O for a total volume of 50µl were mixed together. For positive control cDNA of spleen and/or thymus was used. The primer pairs used are summarized in table 1. The samples were heated to 95°C for 11 min. and then subjected to 35 cycles of the following regimen: 95°C, 30 seconds (denaturation), 51°C, 30 seconds (annealing), and 72°C, 45 seconds (elongation). Final extension was made for 10 min. at 72°C.

Validation of array data

Histological analysis—The animals were perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), brains and spinal cords were dissected, postfixed in 4% PFA/PBS for 24 hrs and paraffine embedded. 2-4µm thick adjacent serial sections were cut on a microtome. The sections were dewaxed in xylol for 1 hour, rehydrated, and treated next for 30 minutes with 0.03% hydrogen peroxide to block the endogenous peroxidase, afterwards heated in 10mM citrate buffer pH6.0 for antigen retrieval, and then rinsed in 0.1M phosphate buffered saline (PBS) and incubated in PBS containing 10% fetal calf serum (PBS/FCS) for 20 minutes. After washing with PBS we incubated the slides at 4°C overnight with the following primary antibodies: ED1 (mouse monoclonal antibody directed against lysosomes of rat macrophages and activated microglia cells; Serotec, Oxford, UK), rabbit anti-human CD3 (polyclonal, cross-reactive to rat CD3, Dakopatts, Denmark), W3/13 (mouse monoclonal antibody against T cells and granulocytes, Harlan SeraLab, Loughborough, UK), or mouse anti-rat CD8 (monoclonal OX-8 directed against CD8α, Serotec, UK) antibodies.

The sections were then washed in PBS and incubated with biotinylated secondary antibodies (1:200; donkey-anti-rabbit; donkey-anti-mouse; Amersham Pharmacia Biotech, Uppsala, Schweden) in PBS/FCS for 2 hours at room temperature followed by avidin-peroxidase complex (1:100; Sigma) for 1 hour. Labeling was visualized with 3,3'-diaminobenzidine-tetra-hydrochloride (Sigma) containing 0,30% hydrogen peroxide. All sections were counterstained with Meyer's hematoxylin, dehydrated and coverslipped.

For conventional staining, the sections were dewaxed in xylol for 1 hour, rehydrated, and stained with hematoxylin/eosin.

Assessment of blood-brain barrier function—The permeability of the blood-brain barrier was measured using the anti-dysferlin antibody Hamlet and anti-rat Ig as described (Hochmeister et al., 2006).

EAE induction and scoring—For the induction of T cell-mediated EAE, 21-day-old wildtype and PLP-transgenic animals were intraperitoneally injected with 1×10^5 activated, myelin basic protein (MBP)-specific T cells. We used MBP-specific T cells, since these cells induce EAE with the same severity and lesion distribution in adult wildtype and PLP-transgenic rats (Aboul-Enein et al., 2004). We injected these cells in low numbers, and only studied early time points of CNS inflammation, because we wanted to avoid that subtle effects of brain-intrinsic chemokines were masked by chemokines liberated from brain infiltrating immune cells. The clinical course of EAE was scored according to the following scale:

0.5 - partial loss of tail tonus; 1 - complete loss of tail tonus; 2 - hind limb or fore limb paresis; 3 - hind limb or fore limb paralysis (our criterium for termination of experiments); 4 - tetraplegia; 5 - moribund; 6 - dead.

Results

The global chemokine expression pattern in the spinal cord changes with age

In first experiments, we studied the chemokine expression in spinal cords of neonatal, juvenile and adult Lewis rats. In the neonatal spinal cords, expression of the chemokines CCL2, CCL6, CCL7, CCL11, CCL27, SCYE1, CX3CL1, CXCL10, and CXCL12 was readily detectable. In the juvenile spinal cords, the chemokine expression pattern was more restricted, with expression of CCL2, CCL6, CCL7, SCYE1, CX3CL1, and CXCL2. In adult spinal cords, finally, only SCYE1 and CX3CL1 were visible (fig. 1a).

The chemokine expression profile of the CNS reflects normal and pathogenic processes

We next compared the global chemokine expression pattern in the myelin degenerative CNS of PLP transgenic juvenile rats to its wildtype counterpart. Similar to the chemokine expression pattern of wildtype juvenile rats, PLP transgenic juveniles expressed CCL2, CCL6, CCL7, SCYE1, CX3CL1, and CXCL2. However, additional transcripts encoding CCL3 (weak signal), CCL11, CCL27, CXCL1, CXCL10, and CXCL12 became detectable (fig. 1b).

Blood vessels of the immature spinal cord blood are leaky

Then, we analyzed whether immature spinal cord vessels have different barrier properties than their adult counterparts. Our read-out for impaired barrier function was the expression of dysferlin, a sensitive marker for “leaky” brain blood vessels (Hochmeister et al., 2006). We found, that neonatal and juvenile rats have high numbers of dysferlin⁺ vessels in the spinal cords, and that the numbers of these vessels decreased with increasing age of the animals. Juvenile PLP tg and wt rats did not differ from each other in the number of dysferlin⁺ blood vessels (fig. 2). We also observed the presence of diffuse perivascular IgG leakage, which was most pronounced in subpial white matter, but was occasionally also observed around gray matter vessels (fig. 2). Hence, the juvenile rat blood-brain barrier is leaky, which might facilitate the access of CNS borne chemokines to the luminal surface of endothelial cells.

Recruitment of immune cells to the intact juvenile spinal cord

We first looked for evidence of macrophage recruitment to the juvenile spinal cord. Our read-out was the presence of CD8⁺ phagocytes which have been described in the rat CNS under conditions of inflammatory demyelination (Schroeter et al., 2003) or focal ischemia (Jander et al., 1998), and which seem to predominantly originate from hematogenous macrophages (Schroeter et al., 2001). CD8⁺ phagocytes were readily detectable in the juvenile PLP transgenic spinal cord white matter. They expressed the ED1 antigen and were negative for CD3 (data not shown). They reached their highest density in the lateral funiculi, but were almost completely absent from the spinal cord of juvenile wildtype animals (fig. 3). CD8⁺ phagocytes are also exceedingly rare in the adult wildtype and transgenic spinal cord (data not shown).

We then determined the degree of T cell infiltration into the juvenile spinal cords of wildtype and transgenic rats. We found that the myelin degenerative spinal cord of PLP transgenic Lewis rats contained more CD3⁺ T cells than its wildtype counterpart, and that the juvenile spinal cord of both genotypes contained more CD3⁺ T cells than the corresponding adult spinal cords (fig. 3).

More rapid EAE development in juvenile PLP transgenic animals

The chemokine transcript pattern observed in the juvenile wildtype and PLP transgenic spinal cords could create a CNS environment facilitating CNS inflammation. Yet, clusters of T cells suggestive of spontaneously developing, early inflammatory foci were not detected in the juvenile spinal cords. This finding raised the question whether the juvenile spinal cord would need an additional inflammatory trigger to reveal the action of its chemokines.

We provided this trigger by inducing EAE with activated, MBP-specific T cells.

The first batch of animals was sacrificed 3 days after T cell transfer. At this timepoint, all animals were clinically healthy. Histological analysis revealed that the PLP tg juvenile rats had significantly more neutrophil granulocytes as markers for initial EAE lesions (McColl et al., 1998; Taub et al., 1996) in the spinal cord than their wildtype counterparts (18.75 $\pm$ 9.57 neutrophils/spinal cord cross section in PLP tg rats vs. 3.5 $\pm$ 2.93 neutrophils/spinal cord cross section in wt rats; $p=0.0006$; data statistically significant according to Mann Whitney W test; fig. 4). We also observed that the juvenile PLP tg rats had a higher index of perivascular CNS inflammation than their wt counterparts (5.23 $\pm$ 1.25 perivascular cuffs/spinal cord cross section in PLP tg rats vs. 1.17 $\pm$ 0.86 perivascular cuffs/spinal cord cross section in wt rats; $p=0.0003$; data statistically significant according to Mann Whitney W test) (fig. 5). In addition, the juvenile PLP tg rats had a significantly higher percentage of parenchymal T cells than their wt counterparts (89.14 $\pm$ 5.11% parenchymal T cells in PLP tg animals vs. 57.59 $\pm$ 28.04% parenchymal T cells in wt animals; $p=0.00498$; data statistically significant according to Mann Whitney W test) (fig. 5).

A second batch of animals was sacrificed 5 days after the transfer of MBP-specific T cells. At this timepoint, the average clinical score of the animals was 0 (0.0625 $\pm$ 0.177 in juvenile PLP tg rats vs. 0.056 $\pm$ 0.167 in wt rats; $p=0.999$; not significant according to Mann Whitney W test). Juvenile PLP tg rats had a higher index of perivascular inflammation than their wt counterparts (5.99 $\pm$ 1.16 perivascular cuffs/spinal cord cross section in juvenile PLP tg rats vs. 3.84 $\pm$ 1.53 perivascular cuffs/spinal cord cross section in wt rats; $p=0.0123$; data statistically significant according to Mann Whitney W test) and higher numbers of T cells (587.81 $\pm$ 84.69 T cells/spinal cord cross section in juvenile PLP tg rats vs 465.44 $\pm$ 133.21 T cells/spinal cord cross section in wt rats; $p=0.048$; data statistically significant according to Mann Whitney W test), but the overall percentage of parenchymal T cells was comparable (88.69 $\pm$ 1.79% parenchymal T cells in PLP tg

animals vs $87.54 \pm 3.80\%$ parenchymal T cells in wt animals; $p=0.5964$; differences not significant according to Mann Whitney W test) (fig. 5).

Taken together, these data indicated that juvenile PLP tg rats have a more rapid EAE development than their age-matched wt counterparts.

Discussion

The data presented above revealed several important points.

First, they clearly showed that the immature spinal cord has a global chemokine expression pattern different from the mature spinal cord. The most likely explanation for this finding is the fact that processes of migration, differentiation and maturation of neurons and glia are still under way in the immature CNS, while they are essentially terminated in the mature, adult CNS. Many of these developmental processes are tightly controlled by chemokines (Rostène et al., 2007). For example, CCL2 can promote neuritogenesis (Edman et al., 2008) and the differentiation of astrocytes (Lawrence et al., 2006), CCL6 is able to induce the migration of microglia and astrocytes (Kanno et al., 2005), and CCL7 regulates neuritogenesis (Edman et al., 2008).

Secondly, our data revealed that the global chemokine expression profile of the CNS changes under pathological conditions like myelin degeneration and repair. For example, we detected additional transcripts for CCL3, CCL11, CCL27, CXCL1, CXCL10, and CXCL12 in the spinal cords of juvenile PLP transgenic rats. CCL3 affects the migration of oligodendrocyte lineage cells (Nguyen et al., 2003), CCL11 favours the proliferation, inhibits the migration, and augments the differentiation of oligodendrocyte precursor cells (Maysami et al., 2006a); CXCL1 supports the proliferation of oligodendrocyte progenitor cells (Wu et al., 2000) and precursors (Robinson and Franic, 2001), and inhibits the migration of oligodendrocyte precursors (Tsai et al., 2002), and CXCL12 directs the migration of neural (Ni et al., 2004) and modulates oligodendrocyte precursor cells (Dziembowska et al., 2005; Maysami et al., 2006b). Hence, the global chemokine expression profile seen in the myelin degenerative spinal cords of PLP transgenic rats closely reflects the myelin repair processes in these animals.

Interestingly, many of the chemokines identified in the immature CNS, for example CCL2, CCL3, CCL6, CXCL1, CXCL2 and CXCL12 also play an important role in the recruitment of monocytes and T cells to inflammatory lesions of the CNS (Matejuk et al., 2002; Elhofy et al., 2005; Fuentes et al., 1995; Asensio et al., 1999; Luo et al., 2000; Anthony et al., 1998; Karpus et al., 1995; Botzug et al., 2002; McCandless et al., 2006 and 2008).

Chemokines readily diffuse through the CNS parenchyma and are then locally retained and accumulated at brain vessels (Fuentes et al., 1995), probably through the binding to proteoglycan-like molecules on the surface of endothelial cells (Rot, 1992; Tanaka et al., 1993). However, in order to attract cells from the immune system, chemokines must be present at the luminal surface of the blood vessel, which is achieved by vesicular-mediated transcellular transport processes (Middleton et al., 1997; Dar et al., 2005; Mordelet et al., 2007; Ge et al., 2008). Several observations suggest that the presence of dysferlin⁺ “leaky” brain endothelial cells of immature rats could be a footprint of such enhanced transendothelial vesicular transport processes. First, dysferlin plays an important role in the fusion of repair vesicles with the plasma membrane of muscle fibres (Bansal and Campbell, 2004; Liu et al., 1998). Secondly, also activated microglia cells/macrophages may express dysferlin (Hochmeister et al., 2006), and these cell types release cytokines in microvesicles (Bianco et al., 2005).

The “leaky” vessels and the particular chemokine profile of the immature spinal cord could facilitate the entry of cells from the immune system into the CNS, and might explain the higher numbers of T cells and the presence of CD8⁺ macrophages in the spinal cord of unmanipulated juvenile PLP transgenic rats, and the earlier onset of EAE in these animals. However, they also raise one important question: Why do animals with leaky brain vessels and immune cell attracting chemokines in the CNS not spontaneously develop CNS inflammation? One possible reason could be the relative immaturity of the rat immune system, which extends from the day of birth to the weaning age (i.e. the age of ~ 3 weeks) (Pérez-Cano et al., 2007), and affects both the innate (Maródi, 2006) and the adaptive arm (Pérez-Cano et al., 2007) of the immune system. Additionally, the immature CNS might have a special buffering capacity for the combined effects of a leaky vasculature and an enhanced chemokine expression, possibly provided by the anti-inflammatory and tolerogenic action of microglia cells/macrophages engaged in apoptotic processes (Magnus et al., 2001; Boven et al., 2006; Liu et al., 2006; Lawson et al., 1997; De Louw et al., 2002).

Although the developmental stages of rats and humans are not fully comparable (the first two weeks of brain development in rodents are roughly comparable to a period spanning from the sixth month of pregnancy to the third year of life in humans (Felderhoff-Mueser et al., 2004; Gerstner et al., 2008; Back et al., 2002; Clancy et al., 2007)), our findings might have important implications for children suffering from neurodegenerative diseases like X-linked adrenoleukodystrophy or globoid cell leukodystrophy. These diseases are characterized by inflammatory reactions in the CNS, involving both T lymphocytes and activated microglia cells/macrophages (Ito et al., 2001; Powers et al., 1992; Itoh et al., 2002), and it is conceivable that the initial recruitment of these cells is facilitated by a disease-specific chemokine expression pattern in the immature CNS.

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Fig. 1.

(a) Chemokine expression in the spinal cords of wildtype Lewis rats aged 0 days (neonates), 21 days (juvenile) and 11 months (adult). The RNA tested was pooled from the complete spinal cords of 2 newborns, and from spinal cord pieces (3 cross sections of 0.4 cm/animal) of 6 juveniles and 3 adults. Further details on changes in the signal intensity of the identified gene products are seen in table 2. (b) Chemokine expression in the spinal cords of wildtype (wt) and PLP transgenic (tg), 21 day-old rat juvenile rats. The RNA tested was pooled from spinal cord pieces (3 cross sections of 0.4 cm/animal) of 6 wt and 8 tg animals. Further details on changes in the signal intensity of the identified gene products are seen in table 3. Peptidylprolyl isomerase A (PP1a) and the ribosomal protein L32 (Rpl32) served as “housekeeping genes” for the calibration of the arrays. Chemokines with known function in the recruitment of macrophages (M), granulocytes (G), or in the course of autoimmune inflammatory CNS disease (E) are labeled with the appropriate letters, (O) labels chemokines with a role in the differentiation of oligodendrocytes. The identified products were confirmed by immunohistochemistry (Scye1), PCR (CCL2, CCL3, CCL6, CCL7, CXCL1, CXCL2, and CXCL10) and additional micorarray analysis (Scye1, CCL3, CCL11, CXCL2, CXCL10, and CX3CL1). The following CC and CXC chemokines were also contained on the arrays, but did not yield hybridization signals with the calibration conditions used: CCL4, CCL17, Scya19 (CCL19), CCL20, CCL21a, CCL22, CCL24, CCL25, CCL28, CXCL9, and CXCL11.

Fig. 2.

Dysferlin expression in the immature spinal cord. Spinal cord cross sections of a newborn wildtype Lewis rat (a), a 3 week-old wildtype Lewis rat (b) and a 3 week-old PLP tg Lewis rat (c) are shown after immunohistochemical stainings with the anti-dysferlin antibody Hamlet (Novocastra). Dysferlin⁺ blood vessels can be identified according to their brown staining. (d) Counts of dysferlin⁺ blood vessels in both lateral funiculi of the wildtype spinal cord. For each mean value (= dysferlin⁺ blood vessels per mm²/age group), spinal cords of newborn (10 rats), 21-25 day-old (juveniles; 14 rats), 2 month-old (4 rats), 7-9 month-old (10 rats) and 10-12 month-old (11 rats) were analyzed. (e) juvenile 21-25 day-old PLP tg (10 rats, total 30 cross sections) and wildtype rats (14 rats, total 42 cross sections) have

comparable numbers of dysferlin⁺ vessels per mm² spinal cord (standard deviations are given). (f,g) Immunoglobulin leakage in the spinal cords of 25 day-old wildtype Lewis rats. Diffuse immunoglobulin leakage is mostly seen in subpial white matter (f), and occasionally perivascularly around gray matter vessels (g).

Fig. 3. Recruitment of CD8⁺ macrophages (a,b) and CD3⁺ T-cells (c-e) in the spinal cord of 25 day-old PLP transgenic Lewis rats (a,c,e; n=10) and age-matched wildtype control animals (b,c,d; n=14). (a,b) Per group of animals, ~100 spinal cord cross sections have been analyzed, and the exact location of each macrophage found was projected into one spinal cord scheme. (c) The juvenile PLP transgenic spinal cord contains significantly more CD3⁺ T cells than its wildtype counterpart (p=0.018; Mann-Whitney W test), and the spinal cords of juvenile wildtype (d) and juvenile PLP transgenic rats (e) contain more CD3⁺ T cells than their adult 2-9 month-old counterparts (d: p=0.0002; e: p=0.0014; Mann-Whitney-W test). On average, 7 spinal cord sections per animal from 14 juvenile wildtype and 9 juvenile PLP

transgenic rats, and 12-14 spinal cord cross sections per animal from 12 PLP transgenic and 14 wildtype 2-9 month-old Lewis rats were analyzed.

Fig. 4. Granulocyte infiltration in the course of EAE. Shown here are the average numbers of granulocytes/spinal cord cross sections $\pm$ standard deviation on day 3 (a; 12 PLP transgenic and 9 wt, 3 week-old rats) after EAE induction with MBP-specific T cells. Differences in granulocyte infiltration between PLP transgenic and wt animals were statistically significant ($p=0.00056$; Mann-Whitney W test).

fig. 5.

T cell infiltration into the dorsal funiculus in the course of EAE. On day 3 after transfer (a-c), many parenchymal T cells are seen in the juvenile PLP tg rats (a), while T cells are mostly in the meninges and perivascular spaces of wt rats (b). The transgenic spinal cord harbours significantly more T cells than its wildtype counterpart (c; $p=0.00014$; Mann-Whitney W test). On day 5 after transfer (d-f), T cells in the juvenile PLP tg rats are mostly found within the parenchyma (d). In juvenile wildtype rats, T cells are located within the meninges and the perivascular spaces, but also in the parenchyma (e). At this time point, T cell infiltration was also pronounced in the wildtype spinal cord, and the differences in T cell infiltration into the spinal cords of wildtype and transgenic animals were only subtle and barely reached significance (f; $p=0.048$; Mann-Whitney W test). On day 3, the PLP transgenic spinal cord contained significantly more parenchymal T cells than the wt spinal cord, although there was a rather high standard deviation in the wildtype samples (12 juvenile PLP tg rats, 8 juvenile wt rats; $p=0.00498$; Mann-Whitney W test), but on day 5, the percentage of parenchymal T cells was similar in both types of animals (8 PLP tg rats, 9 wt rats; $p=0.5964$; not significant according to Mann-Whitney W test). Throughout figure 5, mean values $\pm$ standard deviation are shown.

Table 1

Primer sequences and PCR conditions:

Gene	primers	size	temp	# cycles
CCL2	for: 5'-AGATGATCCCAATGAGTCGGC-3' rev: 5'-AAGTGCTTGAGGTGGTTGTGG-3'	293bp	51°C	40
CCL3	for: 5'-CCTATGGACGGCAAATCCACG-3' rev: 5'-GTCCCTGGATGTGGCTACTTGG-3'	307bp	51°C	35
CCL6	for: 5'-TGGATAAACTACTAGTCTTTGGG-3' rev: 5'-TGTATTCAAGGTGAGCGAGCG-3'	294bp	51°C	40
CCL7	for: 5'-CTTTCACCGTGCACGTGTGG-3' rev: 5'-TTCTACAGACCGTTCCTACC-3'	372bp	51°C	40
CCL11	for: 5'-GAATGAGTTAGGGGTAAGTACG-3' rev: 5'-GGCACGTTAAAGGCAGAAATAG-3'	327bp	53°C	40
CCL27	for: 5'-ATCTACTGGGGCTGCTTCTAC-3' rev: 5'-TCTGGGGATGAACACAGACAC-3'	336bp	53°C	40
CXCL1	for: 5'-AAGGGTGTCCCAAGTAATGG-3' rev: 5'-CCTTCTTCCCGCTCAACACC-3'	328bp	51°C	35
CXCL2	for: 5'-CACCAACCATCAGGGTACAGG-3' rev: 5'-GAGGCACATCAGGTACGATCC-3'	345bp	51°C	35
CXCL10	for: 5'-GTCCGCATGTTGAGATCATTGC-3' rev: 5'-CACGCTGTAGTGGGAGTACC-3'	278bp	51°C	40
CXCL12	for: 5'-AAGGGGGTTGGAGACATACAG-3' rev: 5'-TCACCACATGCAACACTGAAAC-3'	345bp	53°C	40

Table 2

List of house keeping genes (PPia and Rpl32) and chemokines identified in the complete spinal cords of neonatal, juvenile and adult Lewis rats.

gene product	fold-change of signal intensity	
	(juveniles vs neonates)	(juveniles vs adults)
PPia	1.02	0.99
Rpl32	0.86	0.98
CCL2	0.58	5.25
CCL3	0.60	2.62
CCL5	0.45	0.99
CCL6	0.66	6.51
CCL7	0.20	4.15
CCL11	0.20	1.02
CCL27	0.08	1.24
SCYE1	0.92	1.02
CX3CL1	0.88	1.07
CXCL1	0.43	2.10
CXCL2	1.66	6.18
CXCL10	0.09	0.98
CXCL12	0.00	2.05

Table 3

List of house keeping genes (PPia and Rpl32) and chemokines identified in the complete spinal cords of juvenile wt and tg Lewis rats.

gene product	fold-change of signal intensity (transgenic vs wildtype)
PPia	1.03
Rpl32	1.08
CCL2	2.26
CCL3	1.93
CCL5	2.07
CCL6	1.83
CCL7	2.95
CCL11	5.69
CCL27	4.18
SCYE1	1.07
CX3CL1	1.08
CXCL1	6.27
CXCL2	1.93
CXCL10	8.42
CXCL12	3.97