

# The Role of Macrophages in Demyelinating Peripheral Nervous System of Mice Heterozygously Deficient in P0

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**Abstract.** Mice heterozygously deficient in the p0 gene ( $P0^{+/-}$ ) are animal models for some forms of inherited neuropathies. They display a progressive demyelinating phenotype in motor nerves, accompanied by mild infiltration of lymphocytes and increase in macrophages. We have shown previously that the T lymphocytes are instrumental in the demyelination process. This study addresses the functional role of the macrophage in this monogenic myelin disorder.

In motor nerves of  $P0^{+/-}$  mice, the number of macrophages in demyelinated peripheral nerves was increased by a factor of five when compared with motor nerves of wild-type mice. Immunoelectron microscopy, using a specific marker for mouse macrophages, displayed macrophages not only in the endoneurium of the myelin mutants, but also within endoneurial tubes,

suggesting an active role in demyelination. To elucidate the roles of the macrophages, we crossbred the myelin mutants with a spontaneous mouse mutant deficient in macrophage colony-stimulating factor (M-CSF), hence displaying impaired macrophage activation. In the  $P0$ -deficient double mutants also deficient in M-CSF, the numbers of macrophages were not elevated in the demyelinating motor nerves and demyelination was less severe. These findings demonstrate an active role of macrophages during pathogenesis of inherited demyelination with putative impact on future treatment strategies.

**Key words:** macrophage • macrophage colony-stimulating factor • myelin degeneration • Schwann cell • inherited neuropathies

## Introduction

Inherited neuropathies are chronic disorders of the peripheral nervous system with a prevalence of 1:2,500 (Skre, 1974) including Charcot-Marie-Tooth disease (CMT),<sup>1</sup> hereditary neuropathy with liability to pressure palsies, Dejerine-Sottas syndrome, and congenital hypomyelination. The majority of CMT patients suffer from a demyelinating neuropathy with juvenile or adult onset. There are now seven identified genes with various mutations associated with the family of CMT-like disorders. Historically, the peripheral myelin protein 22 gene was the first identified as the culprit gene for CMT1A (Suter and Snipes, 1995); and myotubularin-related protein-2, N-myc downstream-

regulated gene 1, and neurofilament-light are the most recently discovered genes related to CMT4C, hereditary motor and sensory neuropathy-Lom, and to a distinct variant of CMT2, respectively (Bolino et al., 2000; Kalaydjieva et al., 2000; Mersiyanova et al., 2000). The remaining genes related to CMT-like disorders encode the gap-junction protein connexin 32, the transcription factor designated early growth response 2 gene, and the cell adhesion molecule P0 (for reviews see Nelis et al., 1999; Martini, 2000). A good model for some CMT-like disorders are mice heterozygously deficient in the p0 gene. We have shown recently that in this model immune cells are involved in demyelination that is primarily caused by an abnormal dosage of P0. The experimental proof that lymphocytes are not only innocent bystanders, but functionally involved in demyelination was provided by crossbreeding P0 mutants ( $P0^{+/-}$ ) with mice deficient in recombination activating gene-1 that lack mature T and B lymphocytes. These double mutants showed a clear amelioration of the neuropathological changes in peripheral nerves and of nerve conduction properties compared with P0 mutants having wild-type T lymphocytes (Schmid et al., 2000). The same effect was observed

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<sup>1</sup>Abbreviations used in this paper: CMT, Charcot-Marie-Tooth disease; MHC, major histocompatibility complex; M-CSF, macrophage colony-stimulating factor; *op/op*, osteopetrotic;  $P0^{+/-}$ , P0 mutant.

in mice deficient in the  $\alpha$ -subunit of the T cell receptor, reflecting a selective involvement of T cells in the pathogenesis of this inherited demyelinating disease (Schmid et al., 2000). Based on these observations, we proposed that a secondary immunopathological mechanism may contribute to nerve pathology (Schmid et al., 2000).

In the present study, we focussed on the potential pathogenic impact of macrophages in inherited neuropathies. We demonstrate that in the animal models macrophages are present in a pattern reminiscent of chronic human and experimental inflammatory neuropathies (Schmidt et al., 1996; Ho et al., 1998; Gold et al., 1999). Moreover, myelin mutants with an additional defect in microglia/macrophage activation show less nerve pathology than  $P0^{+/-}$  mice with an intact macrophage system. These findings are potentially important in the context of the understanding of pathomechanisms in hereditary neuropathies and designing disease modifying treatments.

## Materials and Methods

### Animals and Determination of Genotypes

$P0^{+/-}$  mice backcrossed to the C57/Bl6 background have been taken from our own breeding colony (Schmid et al., 2000). *Osteopetrosis* is a spontaneous autosomal recessive mutation with the homozygous mice (*op/op*) lacking biologically active M-CSF due to a single base insertion (T), creating a premature stop codon (Yoshida et al., 1990). Lack of incisor tooth eruption is the most definitive phenotypic marker of the *op/op* genotype. The heterozygous *op/wt* mice are phenotypically normal and these mice were maintained as a breeding colony on a mixed C57/Bl6/C3 background. Double mutants were obtained by crossing *op/wt* with  $P0^{+/-}$  mice, leading to ~25% of the F1 progeny with the  $P0^{+/-}$  *op/wt* genotype. Phenotypically normal *op/wt*, *wt/wt*, and osteopetrotic (*op/op*) animals with  $P0^{+/-}$  genotype ( $P0^{+/-}$  *op/op*) and normal  $P0^{+/-}$  were the F2 product of the double heterozygous mice and were investigated at the age of 6 mo. Later stages have not been investigated, since *op/op* mice often die at a premature age. To avoid effects of the different genetic backgrounds, only littermates were analyzed.

Genotypes of  $P0$  mutants were determined by conventional PCR as described (Schmid et al., 2000). Mice of the *op/op* genotype were determined by their typical absence of incisors (Yoshida et al., 1990) and by a PCR protocol generously provided by Dr. S. Oehen (University of Zürich, Zürich, Switzerland). Since *op/wt* mice display a normal phenotype and behavior, their genotype had to be determined. The presence of wild-type and mutant allele was detected by using oligonucleotides 5'-TGTGTC-CCTTCCTCAGATTACA-3' and 5'-GGTCTCATCTATTATGTCTTG-TACCAGCCAAAA-3', which introduce an additional BglII restriction site into the mutant allele. The PCR was performed in a final volume of 20  $\mu$ l; 300 ng genomic DNA was used as template. The reaction product was denatured at 95°C for 3 min, followed by 5 cycles (94°C, 1 min; 56°C, 45 s; 72°C, 45 s), 35 cycles (94°C, 30 s; 52°C, 30 s; 72°C, 30 s), and a final extension at 72°C for 10 min with a PE Biosystems thermal cycler. The PCR product was digested with BglII (20 IU; Boehringer) for 1 h at 37°C. After digestion, the characteristic fragments indicating the genotypes could be detected on a polyacrylamide gel by autoradiography. The wild-type was represented by a 99- and 96-bp fragment, *op/wt* mice by a 99-, 96-, 70-, and 30-bp fragment, and *op/op*-mice by a 96-, 70-, and 30-bp fragment.

### Immunohistochemistry and Immunoelectron Microscopy

For histological analyses, we have chosen the two major branches of the femoral nerve, the quadriceps muscle nerve, and the cutaneous saphenous nerve of the mouse. In some experiments, sciatic nerves have been investigated at the level of the sciatic notch. In addition, we investigated the spinal roots of the third and fourth lumbar segment.

For detection and quantification of T lymphocytes, antibodies to CD8 (a gift from R. Zinkernagel, University of Zürich, Zürich, Switzerland) have been used on serial nerve cryosections as described previously (Schmid et al., 2000).

For detection of macrophages, antibodies to mouse F4/80 (1:300; Serotec) were applied overnight on 14- $\mu$ m-thick serial sections from fresh frozen femoral nerves and spinal roots. To visualize primary antibodies, a biotinylated secondary antibody to rat IgG was applied for 1 h, followed by avidin/biotin reagent (Dako) and staining with diaminobenzidine-HCl and  $H_2O_2$ . For negative control, the primary antibody was omitted. In femoral nerves, immunoreactive profiles were counted on serial cryosections as described (Schmid et al., 2000). In addition, immunolabeling of single fiber preparations of spinal roots from transcardially perfused mice was performed on free-floating tissue as described (Guénard et al., 1996), with the only modification that primary antibodies were detected by biotinylated secondary antibodies, followed by avidin/biotin reagent as indicated above. In some experiments, immunolabeled fibers were embedded on slides and investigated under the light microscope. In other experiments, the immunolabeled fibers were processed for electron microscopy as described (Guénard et al., 1996).

For double immunofluorescence with the monoclonal rat antibodies to mouse F4/80 and mouse major histocompatibility complex (MHC) class II, fresh frozen sections of femoral nerves and spinal roots were first incubated with the MHC class II antibodies (rat anti-mouse I-Ab, 1:400, 1 h; BD PharMingen), followed by incubation with anti-rat Ig antibodies coupled to FITC (1:200, 30 min; Leinco Technologies). Then, incubation with a blocking reagent followed to avoid nonspecific binding of biotin/avidin system reagents (Vector-Sp-2001, 30 min; Vector Laboratories). As a next step, sections were incubated with a biotinylated F4/80 antibody (1:300, 1 h; Serotec), which was visualized with streptavidin coupled to the red fluorescent dye Cy3 (30 min; Dianova). Control experiments were performed omitting primary antibodies, resulting in complete absence of immunoreactivity.

Double immunofluorescence with polyclonal antibodies to neural cell adhesion molecule from rabbit (1:500; Carenini et al., 1999) and with monoclonal antibodies to mouse MHC class I (ER-HR 52, 1:100; Dianova) from rat was performed by simultaneous incubation of primary antibodies for 1.5 h, followed by simultaneous incubation of secondary antibodies (goat anti-rabbit Cy3, 1:500; Dianova; goat anti-rat FITC, 1:500; Leinco Technologies) for 1.5 h. Control experiments were performed omitting primary antibodies resulting in complete absence of immunoreactivity.

For localizing M-CSF receptor expression, double immunofluorescence using rabbit anti-mouse M-CSF receptor antibody (06-175, 1:5,000; UBI) and the macrophage- and microglia-specific rat antibody to mouse  $\alpha$ M $\beta$ 2 integrin (5C6, 1:5,000; Serotec) has been performed as described (Raivich et al., 1998), with the only exception that teased ventral root fibers instead of free-floating brain sections have been used. Digital micrographs of the FITC and Cy3 fluorescence were taken using a Leica TCS 4D confocal laser microscope with a 63 $\times$  objective in an 8-bit greyscale (b/w), 1,024  $\times$  1,024 pixel format as described in previous studies (Raivich et al., 1998; Kloss et al., 1999). 10 consecutive, equidistant levels spacing 12  $\mu$ m were recorded and condensed to a single bitmap using the MaxIntens algorithm.

### Tissue Preservation for Light and Conventional Electron Microscopy and Morphometry

Femoral nerves and spinal roots of mice were processed for light and electron microscopy as described previously (Schmid et al., 2000). Analysis of the g-ratio (diameter of axons/diameter of fiber) of randomly selected nerve fibers was performed on electron micrographs of the quadriceps nerve and of the ventral roots at a final magnification of 1,600 $\times$  using Scion Image Software (Scion Corp.).

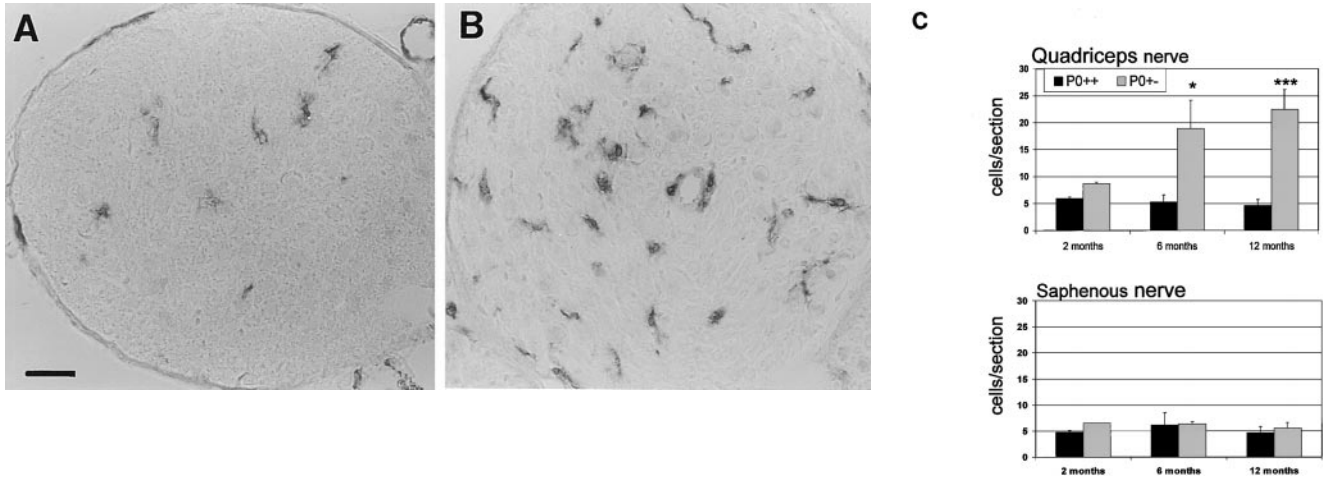
### Statistics

Statistical analysis was performed by using Student's *t* test or Mann-Whitney U test, when appropriate. *P* < 0.05 was considered statistically significant.

## Results

### Immunohistochemistry and Quantification of F4/80- and $\alpha$ M $\beta$ 2-positive Macrophages

In nerve cross sections of normal mice, the F4/80 immunoperoxidase-positive macrophages were usually ramified or showed a few slender processes (Fig. 1, A and B). Most, if not all, F4/80-immunoreactive macrophages in the quad-

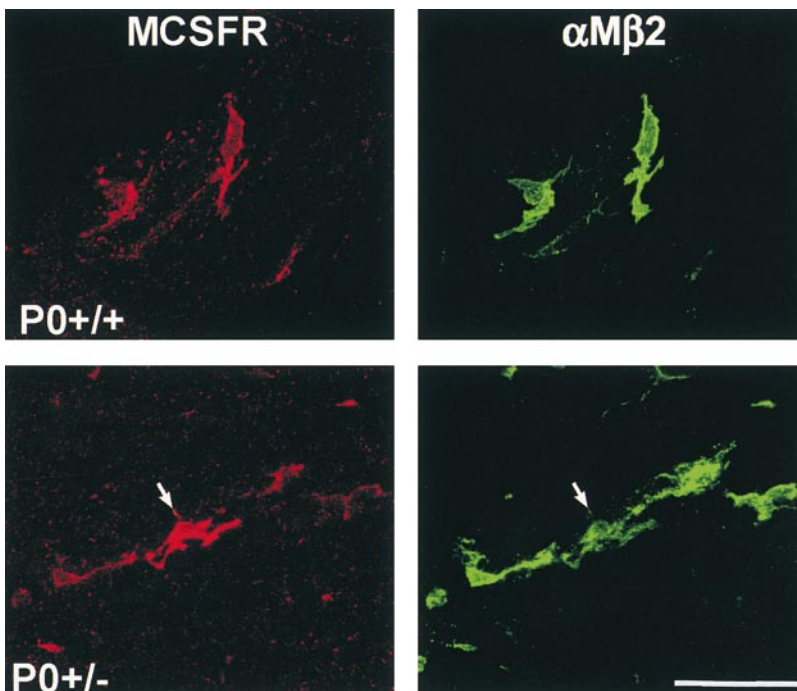


**Figure 1.** (A and B) Immunohistological localization of macrophages in quadriceps nerves of P0<sup>+/+</sup> (A) and P0<sup>+/-</sup> mice (B) at the age of 6 mo using antibodies to F4/80. (A) In quadriceps nerves of P0<sup>+/+</sup> mice some resident macrophages are detectable in the endoneurium. (B) In quadriceps nerves of P0<sup>+/-</sup> mice the number of macrophages is clearly elevated when compared with P0<sup>+/+</sup> mice. Note the larger size of the cells and the close vicinity of two cells to an endoneurial blood vessel. (C) Quantification of F4/80-positive macrophages in quadriceps and saphenous nerves of P0<sup>+/+</sup> (black bars) and P0<sup>+/-</sup> mice (gray bars) at the age of 2 ( $n = 2$  for P0<sup>+/+</sup> and P0<sup>+/-</sup>), 6 ( $n = 3$  for P0<sup>+/+</sup>;  $n = 4$  for P0<sup>+/-</sup>), and 12 mo ( $n = 4$  for P0<sup>+/+</sup> and P0<sup>+/-</sup>). Note elevated number of macrophages in quadriceps nerves of 6- and 12-mo-old P0<sup>+/-</sup> mice. A slight elevation of macrophage numbers is already seen in quadriceps nerves of 2-mo-old P0<sup>+/-</sup> mice. In non-demyelinating saphenous nerves, the number of macrophages is not increased at any age investigated. Bars represent mean values  $\pm$  SD. \* $P < 0.05$ ; \*\*\* $P < 0.001$ . Bar, 50  $\mu$ m.

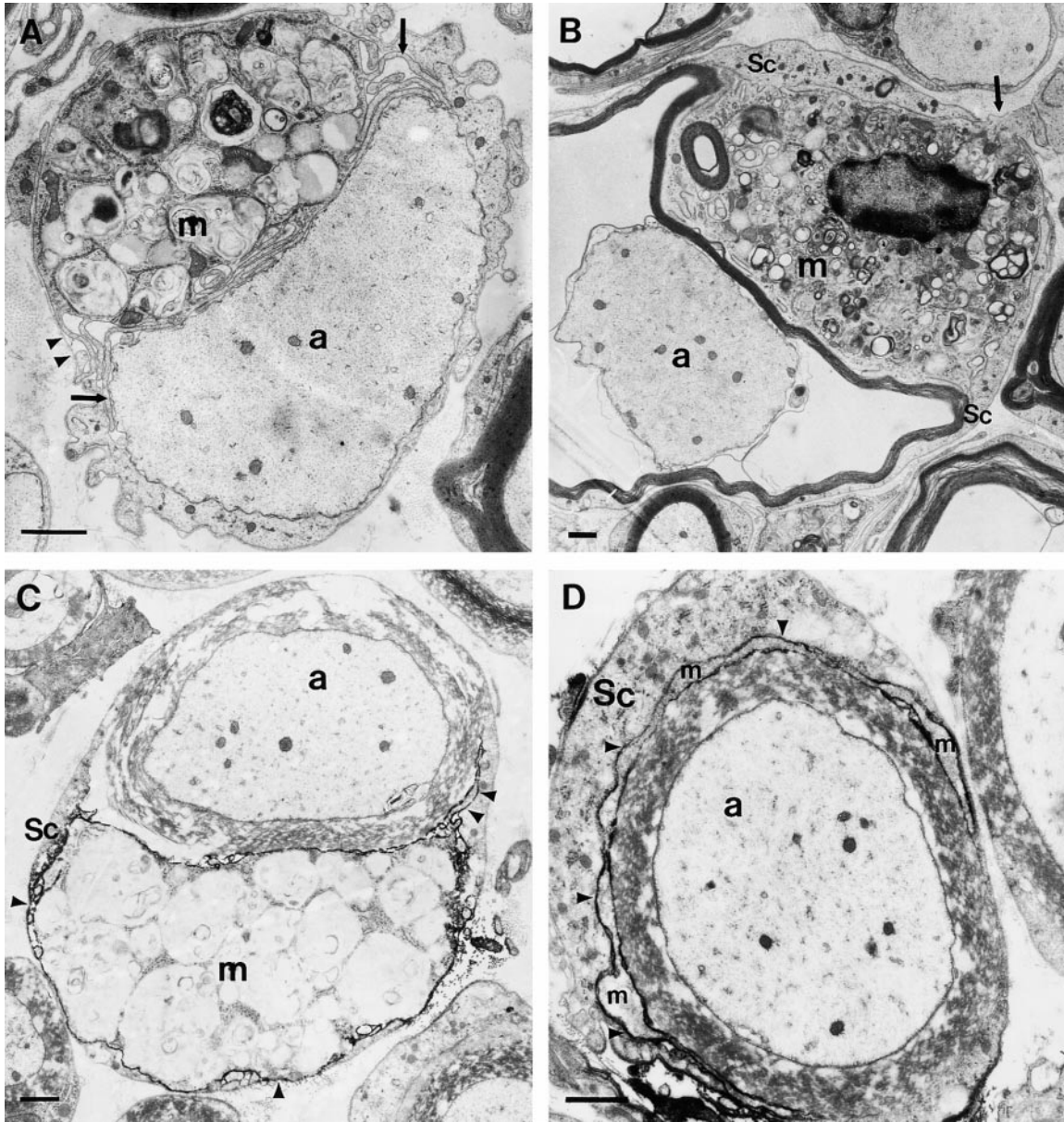
riceps nerve and ventral root were MHC class II–positive, as revealed by double immunofluorescence (not shown). By contrast, MHC class I immunoreactivity was confined to nonmyelinating Schwann cells, as revealed by double immunofluorescence with antibodies to neural cell adhesion molecule, recognizing nonmyelinating Schwann cells in peripheral nerves (not shown).

Localization of the M-CSF receptor was performed on teased fibers from ventral roots of 6-mo-old P0<sup>+/+</sup> and P0<sup>+/-</sup> mice by double immunofluorescence using an M-CSF receptor–specific antibody and the macrophage-

specific marker  $\alpha$ M $\beta$ 2. Labeling for M-CSF receptor was exclusively associated with macrophages, whereas  $\alpha$ M $\beta$ 2-negative cells, such as Schwann cells and endoneurial fibroblasts, were never labeled with antibodies to M-CSF receptor (Fig. 2). Macrophages of the P0<sup>+/+</sup> mice showed a weak labeling for M-CSF receptor (Fig. 2). Interestingly, P0<sup>+/-</sup> mice showed some very strongly labeled macrophages in addition to weakly labeled macrophages (Fig. 2). These data show that M-CSF receptor expression is confined to macrophages and that expression levels are increased in some macrophages of the P0<sup>+/-</sup> mice.



**Figure 2.** Cellular localization of the M-CSF receptor (MCSFR, red) immunoreactivity in teased fiber preparations from ventral roots of P0<sup>+/+</sup> and P0<sup>+/-</sup> mice using antibodies to  $\alpha$ M $\beta$ 2 integrin (green) as a marker for peripheral nerve macrophages.  $\alpha$ M $\beta$ 2-negative cells, such as the adjacent Schwann cells, were never labeled. Note the particularly strongly MCSFR-immunoreactive macrophage in the P0<sup>+/-</sup> mutant (arrow). Bar, 50  $\mu$ m.



**Figure 3.** Conventional electron microscopy (A and B) and immunoelectron microscopy using the macrophage-specific antibody F4/80 (C and D) in quadriceps nerves of 6-mo-old  $P0^{+/-}$  mice. (A) A putative macrophage (m) that is laden with myelin debris is closely apposed to a demyelinated axon (a). Note the slender processes of the macrophage (arrows) and the position of the cell within the endoneurial tube. The arrowheads demarcate the Schwann cell basal lamina. (B) A putative macrophage (m) is in close contact to a thin myelin sheath that is partially detached from the corresponding axon (a). The arrow demarcates a process of the putative macrophage that penetrates the Schwann cell basal lamina. Sc, Schwann cell. (C) An F4/80-positive macrophage (m) containing myelin debris is in close apposition to a myelin sheath. Arrowheads indicate electron-dense immunoreaction product. a, Axon; Sc, Schwann cell. (D) A slender process of a F4/80-positive macrophage (m) has penetrated in between the pericyon of the Schwann cell (Sc) and its normal appearing myelin sheath. Arrowheads indicate electron-dense immunoreaction product. a, axon. Bars, 1.5  $\mu$ m.

We quantified the macrophages in muscular and cutaneous branches of the femoral nerve and in spinal roots of  $P0^{+/+}$  and  $P0^{+/-}$  mice by immunohistochemistry using the F4/80 antibody. In the quadriceps muscle nerves of the  $P0^{+/+}$  mice, approximately five F4/80-positive macrophages per nerve section were found at 2, 6, and 12 mo (Fig. 1 C). In contrast to the normal wild-type mice, the number of macrophages in the quadriceps nerve of heterozygous  $P0^{+/-}$  mice was slightly elevated at 2 mo, increased  $\sim 3.5$ -fold at 6 mo, and 5-fold at 12 mo of age ( $P < 0.001$ ; Fig. 1 C).

Macrophage counts in the cutaneous saphenous nerve of  $P0^{+/+}$  mice revealed a similar number as that in the normal, wild-type quadriceps nerves at any age investigated (Fig. 1 C). The number of macrophages in the  $P0^{+/-}$  saphenous nerves was normal and did not show an age-dependent increase, a finding in line with the still unexplained lack of demyelinating neuropathy in the sensory nerves of  $P0^{+/-}$  mice (Martini et al., 1995; Shy et al., 1997) and of some other myelin mutants (Martini, 1997). This divergence between muscular and cutaneous nerves in the

$P0^{+/-}$  animals also extended to spinal roots, with the macrophages consistently more numerous in the ventral roots than in the dorsal, or in any nerves from the normal,  $P0^{+/+}$  mice. In addition to differences in number, the F4/80-immunoreactive cells in the quadriceps nerves and ventral roots of  $P0^{+/-}$  mice also appeared larger compared with saphenous nerves and dorsal roots of the same genotype, or with spinal roots and femoral nerves of  $P0^{+/+}$  mice (Fig. 1 B). Some of these macrophages from the  $P0^{+/-}$  nerves were also laden with lipid vacuoles or with material reminiscent of myelin debris, a finding that we did not observe in the  $P0^{+/+}$  mice or in the  $P0^{+/-}$  sensory nerves.

### Conventional Electron Microscopy and Immunoelectron Microscopic Localization of Macrophages in Peripheral Nerves of $P0^{+/-}$ Mice

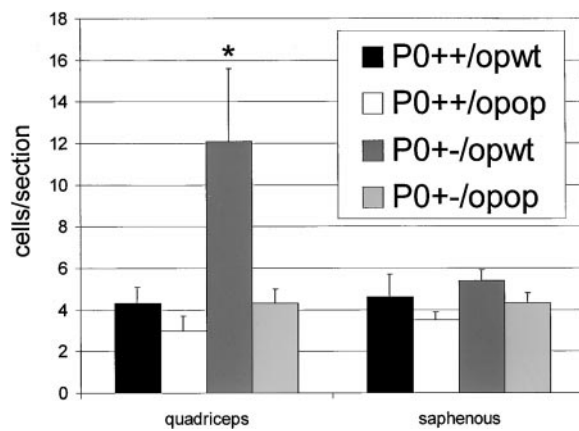
To investigate the spatial relationship of macrophages to demyelinating fibers, we performed conventional electron microscopy and immunoelectron microscopy with F4/80 antibodies in quadriceps nerves and ventral roots of 6-month-old  $P0^{+/-}$  mice.

In ultrathin sections of conventionally prepared quadriceps nerves and ventral roots, endoneurial cells laden with lipid vacuoles or myelin debris were identified as macrophages. They were often found in the endoneurium and were characterized by polymorphic nuclei, abundant heterochromatin, and microvillus-like processes that extended from the surface. Occasionally, such cells were found in the endoneurial tubes, i.e., within the Schwann cell basal laminae (Fig. 3, A and B). In very rare cases, macrophage-like cells were seen just penetrating the basal lamina with a cellular process, reflecting the invasion or leave of the endoneurial tube (Fig. 3 B). However, since Schwann cells can also phagocytose myelin and penetrate basal laminae under pathological conditions, morphological criteria alone appeared insufficient to characterize unequivocally the spatial relationship between macrophages and nerve fibers. Therefore, we performed immunoelectron microscopy using the macrophage-specific antibody F4/80.

The majority of the F4/80-positive macrophages were found in the endoneurial space. Approximately 10–30% of the F4/80-positive cells were found within the endoneurial tubes, i.e., within the inner aspects of the basal laminae (Fig. 3, C and D). Occasionally, the macrophages were in intimate association with myelin sheaths that appeared morphologically normal (Fig. 3, C and D), suggesting an active role of macrophages during demyelination. In other cases, myelin appeared disorganized or was even absent and the demyelinated axons were in direct contact with the macrophages (not shown). No immunoreactivity was observed on axons or on morphologically unequivocally identified Schwann cells.

### *P0/op* Double Mutants: Fewer Macrophages in Femoral Nerves and Less Severe Demyelination

In the current study, we used mice deficient for M-CSF (*op/op*) and heterozygous for *P0* ( $P0^{+/-}$ ) to investigate the role of macrophages in genetically caused demyelination. Myelin mutant littermates with the normal M-CSF phenotype ( $P0^{+/-}$  *op/wt* or  $P0^{+/-}$  *wt/wt*) served as controls.

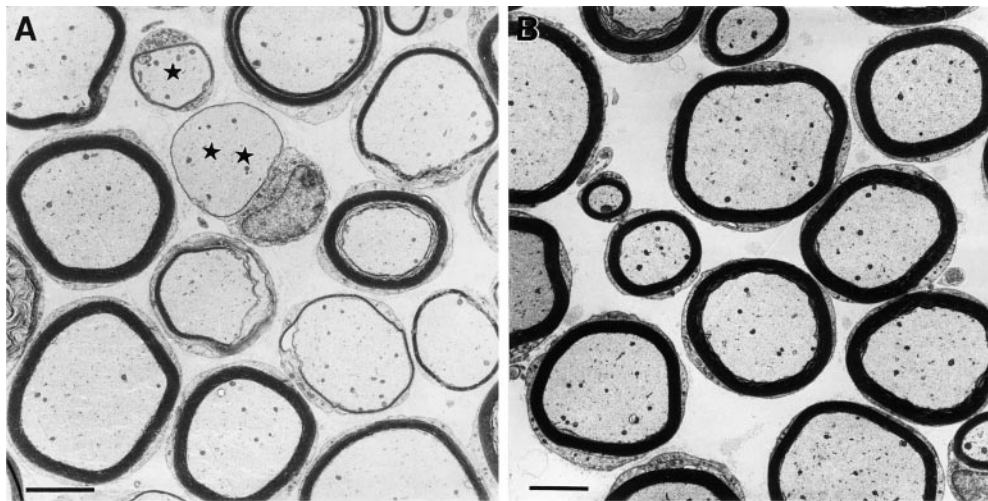


**Figure 4.** Quantification of F4/80-positive macrophages in quadriceps and saphenous nerves of  $P0^{+/+}$  *op/wt* (black bars,  $n = 5$ ),  $P0^{+/+}$  *op/op* (white bars,  $n = 3$ ),  $P0^{+/-}$  *op/wt* (dark gray bars,  $n = 9$ ), and  $P0^{+/-}$  *op/op* mice (bright gray bars,  $n = 3$ ) at the age of 6 mo. The *op* genotype has no influence on the number of resident macrophages in quadriceps nerves of  $P0^{+/+}$  mice. In quadriceps nerves of  $P0^{+/-}$  *op/wt* mice, the number of macrophages is similarly increased, as shown in Fig. 1 C, whereas homozygosity for *op* prevents the increase of macrophage numbers in quadriceps nerves of the myelin mutants ( $P0^{+/-}$  *op/op*). In the nondemyelinating saphenous nerves, the number of macrophages is never increased. Bars indicate mean values  $\pm$  SD. \* $P < 0.05$ .

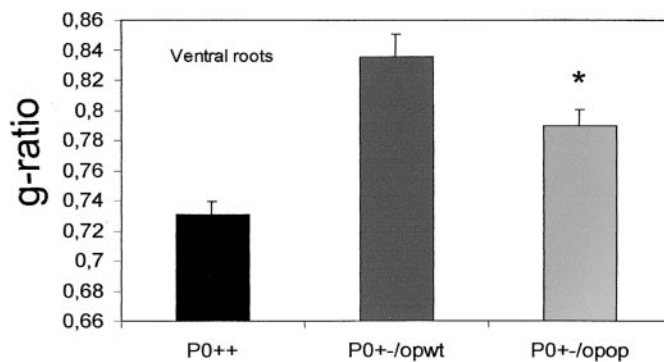
In the quadriceps nerve of M-CSF-deficient, *P0* heterozygous mice ( $P0^{+/-}$  *op/op*), the number of macrophages was not increased compared with mice with normal *P0* dosage ( $P0^{+/+}$  *op/wt* and  $P0^{+/+}$  *op/op*; Fig. 4). In contrast, and similar to the findings obtained from  $P0^{+/-}$  mice (Fig. 1 C), the number of macrophages in the quadriceps nerve was strongly elevated in  $P0^{+/-}$  *op/wt* mice compared with *op* mutants with normal *P0* gene dosage ( $P0^{+/+}$  *op/wt* and  $P0^{+/+}$  *op/op* mice;  $P = 0.01$ ; Fig. 4). Thus, homozygous deficiency in M-CSF leads to a lack of an age-dependent macrophage increase in the motor nerves of  $P0^{+/-}$  mutants.

To investigate whether compromised macrophage increase in  $P0^{+/-}$  *op/op* mice has an influence on the number of T lymphocytes, we also investigated the number of CD8-positive cells in 6-month-old  $P0^{+/+}$  *op/wt*,  $P0^{+/-}$  *op/wt*, and  $P0^{+/-}$  *op/op* mice. Due to the low amount of tissue available from the limited number of mutants, only sciatic nerves could be investigated ( $n = 3$  for each genotype). In the sciatic nerves of  $P0^{+/+}$  *op/wt* mice,  $1.7 \pm 0.2$  T lymphocytes/mm<sup>2</sup> could be detected. There was a clear trend to higher values in  $P0^{+/-}$  *op/wt* mice ( $2.6 \pm 0.4$  cells/mm<sup>2</sup>), but not in the  $P0^{+/-}$  *op/op* mice ( $1.9 \pm 0.3$  cells/mm<sup>2</sup>). The differences between  $P0^{+/-}$  *op/wt* and  $P0^{+/-}$  *op/op* mice failed to reach statistical significance ( $P = 0.06$ ), possibly due to the previously discussed heterogeneous distribution of the lymphocytes (Schmid et al., 2000).

Comparison of semithin sections of quadriceps nerves and ventral roots from 6-month-old  $P0^{+/-}$  *op/wt* mice with those from the  $P0^{+/-}$  *op/op* littermates revealed that all mice with  $P0^{+/-}$  genotype showed pathological alterations indicative of demyelination. However, the severity of the pathological alterations was clearly reduced in mice with the *op/op* genotype. Thus, an investigator who was not



C



**Figure 5.** (A and B) Electron microscopy of ventral roots of 6-mo-old P0<sup>+/-</sup> *op/wt* (A) and P0<sup>+/-</sup> *op/op* mice (B). Myelin thickness in P0<sup>+/-</sup> *op/wt* mice is profoundly reduced compared with P0<sup>+/-</sup> *op/op* littermates. Note that in P0<sup>+/-</sup> *op/wt* mice (A) both small (asterisk) and large caliber fibers (double asterisk) are associated with abnormally thin myelin. The presence of fibers with thicker and thinner myelin in one and the same section in A represents segmental demyelination as has been described previously in longitudinal views of teased fibers (Martini et al., 1995). (C) Quantitative analysis of g-ratios of myelinated fibers in ventral roots of P0<sup>+/-</sup> *op/wt* (dark gray bars, *n* = 3), P0<sup>+/-</sup> *op/op* (bright gray bars, *n* = 3), and P0<sup>+/+</sup> mice (black bars, *n* = 3) at the age of six months. Note the reduction in g-ratios in roots from P0<sup>+/-</sup> *op/op* mice reflecting thicker myelin sheaths, when compared with values from P0<sup>+/-</sup> *op/wt* mice. \**P* < 0.05. P0<sup>+/+</sup> mice display the thickest myelin sheaths. Bars, 5  $\mu$ m.

aware of the genotype (R. Martini) could unequivocally distinguish the nerves from P0<sup>+/-</sup> *op/wt* mice from those of P0<sup>+/-</sup> *op/op* mice.

The thickness of myelin was a particularly striking parameter in the presence or absence of M-CSF. Motor nerves from P0<sup>+/-</sup> *op/op* mice always had thicker myelin sheaths than those from the P0<sup>+/-</sup> *op/wt* littermates. This difference was strongest in the ventral roots (Fig. 5, A and B), but substantial differences in myelin thickness between P0<sup>+/-</sup> *op/wt* and P0<sup>+/-</sup> *op/op* mice were also obtained for the quadriceps nerves (not shown). Semithin sections of quadriceps nerves and ventral roots of P0<sup>+/+</sup> *op/wt* and P0<sup>+/+</sup> *op/op* mice were also investigated at the age of 6 mo. These nerves were of normal phenotype with thick myelin and absence of features indicative of demyelination independent of the *op* genotype (not shown).

To quantify the less reduced myelin thickness in quadriceps nerves and ventral roots of P0<sup>+/-</sup> *op/op* mice versus in P0<sup>+/-</sup> *op/wt* mice, we determined the g-ratio of the myelin sheaths at the electron microscopic level. In agreement with the light microscopic findings, the g-ratios were significantly lower in quadriceps nerves (0.78 in P0<sup>+/-</sup> *op/op* mice versus 0.82 in P0<sup>+/-</sup> *op/wt* mice, *P* < 0.05, *n* = 3). Similar differences in g-ratios were found in ventral roots of P0<sup>+/-</sup> *op/op* mice versus P0<sup>+/-</sup> *op/wt* mice (Fig. 5), thus reflecting thicker myelin in the myelin mutants with compromised macrophage function. Analysis of g-ratios of smaller (<6  $\mu$ m) and larger (>6  $\mu$ m) caliber fibers of ventral roots revealed that in P0<sup>+/-</sup> *op/op* mice, the g-ratios of both fi-

ber groups were decreased, representing rescue of myelin thickness in both smaller and larger caliber fibers (*P* < 0.05 for both size groups; Fig. 5, A and B). Although in P0<sup>+/-</sup> *op/op* mice myelin thickness was clearly increased compared with P0<sup>+/-</sup> *op/wt* mice, normal myelin thickness was not reached as reflected by a lower g-ratio in ventral roots of P0<sup>+/+</sup> mice (Fig. 5 C).

## Discussion

This study focused on the functional roles of macrophages in an established animal model for CMT1B, the P0 heterozygous mice. We found that the number of macrophages was strongly increased and that they showed a frequent association with demyelinating fibers. When the P0<sup>+/-</sup> mice were crossbred with spontaneous mouse mutants deficient in M-CSF, the resulting double mutants lacked the increase in number of macrophages and showed less severe pathological alterations. Our findings show that macrophages are actively involved in demyelination in a model for inherited neuropathies.

### Functional Role of Macrophages in P0<sup>+/-</sup> Mice

Previous studies in M-CSF-deficient (*op/op*) mice revealed an impaired survival, development, and differentiation of the monocyte hematopoietic cell lineage in the absence of M-CSF (Felix et al., 1990; Metcalf, 1991; Witmer-Pack et al., 1993; Stanley et al., 1997; for review see Teitelbaum, 2000). In the central nervous system of the

*op/op* mice, microglial activation is severely impaired under various pathological conditions (Raivich et al., 1994; Berezovskaya et al., 1995). In the peripheral nervous system of the osteopetrotic  $P0^{+/+}$  mice, the number of resident macrophages was normal. However, the number of macrophages did not increase in the myelin-deficient osteopetrotic double mutants, which is reminiscent of findings in the injured central nervous system of osteopetrotic single mutants (Raivich et al., 1994). This lack of macrophage reaction upon the diseased peripheral nervous tissue in *op/op* mice must be considered to be due to a direct and exclusive impairment of the macrophage response, since we show that in  $P0^{+/-}$  mice M-CSF receptor was exclusively found on macrophages, a situation similar to the normal and injured central nervous system (Raivich et al., 1998). Thus, our combined findings not only demonstrate that macrophages are pivotal cellular mediators of myelin damage in this primarily genetically caused demyelinating neuropathy, but also that M-CSF is a critical molecule for the activation of these myelin-phagocytosing cells.

Macrophages play important roles under several pathological conditions, including inflammatory disorders of the nervous system (Hartung et al., 1998; Ho et al., 1998; Kiefer et al., 1998; Gold et al., 1999). In the peripheral nervous system, these functions are particularly well characterized in experimental autoimmune neuritis, the animal model for the Guillain-Barré syndrome. In this disease, macrophages present antigen to autoimmune lymphocytes in the context of MHC class II, which results in activation and proliferation of T lymphocytes (Hartung et al., 1998; Gold et al., 1999). Interestingly, in  $P0^{+/-}$  mice the previously reported increase of the number of T lymphocytes (Schmid et al., 2000) appears to be lacking when macrophage activation is compromised due to the absence of M-CSF. Since T lymphocytes do not carry M-CSF receptors (Raivich et al., 1998), one has to postulate that the reduced number of antigen-presenting and/or cytokine-secreting macrophages might be the reason for the accompanying lack of increase of T lymphocytes.

Macrophages are also involved in the effector phase of the disorders (Craggs et al., 1984; Jung et al., 1993; Hartung et al., 1998; Gold et al., 1999). In electron microscopy studies of Guillain-Barré syndrome and experimental autoimmune neuritis, activated macrophages are seen in direct contact with myelin (Ballin and Thomas, 1969; Lampert, 1969; Ho et al., 1998; Smith, 1999) which is similar to the macrophage-myelin apposition in our animal model for inherited neuropathies. Apart from the direct physical attack, macrophages also synthesize numerous factors, such as arachidonic acid metabolites, oxygen radicals, nitrous oxide, matrix metalloproteinases, and proinflammatory cytokines that can all act to jeopardize myelin stability and the normal function of the myelin-forming Schwann cell (Banati et al., 1993; Ho et al., 1998; Gold et al., 1999).

### **The Role of Macrophages in Human Inherited Neuropathies**

Similar to our animal model, an increased number of endoneurial macrophages has also been reported in nerve biopsies of patients with inherited peripheral neuropathies (Sommer and Schröder, 1995; Schmidt et al., 1996; Stoll et

al., 1998). In many forms of CMT the active phase of demyelination occurs during childhood (Gabreëls-Festen et al., 1995; Garcia et al., 1998; Thomas, 1999), and nerve biopsies were usually not performed. It is conceivable that circulating monocytes enter the peripheral nerve to clear the myelin debris that results from genetically mediated disintegration of myelin. However, macrophages may also actively help to destroy the myelin produced by the mutant Schwann cells. This notion is clearly supported by the data of the current study in  $P0$  mutant mice, as was suggested previously by noting the close association of macrophages with the myelin at the ultrastructural level of inherited human neuropathy (Vital et al., 1992).

What are the possible implications of the current findings for our understanding of human inherited neuropathies? Only few inherited neuropathies are caused by an abnormal gene dosage of  $P0$  (Nelis et al., 1999), but this does not argue against the possibility that similar mechanisms can take place in demyelinating CMT forms that are unrelated to  $P0$  mutations. Indeed, a similar elevation of macrophage numbers as seen in  $P0^{+/-}$  mice has recently been found in *Cx32*-deficient mice (Kobsar, I., M. Mäurer, and R. Martini, unpublished observations), a model for the X-linked form of CMT (Anzini et al., 1997; Scherer et al., 1998). Most interestingly, in some patients with CMT a rather rapid worsening or even overt inflammation (Gregory et al., 1993; Malandrini et al., 1999) with clinical response to corticosteroid treatment (Bird and Slaky, 1991; Crawford and Griffin, 1991; Dyck et al., 1982) or plasma exchange (Malandrini et al., 1999) has been described. It is possible that these clinical cases are at the extreme end of an immunopathologic and chronic disease process that may be unnoticed in the majority of cases. Future studies in the mouse models as well as in human biopsies using appropriate macrophage markers (Kiefer et al., 1998) are needed to substantiate our conclusions with the aim to develop treatment strategies that culminate in the impairment of the detrimental immune cells in peripheral nerves of patients.

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