ANIMAL MODELS

IL-10, IL-4, and STAT6 Promote an M2 Milieu Required for Termination of PO₁₀₆₋₁₂₅-Induced Murine Experimental Autoimmune Neuritis

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The role of the type 2 helper T cell (Th2)—polarizing cytokines IL-4 and IL-10 has not yet been studied in PO₁₀₆₋₁₂₅-induced murine experimental autoimmune neuritis (EAN). We, therefore, addressed the functional relevance of these cytokines and signaling via the IL-4-associated transcription factor STAT6. The clinical course of PO₁₀₆₋₁₂₅-induced EAN in mice deficient for IL-10^{0/0}, IL-4^{0/0}, or STAT6^{0/0} was significantly aggravated compared with that of wild-type control mice. In addition, treatment of PO₁₀₆₋₁₂₅-immunized C57BL/6 mice at the onset of clinical symptoms with a monoclonal IL-10 neutralizing antibody aggravated symptoms and prolonged disease to a similar degree as in IL-10^{0/0} mice. This exacerbated course was attributed to a more prominent Th1 immune response associated with a persistent M1 milieu in the sciatic nerve and in the regional and systemic lymphatic system. These data suggest a Th2-polarized milieu being required to prevent axonal damage of the sciatic nerve and to terminate the PO₁₀₆₋₁₂₅-specific immune response in EAN. Beyond the already known role of macrophages as pathogenic effector cells in EAN, these data suggest that M2-differentiated macrophages do not damage and may even protect neural tissues in EAN. Thus, these data highlight the pathogenetic relevance of the macrophage polarization status in EAN. Therapeutic modulation of immune responses from an M1 toward an M2 milieu may be a promising novel strategy in peripheral nervous system neuritis. (*Am J Pathol* 2014, 184: 2627–2640; <http://dx.doi.org/10.1016/j.ajpath.2014.06.012>)

Guillain-Barré syndrome (GBS) and its animal model experimental autoimmune neuritis (EAN) are autoimmune diseases of the peripheral nervous system. Autoimmune-mediated neuritis may affect the motor, sensory, and autonomous nervous systems. Thus, inadequate ventilation due to respiratory muscle paresis¹ and disturbance of the autonomic nervous system characterized by cardiac arrhythmia, hypertonia/hypotonia, ileus, or urinary retention are severe, potentially life-threatening complications² and may lead to permanent neurologic impairment.

The present model of murine PO₁₀₆₋₁₂₅-induced EAN shares relevant clinical, electrophysiologic, and neuropathologic features with human axonal Guillain-Barré syndrome,^{3,4} including the underlying pathogenesis with CD4⁺ type 1 helper T cells (Th1s) as the decisive cell population

that mediates disease^{4–6} and, finally, induces axonal damage.⁴ Interferon (IFN)- γ is the most important proinflammatory cytokine in PO₁₀₆₋₁₂₅-induced murine EAN.⁴ The proinflammatory cytokines IFN- γ , tumor necrosis factor (TNF), IL-6, and IL-12 contribute to foster Th1-mediated immune reactions in EAN.^{4,7–9} In a model of rat EAN, peak levels of IFN- γ , TNF, IL-6, and IL-12 mRNA coincided with maximal clinical symptoms.^{7,8} Accordingly, in the model of PO₁₈₀₋₁₉₉-induced murine EAN, the frequencies of IFN- γ and TNF-producing cells were reduced in the sciatic nerve of IL-12^{0/0}-deficient mice, whereas the frequency of IL-4-secreting cells was

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Table 1 Primary Antibodies Used for Immunofluorescence Staining and Immunohistochemical Analysis

Antibody	Clone	Company	Species
CD45	M1/9.3.4.HL.2	ATCC	Rat
CD4	H129.19	BD Biosciences	Rat
CD8a	53–6.7	BD Biosciences	Rat
CD19	1D3	BD Biosciences	Rat
F4/80	F4/80	ATCC	Rat
MHC class I	M 1/42.3.9.8	ATCC	Rat
	HLK		
MHC class II; I-Ab,d,q	M5/114.15.2	ATCC	Rat
ICAM-1	3E2	ATCC	Rat
VCAM-1	M/K-2.7	ATCC	Rat
LFA-1	2D7	ATCC	Rat
VLA-4	PS/2	ATCC	Rat
iNOS	Polyclonal	Calbiochem	Rabbit
APP	22C11	Millipore	Mouse
Neurofilament-L	Polyclonal	Millipore	Rabbit
CD40	Polyclonal	Antibodies-online	Rabbit
CD206	MR5D3	Serotec	Rat
SOC51	Polyclonal	Lifespan Biosciences	Rabbit

APP, amyloid precursor protein; ICAM-1, intercellular adhesion molecule-1; LFA-1, leukocyte function–associated antigen-1; MHC, major histocompatibility complex; SOC51, suppressor of cytokine signaling 1; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4.

enhanced,⁹ finally, resulting in reduced axonal damage and demyelination.

In contrast to mechanisms that enhance EAN, immunoregulatory pathways that decrease disease activity are much less characterized. Th1 cells are crucial effector cells in EAN, and Th2 cells negatively regulate the differentiation and expansion of Th1 cells. Therefore, this report aims to elucidate the role of the prototypical Th2 cytokines IL-4 and IL-10 in murine EAN. We also sought to characterize the role of STAT6, the transcriptional activator downstream of IL-4 signaling, in murine EAN. IL-4 signaling activates STAT6, which is required for the transduction of IL-4 signals and the production of IL-4 by antigen-stimulated murine

Table 2 Combination of Antibodies Used for Triple Staining in Flow Cytometry

FITC/Alexa Fluor 488	PE	PerCP
CD19	CD45	CD3
CD8	CD4	CD3
CD19	MHC class II antigen	CD3
F4/80	MHC class II antigen	CD45
CD19	CD40	CD3
IFN-γ	CD4	CD8
IL-10	CD4	CD8
F4/80	IL-10	CD45
CD19	IL-4	CD3

FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex; PE, phycoerythrin; PerCP, peridinin chlorophyll.

splenocytes.¹⁰ In turn, STAT6 itself activates IL-4, thereby fostering an anti-inflammatory milieu by increased production of the Th2-derived cytokine IL-13. Thus, despite our knowledge of IL-10 and IL-4 as important mediators of immune system homeostasis, the finely tuned network of the underlying autoimmune mechanisms and resulting therapeutic strategies have not yet been fully elucidated.

Figure 1 Clinical scores of *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice compared with P0₁₀₆₋₁₂₅-immunized C57BL/6 mice. On day 6 p.i., *IL-10*^{0/0} (A), *IL-4*^{0/0} (B), and *STAT6*^{0/0} (C) mice started to develop neurologic symptoms, thus reaching clinical score 2. On day 7 p.i., clinical disease activity in *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice was significantly higher than that in P0₁₀₆₋₁₂₅-immunized C57BL/6 mice. Recovery was significantly delayed in *IL-4*^{0/0}, *STAT6*^{0/0}, and *IL-10*^{0/0} mice, with persistence of neurologic symptoms up to day 40 p.i., when all P0₁₀₆₋₁₂₅-immunized C57BL/6 mice were already asymptomatic. Data are given as means ± SD. *n* ≥ 4 mice per group and time point. **P* < 0.05, ***P* < 0.005.

Figure 2 Numbers of F4/80⁺ (A), iNOS⁺ (B), CD40⁺ (C), CD206⁺ (D), and SOCS1⁺ (E) macrophages in the sciatic nerve of P0₁₀₆₋₁₂₅-immunized IL-10^{0/0}, IL-4^{0/0}, and STAT6^{0/0} mice compared with P0₁₀₆₋₁₂₅-immunized C57BL/6 mice. **A:** Throughout the study, the number of F4/80⁺ macrophages in the sciatic nerve of P0₁₀₆₋₁₂₅-immunized IL-10^{0/0}, IL-4^{0/0}, and STAT6^{0/0} mice significantly exceeded that of P0₁₀₆₋₁₂₅-immunized C57BL/6 mice. On days 8 and 15 p.i., the number of iNOS⁺ (B) and CD40⁺ (C) macrophages in the sciatic nerve of P0₁₀₆₋₁₂₅-immunized IL-10^{0/0}, IL-4^{0/0}, and STAT6^{0/0} mice already significantly outnumbered that in P0₁₀₆₋₁₂₅-immunized C57BL/6 mice. Beyond day 15 p.i., this difference in numbers of M1 macrophages between P0₁₀₆₋₁₂₅-immunized IL-10^{0/0}, IL-4^{0/0}, and STAT6^{0/0} mice and P0₁₀₆₋₁₂₅-immunized C57BL/6 mice further increased. On days 8 and 15 p.i., the numbers of CD206⁺ (D) and SOCS1⁺ (E) macrophages in the sciatic nerve of all the experimental groups were comparable. Beyond day 15 p.i., the numbers of these M2 macrophage markers of the sciatic nerve of P0₁₀₆₋₁₂₅-immunized IL-10^{0/0}, IL-4^{0/0}, and STAT6^{0/0} mice were significantly below that of P0₁₀₆₋₁₂₅-immunized C57BL/6 mice. Data are given as means $\pm$ SD. $n \geq 20$ HPFs of the sciatic nerve of four mice per group and time point. * $P < 0.05$, ** $P < 0.005$.

Deficiency in IL-10, IL-4, or STAT6 significantly enhanced and prolonged disease activity in EAN. Of note, neutralization of IL-10 in P0₁₀₆₋₁₂₅-immunized mice by a monoclonal antibody at the onset of clinical symptoms

aggravated and prolonged clinical symptoms to the same degree as did genetic deficiency in IL-10. The data reveal the crucial role of IL-10 and IL-4 in controlling type 1 effector cells, including Th2 T cells and M2 macrophages.

Figure 3 F4/80⁺ macrophages (A–D) and their classic activation profile, ie, iNOS⁺ (E–H), CD40⁺ (I–L), and their alternative expression, ie, CD206⁺ (M–P), and SOCS1⁺ (Q–T) in the sciatic nerve of P0₁₀₆₋₁₂₅-immunized IL-10^{0/0} (A, E, I, M, Q), IL-4^{0/0} (B, F, J, N, R), STAT6^{0/0} (C, G, K, O, S), and C57BL/6 (D, H, L, P, T) mice on day 36 p.i. On day 36 p.i., F4/80⁺ macrophages in P0₁₀₆₋₁₂₅-immunized IL-10^{0/0} (A), IL-4^{0/0} (B), and STAT6^{0/0} (C) mice outnumbered F4/80⁺ macrophages in a P0₁₀₆₋₁₂₅-immunized C57BL/6 mouse (D). On day 36 p.i., iNOS⁺ (E–H) and CD40⁺ (I–L) macrophages in P0₁₀₆₋₁₂₅-immunized IL-10^{0/0} (E and I), IL-4^{0/0} (F and J), and STAT6^{0/0} (G and K) mice outnumbered iNOS⁺ and CD40⁺ macrophages in a P0₁₀₆₋₁₂₅-immunized C57BL/6 mouse (H and L). On day 36 p.i., CD206⁺ (M–P) and SOCS1⁺ (Q–T) macrophages in P0₁₀₆₋₁₂₅-immunized IL-10^{0/0} (M and Q), IL-4^{0/0} (N and R), and STAT6^{0/0} (O and S) mice, respectively, are decreased compared with CD206⁺ (P) and SOCS1⁺ (T) macrophages in a P0₁₀₆₋₁₂₅-immunized C57BL/6 mouse. IHC analysis was performed with anti-F4/80 (A–D), anti-iNOS (E–H), anti-CD40 (I–L), anti-CD206 (M–P), and anti-SOCS1 (Q–T); slight counterstaining with hemalum. Original magnification, $\times 400$.

Figure 4 Numbers of CD4⁺ (A–C) and CD8⁺ (D–F) T cells in the sciatic nerve of $IL-10^{0/0}$ (A and D), $IL-4^{0/0}$ (B and E), and $STAT6^{0/0}$ (C and F) mice compared with $P0_{106-125}$ -immunized C57BL/6 mice. Numbers of CD4⁺ T cells in the sciatic nerve of $P0_{106-125}$ -immunized $IL-10^{0/0}$ (A), $IL-4^{0/0}$ (B), and $STAT6^{0/0}$ (C) mice were similar to those of $P0_{106-125}$ -immunized C57BL/6 mice. On days 22, 36, and 43 p.i., endoneurial CD4⁺ T cells in $P0_{106-125}$ -immunized $IL-10^{0/0}$ (A), $IL-4^{0/0}$ (B), and $STAT6^{0/0}$ (C) mice decreased more slowly compared with $P0_{106-125}$ -immunized C57BL/6 mice, in which endoneurial CD4⁺ T cells decreased rapidly, thus differing significantly from $P0_{106-125}$ -immunized $IL-10^{0/0}$ (A), $IL-4^{0/0}$ (B), and $STAT6^{0/0}$ (C) mice. On days 8 and 15 p.i., numbers of CD8⁺ T cells in the sciatic nerve of $P0_{106-125}$ -immunized $IL-10^{0/0}$ mice (D) were similar to those in the sciatic nerve of $P0_{106-125}$ -immunized C57BL/6 mice. However, on days 22, 36, and 43 p.i., endoneurial CD8⁺ T cells in $P0_{106-125}$ -immunized $IL-10^{0/0}$ mice (D) significantly exceeded CD8⁺ T cells in $P0_{106-125}$ -immunized C57BL/6 mice. Differences in the numbers of endoneurial CD8⁺ T cells between $P0_{106-125}$ -immunized $IL-4^{0/0}$ (E), $STAT6^{0/0}$ (F), and C57BL/6 mice were not significant. Note the different scaling of the y axis in A–C and D–F to highlight differences in the respective cell populations between the experimental groups. Data are given as means $\pm$ SD. $n \geq 20$ HPFs of the sciatic nerve of four mice per group and time point. * $P < 0.05$.

The underlying immunoregulatory networks may prove useful for future therapeutic approaches in human autoimmune diseases of the peripheral nervous system, eg, Guillain-Barré syndrome.

Herein, we clarify the role of the Th2-associated anti-inflammatory cytokines IL-10 and IL-4 as well as the IL-4-regulating transcription factor STAT6 in $P0_{106-125}$ -induced murine EAN. The absence of IL-10, IL-4, or

Figure 5 Neuritis (A–D) and axonal damage (E–L) of the sciatic nerve and neurogenic muscular atrophy of the gastrocnemius muscle (M–T) of $P0_{106-125}$ -immunized $IL-10^{0/0}$ (A, E, I, M, Q), $IL-4^{0/0}$ (B, F, J, N, R), $STAT6^{0/0}$ (C, G, K, O, S), and C57BL/6 (D, H, L, P, T) mice on day 36 p.i. On day 36 p.i., numerous CD4⁺ T cells in the sciatic nerve of $P0_{106-125}$ -immunized $IL-10^{0/0}$ (A), $IL-4^{0/0}$ (B), and $STAT6^{0/0}$ (C) mice compared with only single CD4⁺ T cells resting in the sciatic nerve of a $P0_{106-125}$ -immunized C57BL/6 mouse (D; arrows). On day 36 p.i., axonal damage in $P0_{106-125}$ -immunized $IL-10^{0/0}$ (E), $IL-4^{0/0}$ (F), and $STAT6^{0/0}$ (G) mice was more pronounced compared with that in a $P0_{106-125}$ -immunized C57BL/6 mouse (H). On day 36 p.i., pronounced axonal damage in $P0_{106-125}$ -immunized $IL-10^{0/0}$ (I), $IL-4^{0/0}$ (J), and $STAT6^{0/0}$ (K) mice was demonstrated by numerous APP-expressing axons demonstrating disturbed axonal transport compared with some APP-expressing axons in a $P0_{106-125}$ -immunized C57BL/6 mouse (L). On day 36 p.i., severe neuritis resulted in pronounced neurogenic muscular atrophy in $P0_{106-125}$ -immunized $IL-10^{0/0}$ (M and Q), $IL-4^{0/0}$ (N and R), and $STAT6^{0/0}$ (O and S) mice, respectively, compared with less severe neurogenic muscular atrophy in a $P0_{106-125}$ -immunized C57BL/6 mouse (P and T). IHC analysis was performed with anti-CD4 (A–D) and anti-APP (E–H) and slight counterstaining with hemalum; toluidine blue staining (I–L); enzyme histochemical analysis with mATPase (M–P) and NADH (Q–T). Original magnification, $\times 400$.

Figure 6 IFN- γ mRNA levels in PLNs (A) and spleen (B); numbers of P0₁₀₆₋₁₂₅-specific, IFN- γ -producing T cells in PLNs (C) and spleen (D); and IFN- γ serum levels (E) in P0₁₀₆₋₁₂₅-immunized IL-10^{0/0}, IL-4^{0/0}, STAT6^{0/0}, and C57BL/6 mice. Up to day 15 p.i., IFN- γ mRNA levels in PLNs (A) and spleen (B) were similar in all the experimental groups. Beyond day 15 p.i., IFN- γ mRNA levels in PLNs and spleen of P0₁₀₆₋₁₂₅-immunized IL-10^{0/0}, IL-4^{0/0}, and STAT6^{0/0} mice significantly exceeded those of P0₁₀₆₋₁₂₅-immunized C57BL/6 mice. Up to day 15 p.i., numbers of P0₁₀₆₋₁₂₅-specific, IFN- γ -producing T cells in PLNs (C) and spleen (D) of all the experimental groups were similar. Beyond day 15 p.i., numbers of P0₁₀₆₋₁₂₅-specific, IFN- γ -producing T cells in PLNs and spleen of P0₁₀₆₋₁₂₅-immunized IL-10^{0/0}, IL-4^{0/0}, and STAT6^{0/0} mice significantly exceeded those of P0₁₀₆₋₁₂₅-immunized C57BL/6 mice. E: Up to day 15 p.i., IFN- γ serum levels in all the experimental groups were similar. Beyond day 15 p.i., IFN- γ serum levels in P0₁₀₆₋₁₂₅-immunized IL-10^{0/0}, IL-4^{0/0}, and STAT6^{0/0} mice significantly exceed those in P0₁₀₆₋₁₂₅-immunized C57BL/6 mice. Data are given as means $\pm$ SD. $n \geq 4$ mice per group and time point. * $P < 0.05$.

STAT6 as well as the antagonism of IL-10 in established P0₁₀₆₋₁₂₅-induced murine EAN significantly prolonged EAN. The present data highlight the necessity of these cytokines to recover from EAN and may, thereby, provide insight into possible novel therapeutic strategies.

Materials and Methods

Induction of Autoimmune Neuritis

C57BL/6.129P2-Il10^{tm1Cgn/J} (IL-10^{0/0}) mice, C57BL/6.129P2-Il4^{tm1Cgn/J} (IL-4^{0/0}) mice, and C57BL/6.129S2(C)-Stat6^{tm1Gru/J} (STAT6^{0/0}) mice (The Jackson Laboratory, Bar Harbor, ME) were bred in the specific pathogen-free animal facility of the University of Cologne (Cologne, Germany). IL-10^{0/0} mice were backcrossed for 13 generations, IL-4^{0/0} mice for 12 generations, and STAT6^{0/0} mice for 11 generations to ensure that the genetic background was identical to that of control C57BL/6J wild-type mice.

C57BL/6J mice were bred in the specific pathogen-free animal facility of the University of Cologne and were housed under specific pathogen-free conditions throughout the experiments. The experiments were conducted according to local governmental regulations.

Six- to 8-week-old male IL-10^{0/0}, IL-4^{0/0}, STAT6^{0/0}, and C57BL/6J mice were immunized as described previously.⁴ In brief, 200 μ g of P0₁₀₆₋₁₂₅ 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-Aldrich Chemie GmbH, Taufkirchen, Germany) and 0.5 mg of *Mycobacterium*

tuberculosis (strain H 37 RA; Difco, Detroit, MI) emulsified in 25 μ L of phosphate-buffered saline and 25 μ L of Freund incomplete adjuvant (Difco) was applied s.c. to the hindfoot pads on day 0 and s.c. to the back on day 7 postimmunization (p.i.); 500 ng of pertussis toxin per mouse (Sigma-Aldrich Chemie GmbH) was injected i.p. on days 0 and 2 p.i. Control mice were treated with phosphate-buffered saline instead of P0₁₀₆₋₁₂₅ peptide.⁴

Anti-IL-10 Treatment

To neutralize IL-10, C57BL/6J mice were treated with 280 μ g of monoclonal rat anti-mouse IL-10 antibody (clone JES5-2A5; concentration, 140 μ g/100 μ L) i.p. three times per week starting on day 7 p.i. Control mice were treated with 200 μ L of rat IgG i.p. (Sigma-Aldrich Chemie GmbH; concentration, 1.25 mg/mL) in addition to P0₁₀₆₋₁₂₅ peptide.

Assessment of Clinical Score

Animals of all groups were clinically examined on days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 43, and 48 p.i. in a blinded manner (A.B., M.M., M.C.) to determine disease activity according to the following scale: 0 = normal; 1 = ruffled fur; 2 = floppy tail, weak grip, and slightly reduced motility; 3 = mild paraparesis with impaired motility; 4 = severe paraparesis with significantly reduced motility; 5 = tetraparesis with complete immobilization; and 6 = death due to autoimmune neuritis.

Splenic mRNA of M1-associated mediators

Splenic mRNA of M2-associated mediators

Figure 7 Splenic mRNA levels of iNOS (A), CD40 (B), TNF (C), IL-6 (D), IL-12p40 (E), and CCL2 (F), representing an M1 milieu, as well as IL-4 (G), IL-10 (H), IL-13 (I), TGF- β (J), CD206 (K), and SOCS1 (L), representing an M2 milieu. On days 8 and 15 p.i., splenic mRNA levels of the M1-associated cytokines iNOS (A), CD40 (B), TNF (C), IL-6 (D), IL-12p40 (E), and CCL2 (F) were similar in all the experimental groups. Beyond day 15 p.i., the mRNA levels rested at high values in P0106-125-immunized IL-10^{0/0}, IL-4^{0/0}, and STAT6^{0/0} mice, thus exceeding values in P0106-125-immunized C57BL/6 mice significantly. On day 8 and 15 p.i., splenic mRNA levels of the M2-associated cytokines IL-4 (G), IL-10 (H), IL-13 (I), TGF- β (J), CD206 (K), and SOCS1 (L) were similar in all the experimental groups. Beyond day 15 p.i., the mRNA levels rested at low values in P0106-125-immunized IL-10^{0/0}, IL-4^{0/0}, and STAT6^{0/0} mice, whereas the mRNA values in P0106-125-immunized C57BL/6 mice significantly increased. Data are given as means $\pm$ SD. $n \geq 4$ mice per group and time point. * $P < 0.05$, ** $P < 0.005$.

Histopathologic and Immunohistochemical Analyses

For histopathologic and immunohistochemical (IHC) analyses, animals were intracardially perfused with 0.9% NaCl in deep CO₂ anesthesia on days 8, 15, 22, 36, and 43 p.i.

Sciatic nerve and gastrocnemius muscle were investigated by histopathologic and IHC analyses as described elsewhere.⁴ The rat anti-mouse monoclonal antibodies were obtained from supernatant of hybridomas (ATCC, Manassas, VA). Antibodies are listed in Table 1. IHC analysis was performed using 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-Aldrich Chemie GmbH) as chromogene and H₂O₂ as cosubstrate. Axonal damage was evidenced by application of monoclonal mouse amyloid precursor protein (APP; clone 22C11) and detected using the Animal Research Kit (ARK; Dako, Hamburg Germany) according to the manufacturer's instructions.

IFN- γ Enzyme-Linked Immunospot Assay

The frequency of IFN- γ -producing P0106-125-specific lymphocytes in popliteal lymph nodes (PLNs) and spleen was determined by an IFN- γ -specific enzyme-linked

Figure 8 Clinical scores of $PO_{106-125}$ -immunized anti-IL-10-treated mice compared with $PO_{106-125}$ -immunized rat IgG-treated C57BL/6 mice. Up to day 7 p.i., clinical scores of $PO_{106-125}$ -immunized anti-IL-10-treated mice and $PO_{106-125}$ -immunized rat IgG-treated C57BL/6 mice were similar. Up to day 15 p.i., slightly increased disease activity in $PO_{106-125}$ -immunized anti-IL-10-treated mice became obvious. Beyond day 15 p.i., disease activity significantly differed between $PO_{106-125}$ -immunized anti-IL-10-treated mice and rat IgG-treated C57BL/6 mice. Data are given as means $\pm$ SD. $n \geq 4$ mice per group and time point. * $P < 0.05$, ** $P < 0.005$.

immunospot assay on days 8, 15, 22, 36, and 43 p.i. as described elsewhere.⁴

Flow Cytometry

On days 8, 15, 22, 36, and 43 p.i., mice were intracardially perfused with 0.9% NaCl in deep CO_2 anesthesia. PLN and splenic leukocytes were isolated and analyzed after triple immunofluorescence staining (Table 2), followed by flow cytometry as described elsewhere.⁴ Intracellular staining for IFN- γ and IL-10 was performed after fixation and permeabilization of cells using the BD Cytofix/Cytoperm kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Flow cytometry was performed using a BD FACSCalibur platform (BD Biosciences) with CellQuest Pro software version 5.1 (BD Biosciences).

IFN- γ Enzyme-Linked Immunosorbent Assay

To determine serum levels of IFN- γ , on days 8, 15, and 43 p.i., the enzyme-linked immunosorbent assay (ELISA) BD OptEIA sets for IFN- γ (BD Biosciences) were used according to the manufacturer's recommendation. Samples were analyzed in triplicate; four mice per group and time point were studied.

RNA Extraction and Reverse Transcription

RNA was extracted from cryopreserved spleen of two animals per group on days 8, 15, and 43 p.i. using the TRIzol/chloroform method (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. RNA concentrations were measured fluorometrically. First-strand cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Darmstadt, Germany).

Quantitative RT-PCR

Transcription of IFN- γ (Mm01168134_m1), TNF (Mm00443258_m1), IL-12p40 (IL-12b; Mm00434174_m1), IL-6 (Mm00446190_m1), chemokine ligand 2 (CCL2; also known as monocyte chemoattractant protein-1; Mm00441242_m1), inducible nitric oxide synthase (iNOS; Mm00440502_m1), CD40 (Mm00441891_m1), IL-4 (Mm00445259_m1), IL-10 (Mm00439614_m1), transforming growth factor β (TGF- β ; Mm01178820_m1), IL-13 (Mm00434204_m1), CD206 (Mm00485148_m1), and suppressor of cytokine signaling 1 (SOCS1; Mm00782550_s1) mRNA was analyzed by quantitative RT-PCR using Taqman gene expression assays (Applied Biosystems). Water instead of cDNA was used as negative control. PCR reactions were prepared with a cDNA equivalent of 5 ng of RNA in a final volume of 20 μ L. All the samples were studied in triplicate. For normalization, murine *TATA-box binding protein* and *RNA polymerase II* genes were chosen as reference genes.¹¹ Normalized data were calibrated by comparison with expression values of untreated C57BL/6 mice using the $\Delta\Delta C_T$ method.¹²

Statistical Analysis

To test for differences in clinical disease activity between the experimental groups, a two-tailed *U*-test was applied. Numbers of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, F4/80⁺ macrophages, and iNOS⁺, CD40⁺, CD206⁺, and SOCS1⁺ cells in the sciatic nerve were determined by counting immunoreactive cells in 20 high-power fields (HPFs; microscopic magnification, $\times 400$) of sections stained with CD4, CD8, CD19 F4/80, iNOS, CD40, CD206, or SOCS1, respectively, in samples of at least four animals per experimental group on days 8, 15, 22, 36, and 43 p.i.

Data are presented as means $\pm$ SD. Statistical significance of the differences in $\Delta\Delta C_T$ of mRNA transcripts and antigen-specific T cells in PLNs and spleen, respectively, was determined using a two-tailed *U*-test. $P < 0.05$ was considered significant. All the experiments were performed at least in duplicate with four animals per experimental group, ie, i) $PO_{106-125}$ -immunized *IL-4*^{0/0} mice, ii) $PO_{106-125}$ -immunized *STAT6*^{0/0} mice, iii) $PO_{106-125}$ -immunized *IL10*^{0/0} mice, iv) $PO_{106-125}$ -immunized anti-IL-10-treated C57BL/6J mice, v) $PO_{106-125}$ -immunized C57BL/6J mice, and vi) nonimmunized C57BL/6J wild-type mice (control). *P* values refer to differences between $PO_{106-125}$ -immunized mice and genetically modulated and anti-IL-10-treated mice. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA).

Results

Deficiency of *IL-10*, *IL-4*, or *STAT6* Enhances and Prolongs Murine $PO_{106-125}$ -Induced EAN

In *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice, disease started as early as day 6 p.i. with the development of neurologic

symptoms, including weak grip and slightly reduced motility, ie, clinical score 2, and significantly higher disease activity on day 6 p.i. compared with $PO_{106-125}$ -immunized C57BL/6 mice, which reached clinical score 2 not before day 10 p.i. Up to day 15 p.i., clinical symptoms in $IL-10^{0/0}$, $IL-4^{0/0}$, and $STAT6^{0/0}$ mice progressed similar to those in $PO_{106-125}$ -immunized C57BL/6 mice; some $IL-10^{0/0}$ (Figure 1A), $IL-4^{0/0}$ (Figure 1B), and $STAT6^{0/0}$ (Figure 1C) mice even developed tetraparesis. Recovery was significantly delayed in $IL-10^{0/0}$, $IL-4^{0/0}$, and $STAT6^{0/0}$ mice, with persistence of neurologic symptoms corresponding to clinical score 2 up to day 40 p.i., when all $PO_{106-125}$ -immunized C57BL/6 mice were already asymptomatic (Figure 1).

Increased Disease Activity Coincides with More Intense Inflammatory Infiltrates Composed of Proinflammatory M1 Macrophages and T Cells

In parallel to the enhanced clinical onset of EAN, the densities of F4/80⁺ macrophage infiltration were approximately twofold enhanced in the sciatic nerve of $IL-10^{0/0}$, $IL-4^{0/0}$, and $STAT6^{0/0}$ mice during the whole observation period (Figure 2A and Figure 3, A–D). Almost 100% of infiltrating macrophages were of the classically activated M1 proinflammatory subtype as judged by the expression of iNOS and CD40 in $IL-10^{0/0}$, $IL-4^{0/0}$, and $STAT6^{0/0}$ mice from day 15 p.i. up to the end of the observation period. In contrast, only on day 15 p.i., almost all infiltrating macrophages were of the classically activated M1 subtype in control mice; their frequency rapidly dropped to approximately 60% by day 22 p.i. and to <20% by day 43 p.i. (Figure 2, B and C, and Figure 3, E–L). Regarding the frequencies of infiltrating anti-inflammatory M2

macrophages, ie, CD206⁺ and SOCS1⁺ macrophages, in sciatic nerves, in control mice and in all three engineered genotypes, similarly high numbers of M2 macrophages were detectable on day 8 p.i. On day 15 p.i., these numbers rapidly dropped to identically low numbers of <100 M2 macrophages per 20 HPFs in controls and in engineered mice. However, beyond day 22 p.i., this constellation dramatically changed: in $IL-10^{0/0}$, $IL-4^{0/0}$, and $STAT6^{0/0}$ mice, the frequencies of M2 macrophages remained below <100 cells per 20 HPFs, whereas in control mice, the frequencies steadily increased to approximately 200 M2 cells per 20 HPFs on day 43 p.i. (Figure 2, D and E, and Figure 3, M–T).

On day 8 p.i., endoneurial CD4⁺ T cells were almost absent from the sciatic nerve in any of the groups of mice studied herein. On day 15 p.i., CD4⁺ T cells had infiltrated the sciatic nerves to reach similar densities of 100 to 130 cells per 20 HPFs in all groups of mice. However, beyond day 8 p.i., frequencies of infiltrating CD4⁺ T cells rapidly declined in control mice and reached almost background level on day 43 p.i. In contrast, in $IL-10^{0/0}$, $IL-4^{0/0}$, and $STAT6^{0/0}$ mice, densities of CD4⁺ T cells persisted at high levels up to day 22 p.i. Thereafter, the frequencies declined slowly, remaining still 5- to 8-fold higher than in control mice by day 43 p.i. (Figure 4, A–C, and Figure 5, A–D).

The kinetics of sciatic nerve infiltrating CD8⁺ T cells in control mice resembled that of CD4⁺ T cells, albeit at lower absolute levels (Figure 4, D–F). In contrast, in $IL-10^{0/0}$, $IL-4^{0/0}$, and $STAT6^{0/0}$ mice, frequencies of infiltrating CD8⁺ cells tended to remain at elevated levels after day 15 p.i. This effect reached statistical significance in only $IL-10^{0/0}$ mice (Figure 4D).

Figure 9 Numbers of F4/80⁺ (A), iNOS⁺ (D), CD40⁺ (G), CD206⁺ (J), and SOCS1⁺ (M) macrophages and IHC analysis of F4/80⁺ (B and C), iNOS⁺ (E and F), CD40⁺ (H and I), CD206⁺ (K and L), and SOCS1⁺ (N and O) macrophages on day 36 p.i. in the sciatic nerve of $PO_{106-125}$ -immunized anti-IL-10–treated (A, B, D, E, G, H, J, K, M, N) and $PO_{106-125}$ -immunized rat IgG–treated (A, C, D, F, G, I, J, L, M, O) mice. A: On day 8 p.i., the number of F4/80⁺ macrophages in $PO_{106-125}$ -immunized anti-IL-10–treated and rat IgG–treated mice was similar. In $PO_{106-125}$ -immunized anti-IL-10–treated mice, the number of F4/80⁺ macrophages strongly increased thereafter, thus significantly outnumbering F4/80⁺ macrophages in $PO_{106-125}$ -immunized rat IgG–treated mice beyond day 8 p.i. On day 36 p.i., numerous endoneurial F4/80⁺ macrophages in a $PO_{106-125}$ -immunized anti-IL-10–treated mouse (B) outnumbered F4/80⁺ macrophages in a $PO_{106-125}$ -immunized rat IgG–treated mouse (C). On day 8 p.i., numbers of iNOS⁺ macrophages in $PO_{106-125}$ -immunized anti-IL-10–treated and rat IgG–treated mice were similar. In $PO_{106-125}$ -immunized anti-IL-10–treated mice, the number of iNOS⁺ macrophages strongly increased thereafter, thus significantly outnumbering iNOS⁺ macrophages in $PO_{106-125}$ -immunized rat IgG–treated mice beyond day 8 p.i. On day 36 p.i., numerous endoneurial iNOS⁺ macrophages in a $PO_{106-125}$ -immunized anti-IL-10–treated mouse (E) outnumbered iNOS⁺ macrophages in a $PO_{106-125}$ -immunized rat IgG–treated mouse (F). G: On day 8 p.i., the number of CD40⁺ macrophages in $PO_{106-125}$ -immunized anti-IL-10–treated and rat IgG–treated mice was similar. In $PO_{106-125}$ -immunized anti-IL-10–treated mice, the number of CD40⁺ macrophages strongly increased thereafter, thus significantly outnumbering CD40⁺ macrophages in $PO_{106-125}$ -immunized rat IgG–treated mice beyond day 8 p.i. On day 36 p.i., numerous endoneurial CD40⁺ macrophages in a $PO_{106-125}$ -immunized anti-IL-10–treated mouse (H) outnumbered CD40⁺ macrophages in a $PO_{106-125}$ -immunized rat IgG–treated mouse (I). On day 8 p.i., there were comparable numbers of endoneurial CD206⁺ macrophages in $PO_{106-125}$ -immunized anti-IL-10–treated and rat IgG–treated mice, which decreased up to day 15 p.i. on similar levels. Beyond day 15 p.i., the number of endoneurial CD206⁺ macrophages in $PO_{106-125}$ -immunized anti-IL-10–treated mice strongly increased, thus significantly exceeding endoneurial CD206⁺ macrophages in $PO_{106-125}$ -immunized rat IgG–treated mice throughout the rest of the study. On day 36 p.i., numerous endoneurial CD206⁺ macrophages in a $PO_{106-125}$ -immunized rat IgG–treated mouse (K) outnumbered CD206⁺ macrophages in a $PO_{106-125}$ -immunized anti-IL-10–treated mouse (L). On day 8 p.i., there were comparable numbers of endoneurial SOCS1⁺ macrophages in $PO_{106-125}$ -immunized anti-IL-10–treated and rat IgG–treated mice, which decreased up to day 15 p.i. on similar levels. Beyond day 15 p.i., the number of endoneurial SOCS1⁺ macrophages in $PO_{106-125}$ -immunized anti-IL-10–treated mice strongly increased, thus significantly exceeding endoneurial SOCS1⁺ macrophages in $PO_{106-125}$ -immunized rat IgG–treated mice throughout the rest of the study. On day 36 p.i., numerous endoneurial SOCS1⁺ macrophages in a $PO_{106-125}$ -immunized rat IgG–treated mouse (N) outnumbered SOCS1⁺ macrophages in a $PO_{106-125}$ -immunized anti-IL-10–treated mouse (O). IHC analysis was performed with anti-F4/80 (B and C), anti-iNOS (E and F), anti-CD40 (H and I), anti-CD206 (K and L), and anti-SOCS1 (N and O) and slight counterstaining with hemalum. * $P < 0.05$; ** $P < 0.005$. Original magnification, $\times 400$.

Figure 10 Numbers of CD4⁺ (A) and CD8⁺ (B) T cells in the sciatic nerve of P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated and rat IgG-treated mice. **A:** On days 8 and 15 p.i., numbers of CD4⁺ T cells in the sciatic nerve of P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated and rat IgG-treated mice were similar. Beyond day 15 p.i., endoneurial CD4⁺ T cells in P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated mice rested at high levels, thus significantly outnumbering endoneurial CD4⁺ T cells in P0₁₀₆₋₁₂₅-immunized rat IgG-treated mice. **B:** Throughout the study, the number of endoneurial CD8⁺ T cells was similar in both experimental groups. Data are given as means $\pm$ SD. $n \geq 20$ HPFs of the sciatic nerve of four mice per group and time point. Note different scaling of the y axis in A and B to highlight differences in the respective cell populations between both experimental groups. * $P < 0.05$, ** $P < 0.005$.

Axonal Damage and Neurogenic Muscular Atrophy Are Aggravated in *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} Mice

On day 43 p.i., axonal damage of the sciatic nerve of *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice was prominent (Figure 5, E–G). This was evidenced by strong APP expression, indicating disturbed axonal transport (Figure 5, I–K). Axonal damage with disturbed axonal transport was less pronounced in P0₁₀₆₋₁₂₅-immunized C57BL/6 mice at this time point (Figure 5, H and L). Consequently, neurogenic muscular atrophy of the gastrocnemius muscle in *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice was more severe compared with that in P0₁₀₆₋₁₂₅-immunized C57BL/6 mice at this time point (Figure 5, M–T).

IFN- γ Levels in PLNs, Spleen, and Serum Are Significantly Increased in *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} Mice

Beyond day 15 p.i., IFN- γ mRNA levels in PLNs and spleen (Figure 6, A and B); the number of P0₁₀₆₋₁₂₅-specific, IFN- γ -producing T cells in PLNs and spleen (Figure 6, C and D); and IFN- γ serum levels (Figure 6E) in *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice significantly exceeded IFN- γ levels in P0₁₀₆₋₁₂₅-immunized C57BL/6 mice (Figure 6).

mRNA Levels of Mediators Associated with Classically Activated M1 Macrophages Rest High in the Spleen of *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} Mice

At the mRNA level, markers of classically activated M1 proinflammatory macrophages, ie, iNOS, CD40, TNF, IL-6,

IL-12p40, and CCL2, were similarly low on day 8 p.i. and reached maximal levels on day 15 p.i. in spleens of controls and of *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice (Figure 7, A–F). Afterward, expression of these markers declined rapidly in control mice to background levels. In contrast, most markers

Figure 11 Neuritis (A and B) and axonal damage (C–F) of the sciatic nerve and neurogenic muscular atrophy of the gastrocnemius muscle (G–J) of P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated (A, C, E, G, I) and rat IgG-treated (B, D, F, H, J) mice on day 36 p.i. On day 36 p.i., numerous CD4⁺ T cells in the sciatic nerve of a P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated mouse were seen (A) compared with only single CD4⁺ T cells resting in the sciatic nerve of a P0₁₀₆₋₁₂₅-immunized rat IgG-treated mouse (B). On day 36 p.i., axonal damage in a P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated mouse (C) was more pronounced compared with a P0₁₀₆₋₁₂₅-immunized rat IgG-treated mouse (D). On day 36 p.i., pronounced axonal damage in a P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated mouse (E) was illustrated by numerous APP-expressing axons demonstrating disturbed axonal transport compared with a few APP-expressing axons in a P0₁₀₆₋₁₂₅-immunized rat IgG-treated mouse (F). **G, H, I, J:** On day 36 p.i., severe neuritis results in pronounced neurogenic muscular atrophy in a P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated mouse (G and I) compared with less severe neurogenic muscular atrophy in a P0₁₀₆₋₁₂₅-immunized rat IgG-treated mouse (H and J). IHC analysis was performed with anti-CD4 (A and B) and anti-APP (E and F) and slight counterstaining with hemalum; toluidine blue staining (C and D); enzyme histochemical analysis with mATPase (G and H) and NADH (I and J). Original magnification, $\times 400$.

Figure 12 IFN- γ mRNA levels in PLNs (A) and spleen (B); number of P0₁₀₆₋₁₂₅-specific, IFN- γ -producing T cells in PLNs (C) and spleen (D); and IFN- γ serum levels (E) in P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated and rat IgG-treated C57BL/6 mice. Up to day 15 p.i., IFN- γ mRNA levels in PLNs (A) and spleen (B) were similar in both experimental groups. Beyond day 15 p.i., IFN- γ mRNA levels in PLNs and spleen of P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated mice significantly exceeded those of P0₁₀₆₋₁₂₅-immunized rat IgG-treated C57BL/6 mice. Up to day 15 p.i., numbers of P0₁₀₆₋₁₂₅-specific, IFN- γ -producing T cells in PLNs (C) and spleen (D) of both experimental groups were similar. Beyond day 15 p.i., numbers of P0₁₀₆₋₁₂₅-specific, IFN- γ -producing T cells in PLNs and spleen of P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated mice significantly exceeded numbers in P0₁₀₆₋₁₂₅-immunized rat IgG-treated C57BL/6 mice. E: Up to day 15 p.i., IFN- γ serum levels in both experimental groups were similar. Beyond day 15 p.i., IFN- γ serum levels in P0₁₀₆₋₁₂₅-immunized anti-IL-10-treated mice significantly exceeded those in P0₁₀₆₋₁₂₅-immunized rat IgG-treated C57BL/6 mice. Data are given as means $\pm$ SD. $n \geq 4$ mice per group and time point. * $P < 0.05$, ** $P < 0.005$.

remained at high levels in *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice up to the end of the experiment (Figure 7, A–F).

At the mRNA level, markers of alternatively activated M2 anti-inflammatory macrophages, ie, IL-4, IL-10, IL-13, TGF- β , CD206, and SOCS1, were low in all groups of mice used except for IL-10 on days 8 and 15 p.i. (Figure 7G, I–L). However, thereafter, all the markers rose steadily in control mice to reach maximal expression at the end of the experiment. In contrast, in *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice, expression of all the markers tested remained at background levels already detected on day 8 p.i. up to the end of the experiment (Figure 7, G–L).

Altogether, the pattern and kinetics of type 1 markers of T cells as well as macrophages in local and systemic lymphatic organs nicely resembled the data observed for inflammatory infiltrates in the sciatic nerves (Figures 2, 3, and 4).

Therapeutic Neutralization of IL-10 Increases Disease Activity and Prolongs EAN

Constitutive IL-10 deficiency had the most pronounced incremental effects on the course of EAN in this study (Figure 1). To assess whether blocking IL-10 after the onset of clinical symptoms is also effective, P0₁₀₆₋₁₂₅-immunized mice were treated with IL-10-neutralizing monoclonal antibody commencing on day 7 p.i., ie, at the end of the induction phase.

As soon as 3 days after the first injection of IL-10-neutralizing monoclonal antibody, mice started to develop increased disease activity (Figure 8). The treatment was terminated after only three consecutive injections of monoclonal antibody by day 13 p.i.; however, the treatment group showed enhanced and prolonged disease up to at least day 48 p.i. Furthermore, neutralizing IL-10 had similar effects as constitutive genetic deficiency in IL-10 on density and differentiation status of macrophages infiltrating the sciatic nerve (Figure 9), infiltration of CD4 and CD8⁺ T cells (Figure 10 and Figure 11, A and B), and, accordingly, axonal damage and neurogenic muscular atrophy (Figure 11, C–J). Along these lines, the kinetics of local and systemic expression of IFN- γ (Figure 12) and the kinetics and expression profiles of M1 and M2 markers in the spleens after late-onset neutralization of IL-10 (Figure 13) resembled the observations in constitutively *IL-10*-deficient mice. Thus, immunotherapeutically IL-10 blocking during ongoing EAN as a paradigmatic inflammatory disease of the peripheral nervous system might exacerbate the clinical course of the disease.

Taken together, P0₁₀₆₋₁₂₅-induced EAN in *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice and in anti-IL-10-treated mice exhibited a significantly prolonged course of disease compared with P0₁₀₆₋₁₂₅-immunized C57BL/6 mice that was paralleled by a delayed shift from an M1 milieu toward an M2 milieu in P0₁₀₆₋₁₂₅-immunized *IL-10*^{0/0}, *IL-4*^{0/0}, and *STAT6*^{0/0} mice and in anti-IL-10-treated mice. Thus, these

Figure 13 Splenic mRNA levels of iNOS, CD40, TNF, IL-6, IL-12p40, and CCL2, representing a splenic M1 milieu (A and B), and IL-4, IL-10, IL-13, TGF-β, CD206, and SOCS1, representing a splenic M2 milieu (C and D), in $PO_{106-125}$ -immunized anti-IL-10-treated mice (A and C) and $PO_{106-125}$ -immunized rat IgG-treated mice (B and D). On days 8 and 15 p.i., splenic mRNA levels of the M1-associated cytokines iNOS, CD40, TNF, IL-6, IL-12p40, and CCL2 were comparable in $PO_{106-125}$ -immunized anti-IL-10-treated (A) and $PO_{106-125}$ -immunized rat IgG-treated (B) mice. Beyond day 15 p.i., the mRNA levels rested on high values in $PO_{106-125}$ -immunized anti-IL-10-treated mice, thus exceeding values in $PO_{106-125}$ -immunized rat IgG-treated mice significantly. On days 8 and 15 p.i., splenic mRNA levels of the M2-associated cytokines IL-4, IL-10, IL-13, TGF-β, CD206, and SOCS1 were similar in $PO_{106-125}$ -immunized anti-IL-10-treated (C) and $PO_{106-125}$ -immunized rat IgG-treated (D) mice. Beyond day 15 p.i., the mRNA levels rested on low values in $PO_{106-125}$ -immunized anti-IL-10-treated mice, whereas the mRNA values in $PO_{106-125}$ -immunized rat IgG-treated mice significantly increased. Data are given as means $\pm$ SD. $n \geq 4$ mice per group and time point. $**P < 0.005$.

experiments demonstrate a protective role of these mediators in murine EAN.

Discussion

We aimed to elucidate the role of the immunoregulatory cytokines IL-10 and IL-4 in $PO_{106-125}$ -induced murine EAN. EAN was significantly prolonged in $PO_{106-125}$ -immunized $IL-10^{0/0}$, $IL-4^{0/0}$, and $STAT6^{0/0}$ mice and in anti-IL-10-treated mice compared with $PO_{106-125}$ -immunized C57BL/6 mice. This more severe course of disease was attributed to endoneurial macrophages significantly outnumbering $PO_{106-125}$ -immunized C57BL/6 mice, whereas endoneurial $CD4^+$ T cells were not altered in numbers.

IL-10 and IL-4, a prerequisite for Th2 cell development, decrease levels of Th1-derived cytokines together with Th2 cells.^{13,14} In addition to Th2 cells, macrophages are a powerful cellular source of IL-10 in a plethora of inflammatory disorders, including murine *Listeria monocytogenes* meningoencephalitis,¹⁵ serum-induced experimental murine arthritis,¹⁶ and experimental murine colitis,¹⁷ as well as in murine and rat EAN.^{4,18} Macrophages, professional antigen-presenting cells that express major histocompatibility complex class II antigen and costimulatory molecules, including CD40,¹⁹ trigger the autoimmune process by activating Th1 cells and promoting induction of autoimmune neuritis. In addition to macrophages, regulatory B cells produce IL-10 in murine EAN.²⁰ Thus, the interaction between these potent proinflammatory and anti-inflammatory mediators determines the final clinical outcome.^{20,21}

Therefore, endoneurial macrophages, which are well-known as an effector cell population in EAN and Guillain-Barré syndrome,^{22,23} prominently expressed iNOS and CD40 in $IL-10^{0/0}$, $IL-4^{0/0}$, and $STAT6^{0/0}$ mice, indicating their M1 phenotype, which reflects classical activation in contrast to alternatively activated M2 macrophages.^{24,25} Correspondingly, the prolonged clinical course was paralleled by a delayed shift of the M1 toward an M2 milieu in $PO_{106-125}$ -immunized $IL-10^{0/0}$, $IL-4^{0/0}$, and $STAT6^{0/0}$ mice and in anti-IL-10-treated mice compared with the natural course of murine EAN (Figure 9). M1 macrophages activated by IFN-γ promote proinflammatory Th1 immune responses, whereas M2 macrophages activated by IL-4 or IL-10 promote Th2 immune responses.^{26,27} Thus, one may suggest that M1 macrophages foster a vicious circle promoting ongoing Th1 immune responses. Enhanced Th1 immune responses allow prolonged homing of $CD4^+$ T cells to the sciatic nerve, where $CD4^+$ T cells may secrete high levels of IFN-γ. High IFN-γ levels, in turn, promote further activation of macrophages, allowing their increased iNOS production. Finally, iNOS is able to promptly and reversibly block axonal conduction, which was previously shown to be impaired in $PO_{106-125}$ -induced EAN,³ by affecting several axonal channels, including sodium, potassium, and calcium channels.²⁸ In addition, iNOS is directly toxic for axons.^{28,29}

These data indicate that macrophages not only function as effector cells in EAN but also contribute decisively to immunoregulatory mechanisms to terminate the autoimmune reaction. These results extend recent observations of increased IL-10 production by macrophages and decreased

levels of IFN- γ and IL-12 in the absence of TNF, resulting in attenuated EAN.³⁰ In the present study, a persistent M1 milieu with low levels of IL-10, IL-4, IL-13, TGF- β , CD206, and SOCS1 potentially prevents recovery from EAN, thus demonstrating the critical relevance of the combined effect of these Th2-derived immunoregulatory mediators in established EAN. In this regard, EAN may be similar to experimental autoimmune encephalomyelitis, in which alternatively activated M2 macrophages contribute to remission.^{31,32} Nevertheless, the dual role of macrophages during initiation and recovery from EAN remains to be defined precisely, especially regarding a disturbed immune homeostasis in autoimmune inflammation and the net effect of various immunoregulatory mediators, which has to be further dissected by studying the individual role of IL-10, IL-4, and STAT6. The absence of a single anti-inflammatory Th2-derived cytokine, ie, IL-10 or IL-4, and even the absence of a single downstream signaling molecule of the IL-4 pathway, namely, STAT6, markedly interferes with recovery from EAN. These data further highlight the sophisticated autoimmune network in which even the absence of a single transcription factor, STAT6, shifts the immune milieu toward M1 at the cost of M2. This is reflected by significantly increased IFN- γ and iNOS mRNA levels in *IL-4*^{0/0} and *STAT6*^{0/0} mice, whereas IL-13 mRNA levels were significantly decreased. This aspect is supported by *in vitro* studies of bone marrow–derived macrophages in which activated macrophages decreased nitric oxide production via IL-13³³; furthermore, i.p. treatment of mice with IL-13 dramatically down-regulated iNOS mRNA transcription in peritoneal macrophages having been stimulated with IFN- γ and lipopolysaccharide. This iNOS suppression was impaired in *STAT6*^{0/0} mice.³⁴

Thus, one may conclude that an M2 milieu is of special importance in established EAN. Even anti-IL-10 treatment beyond day 7 p.i. was sufficient to suppress the shift toward an M2 milieu sustaining Th1 immune responses with perpetuation of disease. These observations render IL-10 an interesting potential target of immunomodulatory therapy. IL-10 disease-attenuating properties shown in this study are in good agreement with studies in P2-induced rat EAN in which application of IL-10 attenuated disease with low expression of IFN- γ and TNF mRNA in lymph nodes.²¹ In murine myelin oligodendrocyte glycoprotein–induced experimental autoimmune encephalomyelitis, fasudil, a selective Rho kinase inhibitor, altered the M1/M2 balance in remitting disease.³⁵ In fully established myelin oligodendrocyte glycoprotein–induced murine experimental autoimmune encephalomyelitis, treatment with fasudil increased the frequency of CD4⁺ IL-10⁺ T cells and IL-10 production by CD4⁺ T cells as well as by macrophages. Consequently, a shift to an M2 milieu characterized by increased CD206 and IL-10 levels was observed. In contrast, expression levels of typical M1 markers, ie, iNOS, IL-12, CD40, MCP-1, and TNF, were reduced.³⁵

Taken together, these data indicate a central role for a Th2-directed milieu necessary to recover from EAN and to protect the sciatic nerve from axonal damage. In murine EAN, the macrophage polarization status is critical. These data support that Th2-triggered modulation of immune responses to induce a shift from an M1 toward an M2 milieu may be a promising novel therapeutic strategy in neuritis of the peripheral nervous system.

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