CD4 T Cells Mediate Axonal Damage and Spinal Cord Motor Neuron Apoptosis in Murine P0_{106–125}-Induced Experimental Autoimmune Neuritis

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The pathogenesis of inflammatory autoimmune diseases of the peripheral nervous system, leading to demyelination and/or axonal damage, remains incompletely understood. In particular, it is controversial regarding the extent to which (i) autoimmune-mediated destruction of peripheral nerves results in secondary damage of the central nervous system, and (ii) CD4 and CD8 T cells contribute to disease. To address these issues, we applied the murine model of P0_{106–125}-induced experimental autoimmune neuritis. Immunization of C57BL/6 mice with P0_{106–125} resulted in severe axonal damage and mild demyelination. Importantly, these mice developed a “dying-back” axonopathy with apoptosis of a large fraction of neurons in the anterior horn of the lumbar and thoracic spinal cord and a progressive neurogenic muscular atrophy. T cell-depletion experiments identified CD4, but not CD8, T cells as important mediators of experimental autoimmune neuritis. CD4 T cells represented the major cellular source of antigen-specific interferon- γ and interleukin-17 production, regulated the number of tumor necrosis factor-positive and inducible nitric oxide synthase-positive macrophages in the diseased sciatic nerve, and mediated axonal damage and subsequent neuronal apoptosis and neurogenic muscular atrophy. In contrast, the demyelination of peripheral nerves was only slightly ameliorated in CD4 T cell-depleted mice. In conclusion, P0_{106–125}-induced experimental autoimmune neuritis is a CD4 T cell-mediated autoimmune disease that affects both the peripheral and central nervous systems. (*Am J Pathol* 2008, 173:93–105; DOI: 10.2353/ajpath.2008.071101)

Autoimmune-mediated diseases of the peripheral nervous system are a heterogeneous group of clinical entities, which are pathologically characterized by demyelination and/or axonal damage. The various immunopathological mechanisms, which underlie these diverse clinical manifestations, as well as the relative importance of axonal damage and demyelination, are still incompletely defined. In particular, the relative contribution of axonal degeneration and/or demyelination to the pathology is still controversially discussed. This also holds true for inflammatory demyelinating diseases of the central nervous system, ie, multiple sclerosis in humans and experimental autoimmune encephalomyelitis in rodents. In these disorders the extent and severity of inflammation has to some degree observed to be related to the amount of axonal damage.¹ While some authors reported that demyelination precedes and triggers axonal damage (outside-in damage), the extent of which determines the final clinical outcome and degree of neurological disability in multiple sclerosis and experimental autoimmune encephalomyelitis, other groups provided evidence for primary axonal injury, followed by secondary demyelination (inside-out damage).^{1–3} Interestingly, a recent study indicated that axonal injury might herald or trigger demyelination in Theiler's murine encephalomyelitis virus infection.² So far, this issue has not been resolved for autoimmune inflammation of the peripheral nervous system. However, a sound understanding of the underlying immunological mechanisms and pathological responses is a prerequisite to improve or to establish rational therapeutic strategies. In this context, animal models of autoimmune neuritis are useful to prove basic pathogenetic concepts of the corresponding human diseases.

The hallmark of rat experimental autoimmune neuritis (EAN) is the infiltration of the peripheral nervous system by lymphocytes and macrophages. However, it is still controversial whether CD4 T cells and macrophages are sufficient to induce EAN or whether the integrated attack

Supported by Köln-Fortune Program (grant no. 60/2006).

Accepted for publication April 3, 2008.

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of CD4 and CD8 T cells, B cells, and macrophages is crucial for immunopathology of EAN.^{4–7}

Traditionally, most studies of EAN were performed in rat and disease was induced by either adoptive transfer of autoimmune T cells^{8,9} or by immunization with neuritogenic epitopes of the P2 or the P0 protein,^{10–13} respectively. Recently, we have identified the P0_{106–125} peptide as a neuritogenic epitope in mice.¹⁴ Immunization of C57BL/6 mice with P0_{106–125} induced a well reproducible and robust EAN with axonal neuritis and mild demyelination of peripheral nerves.¹⁴ Clinically, P0_{106–125}-induced EAN was more severe and homogenous compared to murine EAN induced by immunization with the traditionally used P0_{180–199} peptide.^{14–16} Therefore, the improved P0_{106–125}-model of EAN should allow a clear dissection of pathogenetic mechanisms.

The aim of the present study was to selectively study the role of CD4 and CD8 T cells in P0_{106–125}-induced murine EAN and to determine their impact on the autoimmune-disease manifestation in the peripheral nerve, and also in its target organ, ie, the gastrocnemius muscle, as well as in its origin, ie, the spinal cord. EAN of P0_{106–125}-immunized mice was characterized by a combined axonal and demyelinating inflammatory neuritis resulting in a severe dying back axonopathy with apoptosis of a large fraction of spinal cord motor neurons and a rapidly progressive neurogenic muscular atrophy. T cell depletion experiments identified CD4 T cells as major pathogenetically relevant T cell population with a predominant impact on axonal damage and, subsequently, motor neuron apoptosis and muscular atrophy.

Materials and Methods

Induction of Autoimmune Neuritis

C57BL/6 mice were bred in the specific pathogen-free animal facility of the University of Cologne (Germany). All animals were housed under specific pathogen-free conditions throughout the experiments. The experiments were carried out according to local governmental regulations. Six-to-eight-week-old male mice were immunized with 200 µg of P0_{106–125} peptide (Leids Universitair Medisch Centrum, Leiden, The Netherlands; amino acid sequence: I-V-G-K-T-S-Q-V-T-L-Y-V-F-E-K-V-P-T-R-Y) dissolved in dimethyl sulfoxide (Sigma, Taufkirchen, Germany) and 0.5 mg *Mycobacterium tuberculosis* (strain H 37 RA; Difco; Detroit, MI) emulsified in 25 µl PBS and 25 µl Freund's incomplete adjuvant (Difco) into their hind foot pads on day (d) 0 and into the back on day 7, respectively. In addition, 500 ng pertussis toxin per mouse (Sigma) was injected i.p. at day 0 and day 2. Control mice were pretreated with PBS instead of P0_{106–125}-peptide.

T Cell Depletion

For depletion of CD4 and CD8 T cells, mice were treated with either rat anti-mouse CD4 (clone GK1.5; concentration 5 mg/ml) and rat anti-mouse CD8 (clone 2.43; concentration 2.5 mg/ml) antibodies, respectively, as de-

scribed before.¹⁷ Control mice were treated with rat IgG (Sigma, Deisenhofen, Germany; concentration 1.25 mg/ml). T cells were depleted by i.p. injection of 200 µl of the respective antibody at days –2, –1, 2, 5, and 7. Successful depletion was determined in the spleen and in the popliteal lymph node (PLN) at days 8, 15, 22, and 40 post-immunization (p.i.) by flow cytometric analysis (depletion efficiency always ≥97%).

Assessment of Clinical Score

Before the first depletion and afterward, at days 0, 2, 5, 7, 8, 9, 12, 15, 18, 22, and 40 p.i., mice were clinically examined in a blinded fashion to determine disease activity according to a previously published scale¹⁴: 0 = normal; 1 = floppy tail, weak grip, ruffled fur and slightly reduced motility; 2 = mild paraparesis with impaired motility; 3 = severe paraparesis with significantly reduced motility; 4 = tetraparesis with complete immobilization; 5 = death due to autoimmune neuritis.

Flow Cytometry

At days 8, 15, 22, and 40 p.i., mice were intracardially perfused with 0.9% NaCl in deep CO₂ anesthesia. Splenic and PLN leukocytes were isolated and analyzed by triple immunofluorescence staining followed by flow cytometry as described.¹⁷ Triple stainings were performed as follows: (1) rat anti-mouse CD25-fluorescein isothiocyanate (FITC; clone 7D4), rat anti-mouse CD4-phosphatidylethanolamine (clone GK1.5), and rat anti-mouse CD8a-peridinin-chlorophyll-protein complex (clone 53-6.7); (2) rat anti-mouse CD4-FITC (clone GK1.5), rat anti-mouse CD45R/B220-phosphatidylethanolamine (clone RA3-6B2) and rat anti-mouse CD8a-peridinin-chlorophyll-protein complex; (3) rat anti-mouse F4/80-FITC (clone BM8), mouse anti-mouse major histocompatibility complex (MHC) class II-antigen-phosphatidylethanolamine (clone AF6-120.1), and rat anti-mouse CD45-peridinin-chlorophyll-protein complex (clone 30-F114); rat anti-mouse CD44-FITC (clone IM7), rat anti-mouse CD62L-phosphatidylethanolamine (clone MEL-14), and rat anti-mouse CD3-peridinin-chlorophyll-protein complex (clone 145-2C11). All antibodies were purchased from BD Biosciences (Heidelberg, Germany), except for anti-F4/80, which was obtained from Caltag (Hamburg, Germany). Flow cytometry was performed on a BD FACSCalibur (BD Biosciences) and analyzed with the CellQuest Pro software (BD Biosciences).

Enzyme-Linked Immunospot Assay

The frequency of P0_{106–125}-specific lymphocytes in spleen and PLN was determined by use of an interferon (IFN)-γ- and interleukin (IL)-17-specific enzyme-linked immunospot assay at days 8, 15, 22, and 40 p.i. as described previously.¹⁷ Triplicates of freshly isolated leukocytes at densities of 2 × 10³/well, 2 × 10⁴/well, and 2 × 10⁵/well, respectively, were placed in an enzyme-linked immunospot assay plate (Nunc, Wiesbaden, Germany)

coated with rat anti-mouse IFN- γ (clone RMMG-1 (10 μ g/ml; Biosource, Camarillo, CA) or rat anti-mouse IL-17A (clone eBio17CK15A5, 10 μ g/ml, eBiosciences). Cells were cocultured with syngeneic C57BL/6 spleen cells from non-immunized mice (4×10^5 /well) preloaded with PO_{106–125} peptides at a concentration of 1×10^{-6} M overnight. Plates were developed with biotin-labeled rat anti-mouse IFN- γ (BD Pharmingen) or biotin-conjugated rat anti-mouse IL-17A (clone eBio17B7, eBiosciences) followed by peroxidase-conjugated streptavidin (Dianova, Hamburg, Germany), and amino-ethylcarbazole dye solution (Sigma). Spots were counted microscopically. The number of the corresponding epitope-specific lymphocytes was expressed as the number of spots per leukocytes seeded. Isolated spleen and lymph node cells from immunized or PBS-injected mice incubated with non-stimulated spleen cells (negative control) and spleen and lymph node cells from PBS-injected mice incubated with PO_{106–125}-stimulated spleen cells served as controls.

Histopathology and Immunohistochemistry

For histopathology and immunohistochemistry, animals were intracardially perfused with 0.9% NaCl in deep CO₂ anesthesia at days 8, 15, 22, and 40 p.i.

One sciatic nerve per animal was excised from the lumbar spinal cord to the periphery including parts of the gastrocnemius muscle, which is innervated by the sciatic nerve, mounted on thick filter paper with Tissue Tek OTC compound (Miles Scientific, Naperville, IL), snap-frozen in isopentane (Fluka, Neu-Ulm, Germany) precooled on dry ice, and stored at -80°C until preparation of serial 10 μ m frozen sections. The other sciatic nerve and corresponding gastrocnemius muscle were postfixed in 3.9% phosphate buffered glutaraldehyde (Merck, Darmstadt, Germany) for 48 hours, osmicated, and embedded in epoxy resin (Serva, Heidelberg, Germany) for preparation of 1 μ m semithin sections. The spinal cord including the meninges was prepared and postfixed with 4% buffered paraformaldehyde before dissection, embedding in paraffin, and preparation of 4- μ m paraffin sections.

Sections of the respective tissues were stained with H&E, Masson-Goldner, 0.1% toluidine blue O (Merck) in 2.5% sodium carbonate (Merck), silver impregnation (Bielschowsky), luxol fast blue, cresyl violet, enzyme histochemistry with reactions for myofibrillary ATPase pH at 9.4, and following preincubation at pH 4.6 in acid phosphatase, and reduced nicotinamide adenine dinucleotide-tetrazolium reductase. In addition, immunohistochemistry with the following rat anti-mouse monoclonal antibodies obtained from hybridomas (American Type Culture Collection, Manassas, VA) were performed: CD45 (clone M1/9.3.4.HL.2), F4/80 (clone F4/80), Mac-1 (clone WEHI-234.1), Mac-3 (clone M3/84), MHC class I-antigen haplotypes (clone M 1/42.3.9.8 HLK), and MHC class II-antigen (I-A^{b,d,q} haplotypes; clone M5/114.15.2). CD4 (clone H129.19), CD8a (clone 53–6.7), CD19 (clone 1D3), and tumor necrosis factor (TNF; clone MP6/XT22) were purchased from BD Biosciences. In addition, the following primary antibodies were used: glial fibrillary

acidic protein (GFAP, Dako, Hamburg, Germany), neurofilament (NF-L, Chemicon/Millipore, Schwalbach, Germany), inducible nitric oxide (iNOS, Calbiochem, Darmstadt, Germany), CD95 (FAS, LabVision, Fremont, CA), CD79a (Zytomed Systems, Berlin, Germany), and synaptophysin antibody (clone SP11, DCS, Hamburg, Germany). Axonal and neuronal damage, respectively, was evidenced by application of monoclonal mouse α -amyloid precursor protein (α -APP, clone 22C11, Chemicon) and monoclonal mouse NeuN (clone A60, Chemicon) and detected by use of the Animal Research Kit (Dako) according to the manufacturer's instructions.

Immunohistochemistry was performed by use of the avidin-biotin complex technique with appropriate biotinylated secondary antibodies (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA). Peroxidase reaction product was visualized using 3,3'-diaminobenzidine (Sigma) as chromogene and H₂O₂ as cosubstrate.

The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit (Roche, Mannheim, Germany) was applied according to the manufacturer's instructions.

Statistical Analysis

To test for differences in the clinical disease activity a two-tailed Mann-Whitney *U*-test was applied. The number of CD4 and CD8 T cells and macrophages was determined by counting immunoreactive cells in high power fields (microscopic magnification $\times 400$) of CD4, CD8, or Mac3 immunostained sections, respectively, of the spinal cord and the sciatic nerve in at least four animals per experimental group. The number of apoptotic neurons, identified by eosinophilic cytoplasm, and condensed hyperchromatic nuclei, or by TUNEL staining was counted in high power fields of cresyl violet stained spinal cord paraffin sections of at least two animals per experimental group. Data are presented as mean $\pm$ SD. Differences in the number of inflammatory cells and antigen-specific T cells in spleen and PLN, were determined using a two-tailed Student's *t*-test. *P* values <0.05 were considered significant. All experiments were performed at least three times with four animals per experimental group. Thus, overall 840 animals were studied.

Results

Immunization with PO_{106–125} Peptide Causes Severe Axonal Inflammatory Neuritis Resulting in Acute Neurogenic Muscular Atrophy and Dying Back Axonopathy with Spinal Cord Motor Neuron Apoptosis

All animals immunized with PO_{106–125} developed clinical signs of neuritis up to day 15 (Figure 1A) as published previously.¹⁴ Immunized mice having received rat IgG did not differ from animals without application of antibodies. At day 8 p.i., individual mice were diseased and showed a floppy tail, weak grip, ruffled fur, and a slightly reduced motility (Figure 1B; mean 0.87 ± 0.2). At this

Figure 1. Disease incidence and clinical score of EAN in mice receiving anti-CD4 and/or anti-CD8 antibodies or rat IgG. **A:** Mice of all experimental groups develop EAN (100% disease incidence). In CD4- and CD4&CD8-depleted mice disease induction is delayed; however, all mice were diseased. At day 40 p.i., mice did not show any clinical symptoms. **B:** In CD4- and CD4&CD8-depleted mice, the clinical score was significantly reduced as compared to rat IgG-treated animals ($P < 0.05$). In contrast, clinical severity of disease was not significantly different between CD4 and CD4&CD8 injected animals ($P > 0.05$) at days 8, 15, 22, and 40 p.i.

time point, single CD4 T cells had homed to the sciatic nerve. Myelin sheaths, axons, and the gastrocnemius muscle were normal.

At day 15 p.i., ie, the maximum of clinical disease activity (Figure 1B; mean 2.83 ± 0.3), the sciatic nerve harbored prominent inflammatory infiltrates consisting of F4/80⁺ macrophages, which expressed TNF and iNOS protein (Figure 2A), CD4 and CD8 T cells (Figure 3A), and single CD19⁺ B cells. Axons were damaged as demonstrated by a reduction in axonal NF protein expression and APP⁺ swelling (Figure 2, B and C). Semithin sections showed axonal damage evidenced by myelin ovoids within the nerve fibers as an indication of axonal degeneration forming bands of Bungner (Figure 4A). Remarkably, damaged axons were associated with macrophages (Figure 4, A and B). These alterations caused a severe and progressive neurogenic muscular atrophy with macrophages phagocytosing damaged muscle fibers (Figure 4C). However, demyelination of axons was mild (Figure 4, A and B).

From day 15 p.i. to day 22 p.i., clinical disease activity had regressed (Figure 1B; mean 2.5 ± 0.5). In the sciatic nerve, CD4 and CD8 T cells, and CD19⁺ B cells had significantly declined ($P < 0.05$, Figure 3B), whereas F4/80⁺ macrophages were more numerous as compared to day 15 p.i. ($P < 0.05$, Figure 3B). Segmental demyelination as well as axonal damage including axonal swelling of the sciatic nerve persisted. Leukocytic infiltrates composed of CD4 and CD8 T cells, macrophages, and single CD19⁺ B cells were still present in the gastrocnemius

muscle, which showed prominent neurogenic muscular atrophy. Phagocytic activity in muscle fibers was ongoing.

In addition to these alterations of the sciatic nerve and the gastrocnemius muscle, the spinal cord showed marked pathological alterations. As early as day 8 p.i., coinciding with the clinical onset of disease, in the spinal cord a few Mac-3⁺ MHC class II-antigen⁺ microglial cells and some ubiquitously distributed GFAP⁺ astrocytes were weakly activated. However, inflammatory infiltrates, in particular CD3⁺ T cells were absent from both the parenchyma and the leptomeninges.

From day 8 to 15 p.i., pathology had progressed. In the lumbar and thoracic spinal cord, APP staining revealed axonal swelling, and a strong reduction of NF protein indicated axonal loss. Although some Mac-3⁺ MHC class II-antigen⁺ macrophages were present in the white matter of the spinal cord, demyelination was not obvious (Figure 5A; Figure 6A). Remarkably, anterior horn motor neurons were apoptotic as evidenced by cell shrinkage, a condensed nucleus, an eosinophilic cytoplasm as well as a positive TUNEL reaction (Figure 5A, 6B, and inset in 6B). They did not express Fas (CD95, data not shown). TUNEL-positive neurons did not express NeuN and their synaptic density was markedly reduced compared to controls as evidenced by a reduced synaptophysin expression. Although activation of microglial cells and astrocytes had moderately increased from day 8 to 15 p.i. (Figure 6A), they did neither form clusters nor were they preferentially associated with neurons. CD3⁺ T cells were absent from the spinal cord.

From day 15 to 22 p.i., axonal damage in the spinal cord had progressed. This was paralleled by an increased fraction of apoptotic neurons, which resulted in a prominent loss of neurons in the anterior horn of the lumbar and thoracic spinal cord. Furthermore, axons in the spinal cord were demyelinated. Inflammatory infiltrates were confined to the white matter of the spinal cord and consisted of CD4 and CD8 T cells, macrophages, and single CD79a⁺ B cells. Activation of Mac-3⁺ MHC class II-antigen⁺ microglial cells and GFAP⁺ astrocytes had declined. A few Mac3⁺ microglial cells/macrophages were observed in the vicinity of apoptotic neurons, which were Fas- and NeuN-negative, whereas vital neurons were never associated with Mac 3⁺ microglia/macrophages.

At day 40 p.i., the anterior horn of the spinal cord harbored patchy loss of neurons (Lückenfelder). Remaining neurons were viable, had a triangular shape with a round nucleus, a prominent basophilic nucleolus, without evidence for chromatolysis or nuclear fragmentation, a normal cytoplasm, and a prominent axon (Figures 5C and 6C). Neuronal viability was further confirmed by a prominent nuclear and cell surface expression of NeuN (Figure 6C, inset) and synaptophysin, respectively, indicative of a normal nuclear structure and synaptic density. Negative TUNEL staining lends further support to the absence of neuronal apoptosis (Figure 5A). A strong and ubiquitous NF protein expression evidenced a dense axonal network, and axonal damage as well as demyelination was absent. In the spinal cord parenchyma as well as in

Figure 2. Histopathological alterations in the sciatic nerve in EAN of rat IgG and anti-CD4-treated mice at day 15 p.i. **A:** Endoneurial macrophages are activated in a rat IgG-treated mouse as evidenced by an up-regulation of the F4/80 antigen and strongly express iNOS (**inset**). Anti-F4/80 immunostaining, original magnification $\times 800$; **inset**: anti-iNOS immunostaining, original magnification $\times 400$. **B:** Significant loss of axons in a rat IgG-treated mouse. Immunofluorescence with anti-neurofilament antibodies and FITC-conjugated mouse anti-rat IgG; original magnification $\times 400$. **C:** Swelling of axons in the sciatic nerve of a rat IgG-treated mouse as evidenced by an up-regulation of the α -APP antigen in these axons. Anti- α -APP immunostaining; original magnification $\times 400$. **D:** In an anti-CD4-treated mouse, the number of activated F4/80⁺ endoneurial macrophages is significantly reduced as compared to rat IgG-treated animals (**A**). Correspondingly, iNOS is weakly expressed by only few macrophages (**inset**). Anti-F4/80 immunostaining; original magnification $\times 800$. **Inset**: anti-iNOS immunostaining; original magnification $\times 400$. **E:** Axonal loss is less severe in an anti-CD4-treated mouse as compared to rat IgG-treated animals (**B**). Immunofluorescence with anti-neurofilament antibodies and FITC-conjugated mouse anti-rat IgG; original magnification $\times 400$. **F:** There is no axonal swelling in the sciatic nerve of an anti-CD4-treated mouse as compared to the rat IgG-treated animal (**C**). Anti- α -APP immunostaining; original magnification $\times 400$.

the leptomeninges neither CD3⁺ T cells nor other CD45-expressing leukocytes or Mac3⁺ macrophages were detected (Figure 5B).

At day 40 p.i., inflammatory infiltrates were absent from the sciatic nerve (Figure 3C). Only single Mac3⁺ macrophages were present (Figure 3C). There was neither axonal damage nor segmental demyelination. Accordingly, neurogenic muscular atrophy did not progress in the gastrocnemius muscle. At this late time point, hyper-

trophic muscle fibers and fiber type grouping indicated compensatory collateral re-innervation.

CD4 T Cells are Important for Development and Severity of EAN

To precisely assess the role of CD4 and CD8 T cells in murine EAN, CD4 and CD8 T cell depletion experiments were performed.

The severity of EAN were significantly reduced in anti-CD4 (mean 1.08 ± 0.3) and anti-CD4 plus anti-CD8-treated mice (mean 1.4 ± 0.25) as compared to rat IgG-treated control mice (mean 2.83 ± 0.38 ; Figure 1B; $P < 0.05$ at days 8, 15, and 22 p.i.).

At day 8 p.i., when clinical symptoms were still absent, sciatic nerve, gastrocnemius muscle, and spinal cord of anti-CD4 plus anti-CD8-treated animals were neuropathologically normal.

At day 15 p.i., the sciatic nerve harbored only a few F4/80⁺ macrophages, while T cells were absent. The number of TNF⁺ and iNOS⁺ cells was also strongly reduced as compared to rat IgG-treated animals (Figure 2, A and D). Moreover, depletion of CD4 & CD8 T cells resulted (i) in remarkably less severe axonal damage of the sciatic nerve as evidenced by only a focal reduction of NF expression (Figure 2E), the absence of APP⁺ axonal swelling (Figure 2F), and the absence of myelin ovoids and missing bands of Bungner in semithin sections (Figure 4D). In addition, demyelination was slightly reduced (Figure 4E), and neurogenic muscular atrophy was markedly reduced as compared to rat IgG-treated mice (Figure 4F). Thereafter, inflammatory infiltrates regressed in parallel to the declining disease activity (Figure 1B).

At day 15 p.i., in the spinal cord of mice devoid of CD4 and CD8 T cells, demyelination was absent, and axons were only mildly damaged. In addition, the number of apoptotic neurons was significantly decreased as compared to rat IgG-treated mice ($P < 0.05$; Figures 5A and 6E). These neurons only weakly expressed NeuN. In addition, synaptophysin was expressed at decreased density as compared to control mice. Microglial activation was weak; only exceptional Mac-3⁺ macrophages were detected in the spinal cord of these animals ($P < 0.05$ for CD4 & CD8- versus rat IgG-treated mice, Figure

Figure 3. Numbers of CD4 and CD8 T cells, and macrophages in the sciatic nerve and the spinal cord of P0₁₀₆₋₁₂₅-immunized and non-immunized mice. **A:** Numbers of CD4 and CD8 T cells as well as macrophages were counted in high power fields of CD4, CD8, and Mac3 immunostained sections, respectively, of the sciatic nerve in mice having received anti-CD4 and/or anti-CD8 antibodies or rat IgG at day 15 p.i. as described in Materials and Methods. Non-immunized C57BL/6 mice served as controls. The mean $\pm$ SD is shown. * $P < 0.05$ as compared to rat IgG-treated mice. **B:** Numbers of CD4 and CD8 T cells as well as macrophages were counted in high power fields of CD4, CD8, and Mac3 immunostained sections, respectively, of the sciatic nerve in mice having received anti-CD4 and/or anti-CD8 antibodies or rat IgG at day 22 p.i. as described in Materials and Methods. Non-immunized C57BL/6 mice served as controls. The mean $\pm$ SD is shown. * $P < 0.05$ for CD4 and CD8 T cells and for macrophages as compared to rat IgG-treated mice. **C:** Numbers of CD4 and CD8 T cells, as well as macrophages, were evaluated in high power fields of CD4, CD8, and Mac3 immunostained sections, respectively, of the sciatic nerve in mice having received anti-CD4 and/or anti-CD8 antibodies or rat IgG at day 40 p.i. as described in Materials and Methods. Non-immunized C57BL/6 mice served as controls. The mean $\pm$ SD is shown. * $P < 0.05$ for macrophages as compared to rat IgG-treated mice.

Figure 4. Ultrastructural investigation of the sciatic nerve and the gastrocnemius muscle in P0106-125-immunized rat IgG and anti-CD4-treated mice at day 15 p.i. **A:** Severe loss of axons in a cross section of the sciatic nerve of a rat IgG-treated mouse. *indicates a degenerated nerve fiber with hands of Büngner. **Arrows** point to axonal damage in association with early damage of myelin sheaths, which, however, are not yet disrupted. **B:** In a longitudinal section of the sciatic nerve of a rat IgG-treated mouse, myelin sheaths too thin in relation to their axonal diameter are present (**arrows**). Macrophages (*) are associated with loss of damaged nerve fibers. **C:** Severe neurogenic atrophy of the gastrocnemius muscle in a rat IgG-treated mouse. The endomysial tissue harbors leukocytes, which have also invaded damaged muscle fibers (*). Groups of small, angulated muscle fibers, which are partly or completely atrophic, and single hypertrophic fibers indicate the severe and progressive neurogenic atrophy (**inset**). **D:** As compared to a rat IgG-treated mouse (**A**), the axons in the sciatic nerve of an anti-CD4-treated mouse are only mildly damaged as evidenced by a normal density of nerve fibers with intact central axons. Only occasionally, myelin sheaths are fragmented (**arrow**). **E:** In an anti-CD4-treated mouse, fragments of myelin are phagocytosed (*), whereas only single nerve fibers of the sciatic nerve show condensed axons (**arrow**) indicating a markedly reduced axonal damage as compared to rat IgG-treated animals (**B**). **F:** Corresponding to the reduced axonal damage in the sciatic nerve (**D**, **E**), neurogenic atrophy of the gastrocnemius muscle is less severe in an anti-CD4-treated mouse as evidenced by single, partly atrophic muscle fibers in the absence of myophagia. Note numerous hypertrophic muscle fibers (*) indicating compensation. Semithin sections stained with toluidine blue; original magnification $\times 400$.

5B). These neuropathological alterations persisted unchanged up to day 22 p.i.

At day 40 p.i., mice depleted of CD4 or CD4 and CD8 T cells neither showed axonal damage nor demyelination in their spinal cord as evidenced by a normally dense expression of the NF protein and lack of APP immunoreactivity. The parenchyma and the leptomeninges of the spinal cord did not harbor CD45⁺ leukocytes including CD3⁺ T cells and Mac3⁺ macrophages (Figure 5B). Neurons in the anterior horn of the spinal cord were viable, exhibited a triangular shape, a round nucleus with a prominent basophilic nucleolus without signs of chromatolysis or nuclear fragmentation, a normal cytoplasm, and a prominent axon (Figure 6F, inset in 6F). Correspondingly, there was no evidence for ongoing apoptosis (Figure 5A). However, the absolute number of viable anterior horn neurons was 95.57 ± 5.44 in 5 HPF in CD4⁺ depleted mice, thus, significantly less decreased as compared to rat IgG-treated animals (52.86 ± 3.34 ; $P < 0.00002$; Figure 5C).

At day 40 p.i., inflammatory infiltrates were absent from the sciatic nerve of CD4-depleted mice (Figure 3C). Only single macrophages were detected (Figure 3C). In the absence of ongoing axonal damage or segmental demyelination, the gastrocnemius muscle contained a few hypertrophic muscle fibers and incipient fiber type grouping indicative of collateral re-innervation.

Having established that T cells were decisive for the induction of EAN, we next determined whether CD4 or CD8 T cells were more important. Mice having been depleted of CD4 T cells showed a similar mild clinical course and essentially the same morphological alterations as observed for mice having received anti-CD4 plus anti-CD8 treatment (Figure 1, A and B). In contrast, mice lacking CD8 T cells exhibited the same severe clinical disease activity as rat IgG-treated animals (Figure 1B; $P > 0.05$ at days 8, 15, and 22 p.i.). Accordingly, the morphological characteristics of EAN were similar to the rat IgG-treated group (data not shown). Thus, these data point to an important role of CD4 T cells in murine EAN

Figure 5. Numbers of apoptotic neurons, macrophages and viable neurons in the spinal cord of $P0_{106-125}$ -immunized and non-immunized mice. **A:** The number of apoptotic neurons was evaluated in high power fields (HPF) of cresyl violet stained spinal cord sections in mice having received anti-CD4 and/or anti-CD8 antibodies or rat IgG at days 15, 22, and 40 p.i. as described in Materials and Methods. Non-immunized C57BL/6 mice served as controls. The mean $\pm$ SD is shown. * $P < 0.05$ as compared to rat IgG-treated mice. **B:** The number of macrophages was determined in high power fields of Mac3 immunostained sections of the spinal cord in mice having received anti-CD4 and/or anti-CD8 antibodies or rat IgG at days 15, 22, and 40 p.i. as described in Materials and Methods. Non-immunized C57BL/6 mice served as controls. The mean $\pm$ SD is shown. * $P < 0.05$ as compared to rat IgG-treated mice. **C:** The number of viable neurons in the anterior horn of the spinal cord was evaluated in high power fields of cresyl violet stained spinal cord sections in mice having received anti-CD4 and/or anti-CD8 antibodies or rat IgG at days 15, 22, and 40 p.i. as described in Materials and Methods. Non-immunized C57BL/6 mice served as controls. The mean $\pm$ SD is shown. * $P < 0.05$ as compared to rat IgG-treated mice.

with a particular impact on axonal damage and spinal cord motor neuron apoptosis.

Immunization with $P0_{106-125}$ Induces Antigen-Specific, IFN- γ - and IL-17-Producing CD4 T Cells

In the PLN and spleen of immunized mice, $P0_{106-125}$ -specific IFN- γ -producing cells were induced (Figure 7). In the PLN, maximal numbers of $P0_{106-125}$ -specific cells were reached as early as day 8 p.i. declining thereafter (Figure 7). In contrast, numbers of splenic antigen-specific IFN- γ -producing cells were maximal at day 15 p.i. (Figure 7B).

In CD4 and CD8 T cell-depleted mice, the number of $P0_{106-125}$ -specific IFN- γ -producing cells was massively reduced in PLN and spleen (Figure 7, A–C) illustrating that the immunization with $P0_{106-125}$ induced antigen-specific T cells. To define which further T cell subset was specific for $P0_{106-125}$, either CD4 or CD8 T cells were selectively depleted. In anti-CD4-treated mice, only very small numbers of $P0_{106-125}$ -specific IFN- γ -producing cells were detectable in PLN and spleen, whereas the depletion of CD8 T cells did not significantly reduce numbers of $P0_{106-125}$ -specific IFN- γ -producing cells compared to rat IgG-treated mice (Figure 7, A–C). Thus, CD4 T cells are the major $P0_{106-125}$ -specific T cell population producing the pro-inflammatory cytokine IFN- γ .

In PLN and spleen of immunized mice, $P0_{106-125}$ -specific IL-17-producing cells were induced. At day 8 p.i., numbers of IL-17-producing T cells were low in PLN and spleen of rat IgG-treated and CD8-depleted mice with a rise up to day 15 and 22 p.i. (Figure 8A–C). Antigen-specific IL-17-producing cells were completely absent from the PLN and spleen of CD4- or CD4&CD8-depleted mice throughout the study, further lending support to the importance of CD4 T cells in the pathogenesis of EAN.

Discussion

The present study demonstrates that immunization of mice with a peptide specific for a protein of peripheral nerves induces a CD4 T cell-dependent EAN with a pathology (i) of peripheral nerves, (ii) of muscles innervated by these nerves, and—as an important new finding—(iii) of central nervous system structures functionally linked to the diseased peripheral nerves.

EAN of the peripheral nerve was characterized by a predominant and severe axonopathy with a mild accompanying demyelination. Since the $P0$ -protein expressing Schwann cells are MHC class II negative¹⁸ (and this study), it is likely that the EAN-inducing CD4 T cells, which were activated on $P0_{106-125}$ immunization, homed to the sciatic nerve and activated local resident endoneurial macrophages. In fact, the infiltrates in the diseased sciatic nerve contained CD4 T cells and activated macrophages expressing iNOS and TNF protein. Since both iNOS and TNF are toxic for axons,^{17,19,20} the ax-

Figure 6. Histopathological alterations in the spinal cord in EAN of rat IgG and anti-CD4-treated mice at day 15 p.i. (**A, B, D, and E**) and day 40 p.i. (**C and F**). **A:** In the white matter of the lumbar spinal cord of a rat IgG-treated mouse, Mac3⁺ macrophages (**arrows**) are present. In addition, microglial cells are slightly activated (**arrowheads**). In the anterior horn of the lumbar spinal cord of a rat IgG-treated mouse, GFAP⁺ astrocytes are ubiquitously activated (**inset**). Anti-Mac3 immunostaining, slight counterstaining with hemalum; original magnification, $\times 400$. **Inset:** anti-GFAP immunostaining, slight counterstaining with hemalum; original magnification $\times 400$. **B:** In the anterior horn of the lumbar spinal cord of a rat IgG-treated mouse, many neurons are apoptotic (**arrows**) as evidenced by their condensed, basophilic nucleus, shrinkage, and TUNEL positivity (**inset**). Note the presence of chromatolysis (**inset**). Cresyl violet staining; original magnification $\times 400$. **Inset:** TUNEL staining, slight counterstaining with hemalum; original magnification $\times 1200$. **C:** In the anterior horn of the lumbar spinal cord of a rat IgG-treated mouse there is a patchy loss of neurons at day 40 p.i. (*). However, remaining neurons are viable and contain a nucleus of normal shape and structure and prominently express NeuN (**inset**). Cresyl violet staining; original magnification $\times 400$. **Inset:** anti-NeuN immunostaining, slight counterstaining with hemalum; original magnification $\times 200$. **D:** In the white matter of the lumbar spinal cord of an anti-CD4-treated mouse, Mac3⁺ microglial cells are only slightly activated as evidenced by their short, stunt cellular processes (**arrowheads**), while macrophages are absent. In an anti-CD4-treated mouse, only a few GFAP⁺ astrocytes in the white matter directly adjacent to the anterior horn of the lumbar spinal cord are activated (**inset**) as compared to rat IgG-treated mice (**inset** in **A**). Anti-Mac3 immunostaining, slight counterstaining with hemalum; original magnification $\times 400$. **Inset:** anti-GFAP immunostaining, slight counterstaining with hemalum; original magnification $\times 400$. **E:** As compared with rat IgG-treated mice (**B**), only a few neurons in the anterior horn of the lumbar spinal cord of an anti-CD4-treated mouse are apoptotic (see also Figure 3C). An **arrow** points to a shrunken neuron with a condensed, basophilic nucleus. TUNEL-positivity in neurons is not observed (**inset**). Cresyl violet staining; original magnification $\times 400$. **Inset:** TUNEL staining, slight counterstaining with hemalum; original magnification $\times 1200$. **F:** At day 40 p.i., the anterior horn of the lumbar spinal cord of an anti-CD4-treated mouse harbors numerous viable neurons which strongly express NeuN (**inset**) with only a minimal areas lacking neurons (*). Cresyl violet staining; original magnification $\times 400$. **Inset:** anti-NeuN immunostaining, slight counterstaining with hemalum; original magnification $\times 200$.

Figure 7. Frequencies and kinetics of P0₁₀₆₋₁₂₅-specific IFN- γ -producing cells in PLN and spleen of P0₁₀₆₋₁₂₅-immunized anti-CD4 and/or anti-CD8, rat IgG and non-immunized C57BL/6 mice in PLN and spleen. The frequencies of P0₁₀₆₋₁₂₅-specific T cells were determined by an IFN- γ enzyme-linked immunospot assay at day 8 (A), 15 (B), and 22 p.i. (C). At least four mice per group and time point were analyzed, and the mean $\pm$ SD is shown. * P < 0.05 in PLN and spleen at day 8 p.i. (A), P < 0.05 in spleen at day 15 p.i. (B), P < 0.005 in spleen at day 22 p.i. (C), as compared with rat IgG-treated mice.

onopathy may be caused by these activated macrophages. In fact, nitric oxide can promptly and reversibly block axonal conduction, which is impaired in P0₁₀₆₋₁₂₅-induced EAN as shown previously.¹⁴ Nitric oxide affects several axonal channels, including sodium, potassium, and calcium channels and may block them, thereby impairing conduction.¹⁹ In this regard, murine EAN is similar to multiple sclerosis, in which the extent of axonal destruction is related to the number of activated macrophages, which are present in close apposition with injured axons.^{19,21} The rather mild demyelination of the

Figure 8. Frequencies and kinetics of P0₁₀₆₋₁₂₅-specific IL-17-producing cells in PLN and spleen of P0₁₀₆₋₁₂₅-immunized anti-CD4 and/or anti-CD8, rat IgG and non-immunized C57BL/6 mice in PLN and spleen. The frequencies of P0₁₀₆₋₁₂₅-specific T cells were determined by an IL-17 enzyme-linked immunospot assay at day 8 (A), 15 (B), and 22 p.i. (C). At least four mice per group and time point were analyzed, and the mean $\pm$ SD is shown. * P < 0.05 in PLN and spleen at day 8 p.i. (A), at day 15 p.i. (B), and at day 22 p.i. (C) as compared with rat IgG-treated mice.

sciatic nerve in murine P0₁₀₆₋₁₂₅-induced EAN may occur secondarily to axonal damage or, mutually not exclusive, may also be caused by iNOS- and TNF-producing macrophages, and may render axons more sensitive to nitric oxide toxicity, since demyelinated axons are particularly vulnerable to the axonal conduction blocking effects of nitric oxide.²² This scenario is supported by our finding that a depletion of CD4 T cells resulted in a clinically highly attenuated EAN with significantly reduced numbers of iNOS⁺ and TNF⁺ macrophages in the sciatic nerve.

The severity of sciatic nerve damage $PO_{106-125}$ -induced EAN is further highlighted by the prominent neurogenic muscular atrophy. Importantly, inflammatory infiltrates, in particular CD4 and CD8 T cells, were absent from affected muscles and macrophages were confined to atrophic muscle fibers in the process of myophagia.

Addressing the mechanisms underlying $PO_{106-125}$ -induced EAN, functional T cell depletion experiments unequivocally identified the important role of CD4 T cells for development and severity of disease. $PO_{106-125}$ -specific CD4 T cells were the major cell population responsible for antigen-specific IFN- γ production, while CD8 T cells were less important. In addition, CD4 T cells produced IL-17. Since IL-17-producing CD4 T cells play a crucial role in the development of experimental autoimmune encephalomyelitis,²³ this cytokine may also be important in our EAN model. In contrast to IL-17, IFN- γ is dispensable for the induction of experimental autoimmune encephalomyelitis, but may contribute to propagation of the disease.

Interestingly, induction of EAN was not completely abrogated in mice devoid of CD4 T cells. Even mice having been depleted of both CD4 and CD8 T cells developed EAN on immunization with the $PO_{106-125}$ peptide, although with a significantly milder degree. The remaining low disease activity of CD4 T cell-depleted mice is in accordance with studies in $CD4^{-/-}$, $CD8^{-/-}$, and $CD4^{-/-}CD8^{-/-}$ mutant C57BL/6 mice,^{7,24} which argues against an induction of EAN in our T cell-depleted animals by the remaining small number of T cells ($\leq 3\%$). Furthermore, neuropathological analysis of serial immunostained sections confirmed the absence of the respective T cell population in the sciatic nerve and the spinal cord. Collectively, these clinical and neuropathological observations point to the involvement of other cell populations in the pathogenesis of EAN. As CD4 and CD4 plus CD8 T cell-depleted mice still mounted a weak residual IFN- γ response in the spleen and PLN, one may suggest that $\gamma\delta$ CD8⁺ T cells and NK cells, which are a cellular source of IFN- γ ,²⁵ also play a role in $PO_{106-125}$ -induced EAN. In fact, these cell populations are present in the cauda equina in EAN of Lewis rats.²⁶ However, so far, their role in murine EAN has not been investigated in detail, and the relative role of these cell populations as well as the importance of IFN- γ and IL-17 remain to be determined.

The relative impact of CD4 and CD8 T cells on the various manifestations of EAN is still controversially discussed. The significantly reduced clinical disease activity in mice lacking CD4 T cells was attributed to a remarkably less severe axonal neuropathy of the sciatic nerve, whereas the mild demyelination was less affected. The latter may be caused by iNOS- and TNF-producing endoneurial macrophages, which still were present in both CD4 and CD4 & CD8 T cell-depleted mice, albeit in significantly reduced number. These findings point to a particular impact of CD4 T cells on axonal damage and also highlight the importance of axonal damage for clinical disability in EAN. Clinically relevant, patients with the axonal variant of the Guillain-Barré syndrome have an explosive onset and a more severe clinical course as compared to those with the demyelinating variant.²⁷

Moreover, patients with multifocal motor neuropathy present with more pronounced and progressive neurogenic muscular atrophy,²⁸ which is also a prominent feature of $PO_{106-125}$ -induced EAN. In addition, in multiple sclerosis, axonal loss, but not demyelination, is associated with irreversible neurological disability.^{2,19,29}

A comprehensive neuropathological investigation of the sciatic nerve as target organ of the autoimmune response as well as the spinal cord and the gastrocnemius muscle revealed that in the absence of prominent inflammatory infiltrates a significant fraction, ie, up to 44%, of lumbar and thoracic spinal cord motor neurons underwent apoptosis. This remarkable extent of apoptotic neurons may account for the persistence of clinical symptoms up to day 22 p.i. Our observation of a complete remission of neurological symptoms at later time points, ie, 40 days p.i.¹⁴ and this study, points to the existence of functional regenerative processes. In this scenario, classical compensation by collateral re-innervation may be the most important factor. In fact, collateral re-innervation was prominent in the gastrocnemius muscle of rat IgG and anti-CD8-treated mice at day 40 p.i. Remarkably, although CD4- and CD4&CD8-depleted mice exhibited only minor symptoms of EAN, these slight alterations were sufficient to stimulate collateral innervation, albeit to a remarkably lesser extent as compared to rat IgG-treated mice. In contrast, the persistence of a reduced number of viable anterior horn neurons in rat IgG- and anti-CD8-treated mice argues against a compensatory effect by the generation of new functionally active neurons in these animals. In addition, the production of neurotrophic factors, eg, brain-derived neurotrophic factor, which can also be produced by autoimmune T cells,³⁰⁻³² may play a role. However, CD4 & CD8 T cell depleted mice also recovered from their mild EAN indicating that T cells are more important for disease induction than for recovery.

The extensive neuronal damage in the spinal cord of $PO_{106-125}$ -immunized mice is attributed to a severe dying back axonopathy resulting from the severe axonal damage and has, to the best of our knowledge, not yet been reported for EAN, while it is characteristically observed in spinal cord injury, sciatic nerve axotomy, and facial nerve axotomy.³³⁻³⁶ Our histopathological and immunohistochemical observations viable and structurally unimpaired neurons as well as of a normal neuron-axonal system at day 40 p.i., ie, a late time point when clinical symptoms have subsided, lend further support to the concept of a dying back axonopathy underlying the spinal cord damage as opposed to a primary neuronopathy. Interestingly, in multiple sclerosis, neuronal damage has gained increasing attention recently as a key feature of pathology.^{21,29} In principle, axonal and neuronal injury in the spinal cord as well as other regions of the central nervous system may be caused in a both antigen-specific as well as antigen-nonspecific manner, either by CD8 T cells or macrophages, respectively. T cells aligning along axons and soma of neurons induce substantial neuronal loss in a cell contact-dependent mechanism involving Fas (CD95)-FasL (CD95L) interactions,^{37,38} and blocking CD95 activity prevents motor neurons from undergoing apoptosis

in models of facial nerve axotomy and spinal ischemia.³⁸ Since in our model of EAN, T cells were consistently absent from the gray matter of the spinal cord as well as from the (overlying) leptomeninges and Fas was not up-regulated on motor neurons, a T cell mediated neuronal destruction is very unlikely to account for the prominent loss of neurons, neither by CD8 T cells nor by CD4 T cells, which may also damage neurons via expression of TNF-related apoptosis inducing ligand.²⁹ In addition, IFN- γ -activated macrophages/microglial cells may damage neurons by inducing oxidative stress via their production of TNF and iNOS/ nitric oxide.^{17,39} However, in murine P0_{106–125}-induced EAN, there was no evidence for a direct macrophage-induced damage of spinal cord neurons, since microglial activation was only moderate and iNOS-expressing macrophages/microglial cells were not associated with neurons in the anterior horn. Only rarely, activated microglial cells/macrophages were observed in the vicinity of neurons, which unequivocally showed evidence for apoptosis, and this colocalization was most likely part of the process of neuronophagia. Collectively, these observations indicate that neuronal loss occurs secondarily to inflammation-induced axonal damage of the sciatic nerve. In fact, leukocytic infiltrates were largely confined to the sciatic nerve, the target organ of the autoimmune response. A similar mechanism underlies retinal ganglion cell degeneration in optic neuritis.⁴⁰

Thus, this study identifies experimental murine P0_{106–125}-induced EAN as a disease involving both the peripheral nervous system and central nervous system. These observations have implications for the pathogenesis of autoimmune T cell-mediated diseases of peripheral nerves, and clinically relevance for the development of specific therapeutic strategies aiming to inhibit the autoimmune attack against peripheral nerves and to prevent a dying back axonopathy, in particular, by conferring axonal protection, which is of utmost importance for the final clinical outcome.

Acknowledgments

We thank Anja Tümmersmann for expert technical assistance. We further thank Prof. Hans Lassmann for helpful discussion and suggestions.

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