Complementary Contribution of CD4 and CD8 T Lymphocytes to T-Cell Infiltration of the Intact and the Degenerative Spinal Cord

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The central role of T cells in inflammatory reactions of the central nervous system (CNS) is well documented. However, there is little information about the few T cells found within the noninflamed CNS. In particular, the contribution of CD4⁺ and CD8⁺ T cells to the lymphocyte pool infiltrating the intact CNS, the location of these cells in CNS white and gray matter, and changes in the cellular composition of T-cell infiltrates coinciding with degeneration are primarily undefined. To address these points, we studied T cells in the intact and degenerative rat spinal cord. In the intact spinal cord, T cells were preferentially located within the gray matter. CD8⁺ T cells were more numerous than CD4⁺ lymphocytes. In cases of neuroaxonal degeneration or myelin degeneration/oligodendrocyte death, T cells were predominantly seen in areas of degeneration and were present in increased numbers. These effects were more pronounced for the CD4⁺ than for the CD8⁺ T-cell subset. Collectively, these data provide evidence for a clear cellular and compartmental bias in T-cell infiltration of the intact and degenerative spinal cord. This could indicate that CD4⁺ and CD8⁺ T cells might fulfill complementary roles in the intact and the diseased organ. (*Am J Pathol* 2005, 166:1441–1450)

The presence of low numbers of T cells in all organs and tissues is an important prerequisite to maintain the integrity of an organism. Because only a few T cells are found within the central nervous system (CNS) of healthy animals, information about the infiltration of T cells into the CNS was mostly deduced from inflammatory T cells entering the CNS in the course of experimental autoimmune

encephalomyelitis (EAE).^{1,2} However, this approach did not address the contribution of CD4⁺ and CD8⁺ T cells to the infiltrating lymphocyte pool of the intact or degenerative CNS, nor did it yield any detailed information about the location of these cells within the CNS. To answer these questions, we studied T-cell infiltration in the spinal cord. We focused on this organ because it allows the clear discrimination between gray and white matter areas, and because T-cell entry into the spinal cord is higher than T-cell entry into the cerebrum.³ We found that in spinal cords of naïve, unmanipulated, adult Lewis rats, T lymphocytes were more frequently located within the gray than within the white matter, and that CD8⁺ T cells outnumber their CD4⁺ counterparts in both compartments. We then analyzed T cells in the degenerative spinal cord. For this purpose, we used spinal cord sections of aged Lewis rats with neuroaxonal degeneration in CNS white matter, and of adult hemizygous proteolipid protein (PLP)-transgenic Lewis rats with low-grade, sub-clinical myelin degeneration and oligodendrocyte death in CNS gray matter.^{4,5} We observed an enhanced T-cell infiltration, which was temporally synchronous to neurodegeneration, and which was more pronounced for CD4⁺ than for CD8⁺ T cells. Hence, our results demonstrate a clear cellular and compartmental bias in T-cell infiltration of the intact and the degenerative spinal cord.

Materials and Methods

Animals and Tissues

All rats used in this study were kept in the animal house of the Max-Planck Institute for Neurobiology (Martinsried,

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Germany). They shared cages and were housed under conventional conditions. For tissue preparation, the following animals were used: young adult wild-type Lewis rats with an intact CNS (age, 2 months; four rats); aging wild-type Lewis rats with neuroaxonal degeneration in spinal cord white matter [age, 7 to 9 months (nine rats) or 10 to 12 months (11 rats)]; adult hemizygous transgenic, PLP-overexpressing Lewis rats with low-grade subclinical myelin degeneration in CNS gray matter^{4,5} [age, 2 months (4 rats), 7 to 9 months (8 rats), 10 to 12 months (11 rats), or 13 to 15 months (9 rats)]. The genotype of the transgenic animals was determined by two independently performed polymerase chain reaction analyses as described.⁴ For spinal cord dissection, the animals were sacrificed with an overdose of CO₂, and perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Spinal cords were postfixed in 4% PFA/PBS for 24 hours and embedded in paraffin. Sections (3 to 5 μ m thick) were cut on a microtome.

Staining Conditions and Characterization of the Degenerative or Intact CNS—Methods of Quantification

For immunohistochemical analyses, tissue sections were stained according to standard procedures.⁵ The following antibodies were used: ED1 (directed against lysosomes of rat macrophages and activated microglia cells; Serotec, Oxford, UK), OX-6 (directed against MHC class II molecules; Serotec), rabbit anti-proteolipid protein (Serotec), mouse anti-myelin oligodendrocyte glycoprotein (kindly provided by C. Linington, Martinsried, Germany, and by S. Piddlesden, London, UK), mouse anti-2',3'-cyclic nucleotide-3'-phosphodiesterase (Affinity, Nottingham, UK), and rabbit anti-neurofilament (Chemicon, Temecula, CA). Cell counts were done by using an ocular morphometric grid.

Evidence for neuroaxonal degeneration was the presence of ED1⁺ microglia cells surrounding and engulfing axons. The number of these cells was determined separately for each rat in a 0.25-mm² area within the lateral funiculus of one representative thoracic section. Evidence for myelin degeneration was the presence of oligodendrocytes accumulating myelin in their cell bodies and the presence of ED1⁺ activated microglia cells engulfing myelin debris.⁴ Evidence for degenerative activity in the CNS was the activation of microglia cells, as revealed by reactivity with ED1 and OX-6. For each animal, the numbers of ED1⁺ and OX-6⁺ microglia cells per spinal cord cross-section were counted. The resulting values were used to calculate the average number of ED1⁺ and OX-6⁺ cells per animal. The spinal cord was considered intact in the absence of microglia activation, neuroaxonal degeneration, myelin degeneration, and oligodendrocyte death.

Establishing Staining Conditions to Identify Rat CD4 and CD8 T Cells

CD8-positive rat T cells can be clearly identified in paraffin-embedded, PFA-fixed tissue sections: They react with anti-

CD3 antibodies directed against the T-cell receptor complex, and with anti-CD8 antibodies. For double stainings of T cells for CD3 and CD8 proteins, paraffin sections were deparaffinized in xylol and transferred to 90% ethanol. Endogenous peroxidase was blocked by incubation in methanol with 0.02% H₂O₂ (30 minutes, room temperature). Sections were then transferred to distilled water via a 90%, 70%, and 50% ethanol series. For antigen retrieval, sections were placed in a plastic coplin jar filled with ethylenediamine tetraacetic acid buffer, pH 8.5, and incubated for 60 minutes in a household food steamer device (MultiGourmet FS 20; Braun, Kronberg/Taunus, Germany). Afterward, they were incubated with 10% fetal calf serum in 0.1 mol/L PBS containing rabbit anti-human CD3 (polyclonal, cross-reactive to rat CD3; Dakopatts, Glostrup, Denmark) and mouse anti-rat CD8 (monoclonal OX-8, Serotec) antibodies (4°C, overnight). The sections were then washed in PBS and incubated with a mixture of alkaline phosphatase-conjugated goat anti-mouse antibodies (Dianova, Hamburg, Germany) and biotinylated donkey anti-rabbit (Amersham Pharmacia Biotech, Uppsala, Sweden) in fetal calf serum/PBS with 3% normal human serum (1 hour, room temperature). In a last step, avidin peroxidase (1:100, Sigma, Vienna, Austria) was applied for 1 hour at room temperature. Next, the alkaline phosphatase label was visualized with Fast Blue B base (Sigma), and the avidin peroxidase with 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Sigma). With this staining procedure, we were able to identify CD3⁺8⁺ T cells based on a dark violet staining, and CD3⁺8⁻ T cells based on brown staining. To determine whether the CD3⁺8⁻ T cells were CD4⁺ T lymphocytes, we had to use cryosections, because anti-rat CD4 antibodies do not work on paraffin-embedded material. We performed triple labeling of spinal cord cryosections derived from a Lewis rat with CD4⁺ T-cell-mediated CNS inflammation. The sections were pretreated for 10 minutes with acetone. Then, W3/25 (against rat CD4, Serotec) was diluted 1:50 in diluent (DAKO, Glostrup, Denmark) and applied (4°C, overnight). The slides were washed with PBS, and incubated with a biotinylated sheep anti-mouse antibody (Amersham Pharmacia Biotech), diluted 1:200 in diluent (4 to 6 hours, room temperature). Afterward, the slides were washed and then incubated with streptavidin-Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:75 in diluent containing 10% normal mouse serum (4°C, overnight). Next, slides were washed with PBS and incubated overnight at 4°C with fluorescein isothiocyanate (FITC)-labeled OX-8 (against rat CD8, Serotec) and rabbit anti-human CD3 (cross-reactive with rat CD3, DAKO). Both antibodies were diluted 1:50 in diluent. Double labeling was finished by application of goat anti-rabbit Cy5 (Jackson ImmunoResearch) diluted 1:200 for 1 hour at room temperature. Slides were mounted with PBS/glycerol (1:9) containing 3% DABCO (Sigma) and inspected with a laser confocal microscope (LSM-410; Carl Zeiss, Jena, Germany). Excitation wavelengths of 488 nm (for FITC-labeled CD8), 543 nm (for Cy3-labeled CD4), and 633 nm (for Cy5-labeled CD3) were used to generate fluorescence emission, which was digitally translated to green (FITC), red (Cy3), and blue (Cy5) signals. With this staining protocol, we were able to verify

Figure 1. Confocal microscopy of a spinal cord section on day 5 after EAE induction. The section was reacted with antibodies against CD4 (**a**, Cy3 labeled, red), CD8 (**b**, FITC labeled, green), and CD3 (**c**, Cy5 labeled, blue). Note that in the merged picture (**d**) the CD8⁻CD3⁺CD4⁺ T cells are pink (mixture of red and blue, **open triangles**), and the CD8⁺CD3⁺CD4⁻ T cells are turquoise (mixture of green and blue, **filled triangles**). Scale bars, 5 μm.

that CD3⁺8⁺ T cells were CD4⁻, and CD3⁺8⁻ T cells were CD4⁺ (Figure 1).

In a next step we analyzed whether the data obtained on frozen tissues were comparable to the data obtained on paraffin sections. To do that, we induced EAE in two wild-type Lewis rats by injecting activated CD4⁺, MBP-specific T cells. On day 6, at the peak of EAE, these animals were sacrificed and their spinal cords were dissected. Spinal cord pieces (0.5 cm long) were cut and

every second piece was frozen in isopentane/fluid nitrogen. All other pieces were fixed in 4% PFA.

Frozen spinal cord sections were reacted with rabbit anti-CD3 (Dakopatts) and mouse anti-CD4 or mouse anti-CD8 (clones W3/25 or OX-8, respectively; Serotec). The PFA-fixed spinal cord pieces were processed to paraffin sections, which were subjected to rabbit anti-CD3 (Dakopatts) and mouse anti-CD8 antibodies (clone OX-8, Serotec). Then, the sections were reacted with alkaline

phosphatase-conjugated goat anti-mouse antibodies (Jackson Dianova) and biotinylated donkey anti-rabbit (Amersham Pharmacia Biotech). After the addition of avidin peroxidase for 1 hour at room temperature, alkaline phosphatase was visualized with Fast Blue B base (Sigma), and the avidin peroxidase with DAB.

In all cases, the ratios of CD8⁺ T cells (= CD3⁺CD4⁻ and CD3⁺CD8⁺ cells in frozen, CD3⁺CD8⁺ cells in paraffin sections) in the total CD3⁺ T-cell pool infiltrating spinal cord gray matter was determined. We found that the data obtained from frozen and PFA-fixed sections were comparable (data not shown). Based on these observations, we used paraffin-embedded spinal cord sections throughout this study, double-stained them with antibodies against CD3 and CD8, and identified CD8⁺ and CD4⁺ T cells by their individual staining pattern (CD3⁺8⁺ or CD3⁺8⁻, respectively).

Determining the Location of T Cells within the Spinal Cord Sections

To find out whether the T cells seen in the spinal cord sections were located within the parenchyma or were trapped within capillaries, we used spinal cord sections of four different aged rats (two transgenic, two wild type) and reacted them with a polyclonal rabbit anti-human von Willebrand factor antibody (vWF) (cross-reactive with rat vWF, DAKO) as a marker for blood vessels, and with W3/13 (DAKO), a mouse antibody specific for rat T cells. After the addition of alkaline phosphatase-conjugated goat anti-mouse (Jackson Dianova) and biotinylated donkey anti-rabbit antibodies (Amersham Pharmacia Biotech), the sections were incubated with avidin peroxidase for 1 hour at room temperature. Alkaline phosphatase was visualized with Fast Blue B base (Sigma), and the avidin peroxidase with DAB. We then determined the percentage of T cells trapped within capillaries ($6.5 \pm 4.9\%$ in wild-type animals versus $7 \pm 4.2\%$ in transgenic animals), found perivascularly ($9 \pm 8.5\%$ in wild-type animals versus $4.5 \pm 0.7\%$ in transgenic animals) or located within the parenchyma ($84 \pm 12.7\%$ in wild-type versus $90 \pm 5.7\%$ in transgenic animals). We found that the vast majority of T cells was clearly located outside capillaries, either within the CNS parenchyma or in the perivascular space (Figure 2).

Studying T Cells within the Spinal Cord—Methods of Quantification

At least 10 spinal cord sections per adult animal were evaluated to determine the location of CD4⁺ and CD8⁺ T cells within the spinal cord, and to calculate the average number of CD4⁺ and CD8⁺ T cells per spinal cord section and animal. For all quantitative evaluations, between 4 and 10 animals per age group and genotype were analyzed. Quantification of T cells was done on complete spinal cord sections. Occasionally, hemisections were included.

Because we wanted to further validate the counts based on immunohistochemical stainings of tissue sections, we made an additional experiment in which we isolated T cells

from the spinal cords of aged PLP transgenic rats, essentially as described,⁶ and studied these cells by cytofluorometry. As controls, T cells derived from the peripheral blood of the same animals were used. For staining, the cells were first incubated with CD4-FITC or CD8-FITC, diluted 1:100 in PAB (1× PBS containing 2% fetal calf serum and 0.01% sodium azide) (30 minutes, 4°C). Next, the cells were washed in PAB and reacted with R73 (specific for the rat T-cell receptor, Serotec) or isotype-matched mouse IgG (negative control), both diluted 1:100 in PAB (30 minutes, 4°C). Afterwards, T cells were washed and incubated with RPE-Cy-5-labeled secondary anti-mouse antibody (DAKO) (30 minutes, 4°C). Finally, T cells were washed and cytofluorometrically analyzed as described.⁶ The percentage of R73⁺CD4⁺ or R73⁺CD8⁺ T cells in the R73⁺ T-cell population was determined with Lysis II software (Becton Dickinson, Heidelberg, Germany).

Studying the Numbers of T Cells in Spinal Cord White and Gray Matter at Different Time Points after the Induction of CNS Inflammation

In additional experiments, we determined how long after their entry T cells can be found in spinal cord white and gray matter. We studied the numbers of CD4⁺ T cells in archival tissue material originating from animals, in which EAE had been induced by transfer of myelin basic protein-specific T cells derived from TK-tsA transgenic rats.⁷ This archival material had two important advantages: first, the regimen used to induce EAE resulted in inflammatory lesions in spinal cord gray and white matter; secondly, the genetic marker allowed the identification of disease-inducing CD4⁺ T cells.⁷ Because there are no encephalitogenic CD8⁺ rat T cells available to date, we studied CD8⁺ T cells, which were secondarily recruited into the spinal cord in the course of a CD4⁺ T-cell-mediated EAE. In both experimental settings, the numbers of T cells in spinal cord white and gray matter was determined by counting TK-tsA-labeled T cells (for disease-inducing CD4⁺ T cells; detection of the TK-tsA marker as described⁷) or CD3⁺8⁺ T cells on different days after EAE induction. Complete spinal cord sections of two to three animals per time point were used.

Statistical Evaluation

Statistics was calculated with the Statgraphics Plus program. The Mann-Whitney (Wilcoxon) *W* test (comparison of medians) was used in all cases.

Results

T Cells in the Intact Spinal Cord of Adult Lewis Rats

In the naive, intact spinal cord of adult Lewis rats, CD8⁺ and CD4⁺ T cells are rare. They are distributed throughout the spinal cord, but are more frequently located in gray than in white matter (Figure 2). In both compartments, CD8⁺ T cells

Figure 2. T cells in the intact and myelin-degenerative spinal cord. **a** and **b**: Double staining of spinal cord sections with anti-von Willebrand factor (brown) and W3/13 (blue). Shown here are sections derived from aged PLP-transgenic Lewis rats. Note that spinal cord sections of aged wild-type animals showed a similar location of T cells relative to blood vessels. **c-f**: Double staining of spinal cord sections with anti-CD3 (brown) and anti-CD8 (clone OX-8, blue). CD8⁺ T cells (**c**) are CD3⁺CD8⁺ and appear purple (mixture of brown and blue), CD4⁺ T cells (**d**) are CD3⁺CD8⁻ and appear brown. **g**: Distribution of T cells in cervical (C₁₋₇), thoracic (T₁₋₁₀), and lumbar/sacral (L_{1-S₄}) spinal cord sections of wild-type Lewis rats. Spinal cord sections of four animals (age, 2 months) were analyzed, and the location of each T cell found was projected as a dot into the relevant scheme. The data shown were obtained from 7 cervical, 19 thoracic, and 11 lumbar/sacral sections. T cells found within the meninges were omitted. **h** and **i**: Distribution of T cells in the spinal cord of aged hemizygous transgenic Lewis rats. Double staining of spinal cord sections with anti-CD3 (brown) and anti-CD8 (clone OX-8, blue). CD8⁺ T cells are CD3⁺CD8⁺ and appear purple (mixture of brown and blue), CD4⁺ T cells are CD3⁺CD8⁻ and appear brown. The white line in **h** represents the gray/white matter junction. **j**: Double staining of spinal cord sections with anti-CD3 (brown) and anti-CD8 (clone OX-8, blue). The infiltrating T cells belong to the CD8⁺ (purple) and to the CD4⁺ (brown) T-cell subset. Scale bars: 20 μ m (**a-d**); 100 μ m (**e, f**); 1 mm (**h, i**); 10 μ m (**j**).

Figure 3. The distribution of CD4⁺ and CD8⁺ T cells in white matter (white) and gray matter (gray) of spinal cord sections from wild-type (wt) Lewis rats. The animals were grouped by age (2 months: 4 animals with 59 sections; 7 to 9 months: 10 animals with 141.5 sections; 10 to 12 months: 11 animals with 126.5 sections). **Gray asterisks** show statistically significant differences between spinal cord gray and white matter at the 95% confidence level [*P* values <0.05, Mann-Whitney (Wilcoxon) *W*-test]. **Black asterisks** indicate statistically significant differences at the 95% confidence level [*P* values <0.05, Mann-Whitney (Wilcoxon) *W*-test] in the number of cells to the value determined for the same compartment in younger animals. T cells found within the meninges were omitted from this study.

are more numerous than their CD4⁺ counterparts (Figure 3). This pattern of T-cell distribution is independent of the spinal cord level along the neuro-axis (Figure 2).

The higher numbers of CD4⁺ and CD8⁺ T cells in spinal cord gray matter could have a simple explanation: That T lymphocytes which had entered the CNS are longer detectable in white than in gray matter. To address this point specifically, we determined the numbers of CD4⁺ and CD8⁺ T cells in these two different spinal cord compartments at different time points after induction of CNS inflammation. We could detect both T-cell subsets for comparable time spans in the spinal cord white and gray matter (Figure 4). Hence, there are no differences in the disappearance rate of T cells from spinal cord white and gray matter, which could account for the higher numbers of T cells in the intact gray matter.

Figure 4. The numbers of CD4⁺ and CD8⁺ T cells found in the white (white) or gray (gray) matter of spinal cord sections from wild-type Lewis rats that had been injected with TK-tsA-labeled, CD4⁺ MBP-specific T cells to induce CNS inflammation. The data on CD4⁺ T cells were obtained from *in situ* hybridizations for the TK-tsA transgene and represent the disease-inducing cell population. The data on CD8⁺ T cells represent the cell population that is unspecifically recruited to the spinal cord in the course of inflammation. Note that the data on CD4⁺ and CD8⁺ T cells were obtained from different experiments.

T Cells in the Spinal Cord of Aged Lewis Rats with Neuroaxonal Degeneration in CNS White Matter

Next, we studied T cells in spinal cord sections of aged wild-type Lewis rats. These spinal cord sections were characterized by neuroaxonal degeneration and microglia activation, as indicated by the presence of axonal spheroids (data not shown) and axonophagic ED1⁺ microglia cells (Figure 5) in CNS white matter. The numbers of CD4⁺ and CD8⁺ T cells found within the spinal cords of 7- to 9-month-old animals did not significantly differ from T-cell frequencies observed in the 2-month-old group of animals. This is in marked contrast to the situation in 10- to 12-month-old animals, in which the numbers of T cells in spinal cord white matter were significantly higher (Figure 3) and more pronounced in the CD4⁺ T-cell pool (average numbers per section in 2-month-old versus 10- to 12-month-old animals: 0.15 versus 1.2; *P* = 0.012) than in the CD8⁺ T-cell group (average numbers per section in 2-month-old versus 10- to 12-month-old animals: 0.45 versus 1.5; *P* = 0.169) (Figure 3). Taken together, these results revealed T-cell immigration into the spinal cord, which temporally and spatially coincides with neuroaxonal degeneration and microglia cell activa-

Figure 5. The number of ED1⁺ axonophagic microglia cells of wild-type (gray) and transgenic (black) animals, as detected by staining with the ED1 antibody. The animals were grouped by age. **Black asterisks** indicate statistically significant differences at the 95% confidence level [*P* values <0.05, Mann-Whitney (Wilcoxon) *W*-test] in the number of ED1⁺ microglia cells to the value determined for younger animals.

tion, and showed higher immigration rates of CD4⁺ than of CD8⁺ T cells.

T Cells in the Spinal Cord of Adult Hemizygous Transgenic, PLP-Overexpressing Lewis Rats with Myelin Degeneration in CNS Gray Matter

In further experiments, we screened spinal cord sections of hemizygous-transgenic, PLP-overexpressing Lewis rats. We already knew that the principle pathological features of these animals were low-grade, subclinical degeneration of CNS myelin, accumulation of myelin proteins in CNS gray matter oligodendrocytes, occasional oligodendrocyte death, and activation of microglia cells in CNS gray matter.^{4,5} Moreover, in CNS gray matter, the numbers of dystrophic oligodendrocytes (data not shown) and of activated microglia cells (Figure 6) increase with age.

When we studied the distribution of T cells in spinal cord sections of 7- to 8-week-old transgenic animals, we obtained similar results as seen in age-matched wild-type animals: T cells were found everywhere within the spinal cord, but more so in gray matter. Again, CD8⁺ T cells were more numerous than CD4⁺ T cells (Figure 7). With age, the numbers of infiltrating T cells increased (Figure 7), most pronounced in spinal cord gray matter. The increase in cell numbers was most noticeable in the CD4⁺ T-cell pool (13-fold increase from levels of 2-month-old animals to levels of 10- to 12-month-old animals in spinal cord gray matter), but was also evident for CD8⁺ T lymphocytes (2.7-fold increase from levels of 2-month-old animals to levels of 10- to 12-month-old animals in spinal cord gray matter; Figure 7). As a net result of the different increase rates, similar numbers of CD4⁺ and CD8⁺ T cells were found in the gray matter of aged transgenic rats (Figure 7, Table 1). Taken together, the enhanced T-cell infiltration of the transgenic spinal cord coincided temporally and spatially with the severity of myelin/oligodendrocyte degeneration and the degree of microglia cell activation in the spinal cord gray matter.

Figure 6. The age-related increase in the numbers of ED1⁺ and OX6⁺ microglia cells in white (white) and gray (gray) matter of spinal cord sections from hemizygous transgenic, PLP-overexpressing transgenic Lewis rats. **Black asterisks** indicate statistically significant differences at the 95% confidence level [*P* values <0.05, Mann-Whitney (Wilcoxon) *W*-test] in the number of cells to the value determined for the same compartment in younger animals. The ED1⁺ microglia cells engaged in phagocytic uptake of degenerating axons in the spinal cord white matter were excluded from this study.

Discussion

The presence of T cells within the intact CNS is undisputed. T-cell numbers within the CNS are low in the healthy organism and increase when there are strong immunological reactions within the body³ or degeneration within the CNS.⁸⁻¹¹ Our studies revealed that the T cells found within the intact spinal cord predominantly belong to the CD8⁺ subset of lymphocytes, and are most frequently found in the gray matter. In the degenerative spinal cord, T cells preferentially infiltrated the areas of degeneration. The increase in T-cell numbers was more pronounced for the CD4⁺ than for the CD8⁺ T-cell subset.

As summarized above, in the intact spinal cord, CD8⁺ T cells outnumber their CD4⁺ counterparts both in white and gray matter. Interestingly, these T-cell frequencies are just the opposite of CD4/CD8 ratios in peripheral rat blood, where CD4⁺ T cells are more numerous than CD8⁺ T cells (Table 1).¹² CD8⁺ T-cell numbers reminiscent of our findings were described in the CNS of MBP-specific T-cell receptor-transgenic mice,¹³ and of mice injected with dendritic cells into the CNS parenchyma.¹⁴ Another interesting observation was made in transgenic mice expressing hemagglutinin (HA) in astrocytes and enteric glia, and in their nontransgenic littermates:¹⁵ when both of these animals were injected with activated HA-specific CD8⁺ T cells, inflammatory lesions in the CNS readily developed in the HA-transgenic animals. However, HA-specific CD8⁺ T cells also entered the non-transgenic CNS in significant numbers, and accumulated in this organ. Based on these studies it was speculated that brain infiltration by CD8⁺ T cells might be less re-

Figure 7. The distribution of CD4- and CD8-positive T cells in white matter (white) and gray matter (gray) of spinal cord sections from hemizygous PLP-transgenic (tg) Lewis rats. The animals were grouped by age (2 months: 4 animals with 41.5 sections; 7 to 9 months: 8 animals with 108 sections; 10 to 12 months: 11 tg animals with 164 sections). **Gray asterisks** show statistically significant differences between spinal cord gray and white matter at the 95% confidence level [P values <0.05 , Mann-Whitney (Wilcoxon) W -test]. **Black asterisks** indicate statistically significant differences at the 95% confidence level [P values <0.05 , Mann-Whitney (Wilcoxon) W -test] in the number of cells to the value determined for the same compartment in younger animals. Note that in the last graph (= CD4⁺ T cells in tg spinal cords) standard deviations were omitted for the sake of clarity. The standard deviations for CD4⁺ T cells in spinal cord white and gray matter of 10- to 12-month-old animals were 3.47 and 7.16, respectively. T cells found within the meninges were omitted from this study.

stricted compared to CD4⁺ T cells.¹⁵ CD8⁺ T cells could cross more easily through the blood-brain-barrier than CD4⁺ T cells, if they respond better/differently to chemokines. Several observations support this interpretation. For example, CD8⁺ T cells do express the chemokine receptor CCR9, but CD4⁺ T cells do not.^{16,17} Moreover, in murine models of graft-versus-host disease, MIP-1 α preferentially recruits CD8⁺, but not CD4⁺ T cells to the target organs.¹⁸ The higher number of CD8⁺ T lymphocytes in the spinal cord could also reflect the presence of selective T-cell survival factors. Interestingly,

Table 1. Percentages of CD4⁺ and CD8⁺ T Cells in Peripheral Blood and Spinal Cord of 13- to 15-Month-Old Hemizygous, PLP-Transgenic Lewis Rats

	Peripheral blood	Spinal cord
CD4	61.0 $\pm$ 11.3	49.5 $\pm$ 6.4
CD8	31.0 $\pm$ 2.9	51.0 $\pm$ 7.1

Data shown are the mean values ($\pm$ SD) of two different, independently performed FACS analyses. For the first analysis, cells of four animals were pooled, for the second analysis, cells of five animals were pooled.

CD8⁺ T cells in peripheral tissues are more resistant to apoptosis than those in lymphoid organs.¹⁹ Moreover, CD8⁺ T cells, but not CD4⁺ T lymphocytes express the IL-15R α receptor molecule, which is necessary for long-term survival in the presence of IL-15.^{20,21} IL-15 is constitutively produced in the nervous system by microglia cells²² and astrocytes.²³

As mentioned above, we did not only observe a cellular bias in T-cell infiltration of the intact spinal cord, but also a compartmental bias, which affected the location of CD4⁺ and CD8⁺ T lymphocytes. In the healthy spinal cord, CD4⁺ and CD8⁺ T lymphocytes were more frequently found in the gray than in the white matter parenchyma. It is unlikely that this bias is caused by differences in T-cell retention in spinal cord gray matter, because CD4⁺ and CD8⁺ T cells that had entered white and gray matter in the course of EAE disappear from both sites with essentially the same time kinetics and efficiency. Instead, differences in T-cell entry through the blood-brain barrier into gray or white matter seem to be more reasonable. Most easily, this could be ascribed to the higher capillary densities^{24,25} and blood flow rates²⁶ in spinal cord gray compared to white matter.

The favored infiltration of spinal cord gray matter by CD4⁺ and CD8⁺ T lymphocytes explains findings of Njenga and colleagues.²⁷ In this study, the authors worked with the Theiler's virus-induced murine encephalomyelitis model and made two important observations: first, that the immune system preferentially clears Theiler's virus from spinal cord gray, and not from white matter; secondly, that both CD4⁺ and CD8⁺ T cells play an important role in this process because SCID mice reconstituted with splenocytes depleted of CD4⁺ or CD8⁺ T cells cleared the virus from the gray matter, but allowed viral replication in the white matter.²⁷ Hence, the compartmental bias in T-cell infiltration could have important clinical and therapeutic implications.

As stated previously, the overall number of T cells found within the intact CNS is rather low. However, it is well established that neurodegeneration is frequently accompanied by increased T-cell infiltration into the CNS. Interestingly, the T-cell frequencies observed span a wide range from slightly elevated T-lymphocyte numbers seen in human patients with Alzheimer's¹⁰ or Parkinson's disease⁹ to overt inflammatory reactions in the CNS of patients suffering from the severe cerebral form of adrenoleukodystrophy.²⁸ Similar changes are also seen in animal models, for example in the *twitcher* mouse with globoid cell leukodystrophy,^{29,30} in mice with GM1 and

GM2 gangliosidosis,³¹ or in mice with facial nerve transections, where T cells preferentially infiltrate the axotomized nerve nucleus and aggregate around the sites of neurodegeneration, despite an apparently intact blood-brain barrier.¹¹ We also noted increased T-cell infiltration that was temporally and spatially synchronous to degeneration in two different Lewis rat models: in aged rats with neuroaxonal degeneration, and in hemizygous PLP-transgenic rats with low-grade myelin degeneration. Irrespective of the underlying type of degeneration, these models had two characteristics in common. First, the number of T cells in the affected spinal cord regions was higher, and secondly, the relative increase in T-cell numbers was much more pronounced for CD4⁺ than for CD8⁺ T cells.

This could be the end-result of a nonspecific immigration of T cells from the immune system, because the peripheral immune repertoire of Lewis rats contains more CD4⁺ than CD8⁺ T cells (Table 1).¹² Equally likely, this could point to a specific mechanism of T-cell recruitment into the degenerative CNS, and reflect different responses of CD4⁺ and CD8⁺ T cells to environmental cues like chemokines^{16–18} or proinflammatory cytokines.³²

It is tempting to speculate that CD8⁺ and CD4⁺ T cells might have complementary roles in the intact and the degenerative CNS. In the intact spinal cord, CD8⁺ T cells dominate. Although there is some experimental evidence that CD8⁺ T cells are able to induce CNS inflammation,^{15,33,34} the major function of these cells seems to find and destroy infected³⁵ or malignant cells³⁶ without causing bystander damage to the adjacent tissue,¹⁵ and to down-regulate immune responses.^{37–40} In cases of degeneration, more CD4⁺ than CD8⁺ T lymphocytes are found in the spinal cord. This could have two different consequences.

On the one hand, CD4⁺ T cells drive inflammation and trigger tissue damage. This is particularly evident in Theiler's virus-infected mice.^{41–44} In these animals, CD8⁺ virus-specific T cells are found in the CNS at early stages of the infection,³⁵ but the development of the chronic-progressive T-cell-mediated autoimmune demyelinating disease typical for late stages of Theiler's virus infection critically depends on tissue destruction initiated by virus-specific CD4⁺ T cells, and on the subsequent activation of myelin protein-specific CD4⁺ T cells via epitope spreading.⁴¹ This does not exclude an additional role of CD8⁺ T cells in the induction of neurological deficits and clinical disease once demyelination has started.^{45,46} The crucial role of CD4⁺ T cells in neuroinflammation is also seen in the different animal models of EAE.⁴⁷

On the other hand, CD4⁺ T cells could provide some degree of neuroprotection.^{47,48} They produce and secrete molecules such as brain-derived neurotrophic factor^{49,50} and nerve growth factor,^{51,52} which may both promote neuronal survival⁵³ and down-regulate MHC class II products on microglia cells,⁵⁴ or neurotrophin-3,⁵⁵ which may prevent neuronal cell death,⁵⁶ regulate phagocytosis by microglia cells⁵⁷ and contribute to the proliferation of cells along the oligodendrocyte lineage.⁵⁸ Hence, the preferred recruitment of CD4⁺ T cells to the neurodegenerative spinal cord may pave the way for

either tissue destruction or tissue protection. The actual route taken seems to crucially depend on additional environmental cues, possibly provided by activated microglia cells. Further studies will concentrate on the identification of these signals.

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