Leukemia Inhibitory Factor Deficiency Modulates the Immune Response and Limits Autoimmune Demyelination: A New Role for Neurotrophic Cytokines in Neuroinflammation

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Leukemia Inhibitory Factor Deficiency Modulates the Immune Response and Limits Autoimmune Demyelination: A New Role for Neurotrophic Cytokines in Neuroinflammation

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The neurotrophic cytokines ciliary neurotrophic factor and leukemia inhibitory factor (LIF) play a key role in neuronal and oligodendrocyte survival and as protective factors in neuroinflammation. To further elucidate the potential of endogenous LIF in modulating neuroinflammation, we studied myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis in LIF knockout mice (LIF<sup>−/−</sup> mice). In the late phase of active myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis, LIF<sup>−/−</sup> mice exhibited a markedly milder disease course. The inflammatory infiltrate in LIF<sup>−/−</sup> mice was characterized by an increase in neutrophilic granulocytes early and fewer infiltrating macrophages associated with less demyelination later in the disease. In good correlation with an effect of endogenous LIF on the immune response, we found an Ag-specific T cell-priming defect with impaired IFN-γ production in LIF<sup>−/−</sup> mice. On the molecular level, the altered recruitment of inflammatory cells is associated with distinct patterns of chemokine production in LIF<sup>−/−</sup> mice with an increase of CXCL1 early and a decrease of CCL2, CCL3, and CXCL10 later in the disease. These data reveal that endogenous LIF is an immunologically active molecule in neuroinflammation. This establishes a link between LIF and the immune system which was not observed in the ciliary neurotrophic factor knockout mouse. The Journal of Immunology, 2008, 180: 2204–2213.

Neurotrophic cytokines play a key role in neuronal and oligodendrocyte survival, among them leukemia inhibitory factor (LIF)<sup>3</sup> and ciliary neurotrophic factor (CNTF). In vitro and in vivo, these factors support differentiation and survival of oligodendrocyte precursor cells (1, 2) and prevent oligodendrocyte apoptosis in response to serum withdrawal or cytokine challenge (3, 4). LIF and CNTF also support motoneuron survival in vitro and in vivo under different experimental conditions (5). Thus, neurotrophic cytokines may be of great interest as protective factors not only for neurodegenerative diseases, but also in autoimmune demyelination (6). Recently, the role of CNTF and LIF was investigated in experimental autoimmune encephalomyelitis (EAE), a model disease reflecting some of the typical features of the human disease multiple sclerosis (7). EAE in CNTF knockout (CNTF<sup>−/−</sup>) mice takes a more severe course with enhanced oligodendrocyte apoptosis and axonal damage (8). On the other side, treatment with LIF ameliorates EAE by preventing oligodendrocyte cell death (9), whereas administration of CNTF can also interfere with the immune system (10).

Besides its effects on neuronal and glial cells, LIF possesses pleiotropic functions in many cell types and organs (see Ref. 11 for review) including the inhibition of embryonic stem cell differentiation, promotion of survival of hematopoetic precursor cells or support of blastocyst implantation; the latter resulting in infertility in LIF-deficient (LIF<sup>−/−</sup>) mice (12). Furthermore, LIF seems to interact with the immune system. LIF<sup>−/−</sup> mice display decreased numbers of hematopoietic stem cells in spleen and bone marrow and an impaired Con A-mediated thymocyte stimulation (12). Overexpression of LIF in T cells leads to altered immune organ morphology (13). Analyzing the immune response to peripheral nerve injury in LIF<sup>−/−</sup> mice reveals a role for LIF in macrophage recruitment (14, 15). Likewise, LIF deficiency modulates the microglia/macrophage response in a model of spinal cord injury (16). In summary, these data suggest a proinflammatory function of LIF. Yet, other studies focusing on the immune reaction in LIF<sup>−/−</sup> mice after injection of CFA even point at a prominent anti-inflammatory role for this cytokine (17, 18). So far, little is known on the interaction of LIF with the immune system during autoimmune inflammation of the CNS. Previous studies in EAE models mainly focused on the beneficial impact of exogenous or endogenous LIF on glial cells (9, 19). However, the consequences of LIF deficiency on initiation and...
course of neuroinflammatory diseases have not been investigated so far.

In this study, we have induced the model disease EAE with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG 35–55) in LIF−/− mice. We show that LIF deficiency results in attenuation of disease in the late phase of MOG-EAE with an altered composition and altered maintenance of the inflammatory infiltrate. These data suggest that endogenous LIF is an important regulator that orchestrates T cell and macrophage responses in EAE.

Materials and Methods

Mice

LIF−/− mice were backcrossed on a C57BL/6 background for >10 generations and bred at the in-house animal care facilities of the Institute of Clinical Neurobiology (Würzburg, Germany). Because female LIF−/− mice are infertile, they were maintained on a heterozygous background. Previous studies suggest a gene-dose effect in LIF−/− littersmates (12). Therefore, age- and gender-matched control C57BL/6 animals were purchased from Harlan Laboratories (Harlan Winkelmann) for all experiments. Mice were 8–12 wk old and body weight was in a range of 20–25 g. Animals were housed in a room with controlled light cycle and were given commercial food pellets and water ad libitum. All experiments were approved by the Bavarian and Lower Saxony state authorities for animal experimentation.

Induction and clinical evaluation of active MOG-EAE

For active induction of EAE, mice received a s.c. injection at flanks and tail base of 200 μg of MOG 35–55 peptide (Bio-Rad and Charite) in PBS emulsified in an equal volume of CFA containing Mycobacterium tuberculosis H37RA (Difco) at a final concentration of 1 mg/ml. Two injections of pertussis toxin (400 ng/mouse i.p.; Sigma-Aldrich or List Biochemicals) were given 24 and 72 h later. Animals were weighed and scored for clinical signs of disease on a daily basis. Disease severity was assessed using a scale ranging from 0 to 10; scores were as follows: (8): 0, normal; 1, reduced tone of tail; 2, limp tail, impaired righting; 3, absent righting; 4, gait ataxia; 5, mild paraparesis of hind limbs; 6, moderate paraparesis; 7, severe paraparesis or paraplegia; 8, tetraparesis; 9, moribund; 10, death.

Generation of a MOG 35–55-specific T cell line and induction of adoptive transfer MOG-EAE

MOG 35–55-specific T cells were generated as described earlier (20). Briefly, wild-type (WT) C57BL/6 mice were immunized with 200 μg of MOG 35–55 in CFA. Nine to 12 days later, draining lymph nodes and spleen were harvested and single-cell suspensions were prepared. Lymph node cells were then cultured at a density of 3–6 × 10^6 cells/ml in 35-mm plastic dishes (Nunc) in the presence of 20 μg/ml MOG 35–55 in RPMI 1640 supplemented with 100 μM penicillin, 100 μg/ml streptomycin (Biochrom), 1% L-glutamine, 1% sodium pyruvate, and 1% nonessential amino acids (Invitrogen Life Technologies) and 10% FCS (heat-inactivated FCS; PAA Laboratories). Ag-specific T cells were selected by repeated propagation cycles in medium with 6–10% supernatant from Con A-treated Lewis rat spleen cells and 10% FCS followed by Ag-specific restimulation using irradiated (30 Gy) syngeneic spleen cells at a 6:1 ratio after 7–12 days of primary culture. For induction of adoptive transfer-EAE, WT, or LIF−/− recipients received 4–6 × 10^6 freshly activated MOG-specific T cell blasts from a stable T cell line (MOG.I0) i.v. A total of 400 ng of pertussis toxin was administered i.p. immediately after cell transfer and 2 days later. Disease severity was assessed as above.

Proliferation assay

For lymph node and spleen cell proliferation assays, single-cell suspensions of spleen and inguinal lymph nodes from MOG 35–55-immunized LIF−/− and WT mice were prepared 12 days after immunization of mice with 200 μg of MOG 35–55 (8). A total of 2 × 10^5 cells were seeded in 96-well microtiter plates (Nunc) in 100 μl of medium with addition of Ag. In some assays, T cells were isolated by MACS (see below) and cocultured with freshly prepared APC from spleen of different donors. Ag concentrations were 10–20 μg/ml (except for Con A (1.25–2.5 μg/ml)). Triplicate cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂ for 56 h and harvested following a 16-h pulse with 0.2 μCi/well [3H]TdR (tritiated thymidine; Amersham-Buchler). Cells were collected on fiberglass filter paper with a 96-well harvester (Pharmacia), and radioactivity was measured with a 96-well Beta plate liquid scintillation counter (Pharmacia).

For proliferation assay of propagated MOG-specific T cells in culture, 20,000 or 40,000 T cells were cultured with 125,000 or 250,000 spleen cells, respectively; otherwise, the protocol remained unchanged. In some of these experiments, Ly6G-positive cells were isolated from spinal cord and spleen of EAE diseased WT mice at the first maximum of disease and then titrated into the assay. All experiments were at least repeated once.

ELISA

Lymph node cells were prepared and cultured as above with medium alone or in the presence of 10 μg/ml MOG 35–55 peptide. Supernatants were harvested after 3 days of culture. Cytokines or chemokine (IL-2, IFN-γ, IL-6, IL-12 p35/p70, IL-5, and CCL2 (MCP-1)) were determined by sandwich ELISA, as described (21). mAb pairs and recombinant cytokine standards were purchased from R&D Systems for IFN-γ or BD Pharmingen for all other cytokines and CCL2 (MCP-1). All experiments were at least repeated once.

Histology

Different time points were chosen for histologic analysis (day 13 postinfection (p.i.) for the early phase of MOG-EAE, day 27 p.i. for the intermediate phase, and day 60 p.i. for the late phase of MOG-EAE). Animals were deeply anesthetized with pentobarbital or ketamine and transcardially perfused with saline followed by 4% paraformaldehyde. The complete spinal cord—and, in some mice, also spleen, thymus, and lymph nodes—were carefully removed. Thymus, lymph nodes, spleen, and six to eight axial spinal cord cross-sections per animal were further processed for routine paraffin embedding. Histologic evaluation was done from at least two independent experiments per time point. Paraffin sections were subjected to H&E staining to assess the structure of immune organs or parameters of inflammation. Spinal cords were also stained with Luxol Fast Blue for demyelination.

Immunohistochemistry

Immunohistochemistry was performed with 5-μm paraffin sections as described (8). If necessary, Ag unmasking was achieved by heat pre-treatment of sections for 30 min in 10 mM citric acid buffer (Mac-3, CD3, neutrophilic granulocytes) in a microwave oven (850 W). After inhibition of unspecific binding with 10% BSA, sections were incubated overnight at 4°C with the appropriate primary Ab in 1% BSA. Secondary Abs were used as indicated below. After blocking of endogenous peroxidase with H₂O₂, the peroxidase-based ABC detection system (DakoCytomation) was used with diaminobenzidine as the chromogenic substrate. Specificity of staining was confirmed by omitting the primary Ab as a negative control. T cells were labeled by rat anti-CD3 (1:300; Serotec) and macrophages by rat anti-mouse Mac-3 (1:200; BD Pharmingen), each with a rabbit anti-rat secondary Ab (1:100; Vector via Linaris). Staining for neutrophilic granulocytes was done by immunohistochemistry for the 7/4 Ag (1:300; Serotec MCA 771GA (22)) with a rabbit anti-rat secondary Ab or by Naphthol AS-D chloroacetate reaction (kit no. 91C; Sigma-Aldrich).

FACS analysis

Thymus and lymph node single-cell suspensions were stained in PBS/1% BSA/0.05% NaN₃ and analyzed by triple-color flow cytometry on a FACSCalibur (BD Biosciences). Data were analyzed using FACSscan software (BD Biosciences). The following Abs were used for analysis: FITC-labeled anti-CD8a (clone 53-6.7); PE-labeled anti-CD4 (clone GK 1.5), FITC-labeled anti-CD25 (clone 7D4), and FITC-labeled anti-CD69 (clone H1.2F3, all obtained from BD Biosciences).

Preparation of T cells, neutrophilic granulocytes, macrophages, and bone marrow dendritic cells (bmdDC)

T cells were isolated from mouse spleens using a MACS pan-T cell isolation kit by negative selection (Miltenyi Biotec). Neutrophilic granulocytes were isolated from mouse spinal cord and spleen by MACS using Ly6G beads (Miltenyi Biotec). Resident peritoneal macrophages were obtained after 2–3 ml peritoneal lavage. Murine bmdDC were prepared in adaptation of a protocol by Grauer et al. (23). The generation of mature bmdDC was proven by FACS staining for MHC class II, B7-1, B7-2 CD40, and CD11c expression (all Abs via BD Biosciences).
In vitro migration of murine peritoneal macrophages and bmDC

Recombinant murine LIF was a gift from H. Butzkueven (University of Melbourne, Melbourne, Australia). Murine peritoneal macrophages and bmDC were prepared as described above. Murine bmDC were used on days 10–12 after preparation and maturation for 3 days in the presence of 500 U/ml TNF-α. Chemotactic activity was assayed in multiwell microchambers (Costar/Corning via Omnis Life Science) using a modified protocol according to Ref. 24, with a polystyrene-free polycarbonate filter, pore size 5 μm. After 100- to 180-min incubation at 37°C and 5% CO₂ in a humidified atmosphere, cells that had migrated through the filter into the lower chamber were counted by FACS. Measurements were performed in triplicates, outliers exceeding or dropping below 40% of the respective mean values were not considered for further analysis. Data are pooled from two independent experiments and presented as chemotactic index which is the quotient of cells migrating in the presence of LIF and cells migrating in the presence of medium alone (14).

In vivo migration of murine bmDC

In vivo migration of murine bmDC was investigated following a protocol by Del Prete et al. (25). Briefly, WT mature bmDC after 9 days of culture were used. After 100- to 180-min incubation at 37°C and 5% CO₂ in a humidified atmosphere, cells that had migrated through the filter into the lower chamber were counted by FACS. Measurements were performed in triplicates, outliers exceeding or dropping below 40% of the respective mean values were not considered for further analysis. Data are pooled from two independent experiments and presented as chemotactic index which is the quotient of cells migrating in the presence of LIF and cells migrating in the presence of medium alone (14).

RT-PCR

Total RNA from spinal cord, spleen, or freshly prepared T cells, macrophages, and DCs was purified over RNeasy columns (Qiagen). Reverse transcription was performed with 12 μl of purified RNA with 200 U of Superscript II reverse transcriptase. Quantification of β-actin was achieved with primers β-actin S2 (5'-ATTCGGCACAGGATGCAGAA-3'), β-actin AS2 (5'-GCTGACCACATCTGCTGGAA-3'), and β-actin Son2 (5'-FAM-CAGATCATGCTCCCTGAGCA-TAMRA-3') (26). For quantification of murine CCL2 (MCP-1), CXCL1 (KC), CXCL10 (IFN-γ-inducible protein 10 (IP10)), CCL3 (MIP-1α), CCL5 (RANTES), GM-CSF, IFN-γ, and IL-17, we used predescribed assays from Applied Biosystems. Murine (m) LIFRβ mRNA expression was measured with mLIFR S (5'-GGATACCAACTGTGCTCAGCTAAATT-3'), mLIFR AS (5'-TATCGAGTCTGCCGACGTATCTT-3'), and mLIFR Son (5'-FAMAGAAGCTGCTCCCTGAGCA-TAMRA-3') as primers. All PCR were performed on a 7500 Real-Time PCR System (Applied Biosystems) in quadruplicate; relative quantification was performed according to Livak and Schmittgen (27).

Statistical analysis

Quantitative evaluation of histopathological changes was essentially performed as described (28). Coded sections were counted by blinded observers using means of overlaying a stereological grid onto the sections and counting inflammatory infiltrates per mm² white matter (29). The extent of demyelination was assessed according to Storch et al. (7). CD3, Mac-3-positive cells, and neutrophilic granulocytes were quantified on three representative sections, each one from cervical, thoracic, and lumbar spinal cord by counting two defined areas with the most intense pathology under a 400-fold magnification. For statistical evaluation of the clinical course, data were pooled from different experiments. Analysis was performed using the Mann-Whitney U test or for histology and clinical course and test for ELISA, RT-PCR, proliferation, and migration data (SPSS program; SPSS). Data are given as mean values ± SEM or mean values ± SD as indicated. Values of p were considered significant at *, p < 0.05 and highly significant at **, p < 0.01 or ***, p < 0.001.

Table I. Clinical characteristics of MOG 35–55 EAE in LIF−/− mice

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<tr>
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<th>WT</th>
<th>LIF−/−</th>
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<tr>
<td>Body weight before immunization (g)</td>
<td>22.7 ± 3.6</td>
<td>20.5 ± 3.6***</td>
</tr>
<tr>
<td>Incidence of MOG 35–55 EAE (%)</td>
<td>100%</td>
<td>96.9%</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>7.1%</td>
<td>0%</td>
</tr>
<tr>
<td>Onset of disease (days p.i.)</td>
<td>14.0 ± 3.9</td>
<td>12.8 ± 1.7</td>
</tr>
<tr>
<td>Disease severity day 60 p.i.</td>
<td>4.4 ± 0.9</td>
<td>1.8 ± 0.6*</td>
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</table>

Note: *LIF−/− mice display reduced body weights and a reduced disease severity on day 60 p.i. of MOG-EAE. There was no difference in incidence, mortality, or onset of disease. Data are given as mean ± SD. *, p < 0.05; ***, p < 0.001.

FIGURE 1. Clinical course of active and passive MOG-EAE in LIF−/− mice. A, Clinical course of active MOG-EAE in LIF−/− (n = 32, gray line) vs WT control mice (n = 28, black line). Data are summarized from a total of six experiments. Ten LIF−/− and 9 WT mice from a total of three experiments were followed until the late phase of the disease (day 60 p.i.). At that time point, LIF−/− mice exhibited a significantly milder disease course (p = 0.02), B, Clinical course after adoptive transfer of 4–6 million MOG 35–55-specific WT T cell blasts (MOG.10 T cell line) into LIF−/− mice (n = 8, gray line) and WT (n = 7, black line) recipients. Both groups displayed a similar disease course early and late during MOG-EAE with gait impairment. Data are pooled from a total of two experiments; error bars represent SEM.

FIGURE 2. RT-PCR analysis for LIFRβ expression in different resting and activated immune cell types. LIFRβ mRNA was present in macrophages (□) and bmDC (■) at baseline and after stimulation with 100 ng/ml LPS or 500 U/ml TNF-α (TNF), respectively. Naïve T cells did not display LIFRβ mRNA while it was clearly present in MOG-specific T cell blasts (□).
umns from spinal cord cross-sections are shown. Representative anterior columns from spinal cord cross-sections from the anterior columns of a WT control mouse (A) and a LIF−/− mouse (B) are shown. Although WT mice displayed some granulocytic infiltration, numbers of neutrophilic granulocytes were clearly increased in the spinal cord of LIF−/− mice (infiltrate extension is marked by arrows; bar, 100 µm). 7/4-positive cells were also labeled by histochemistry for chloroacetate-esterase (see inset in B). C and D, Anti-Mac-3 staining for macrophage infiltration in LIF−/− mice in the late phase of active MOG-EAE (day 60 p.i.). Representative anterior columns from spinal cord cross-sections are shown. LIF−/− mice (D) exhibited a clearly reduced infiltration of Mac-3-positive cells in comparison to WT control mice (C, infiltrate marked by arrows; bar, 100 µm). E and F, Luxol Fast Blue staining of spinal cord cross-sections from WT (E) and LIF−/− mice (F) revealed a clearly reduced demyelination (marked by arrows) in LIF−/− mice on day 60 p.i. Representative sections are shown; bar, 200 µm.

Results

Milder disease course of active MOG 35–55 EAE in LIF−/− mice

In a first set of experiments, LIF−/− mice on a C57BL/6 background were compared with age- and gender-matched WT control mice for their susceptibility to EAE induction. As described earlier (12), LIF−/− mice displayed an ~10% reduction in body weight (Table I), but otherwise appeared grossly normal. MOG 35–55 EAE was induced in 32 LIF−/− and 28 WT mice in a total of six independent experiments. Disease incidence and mortality did not differ between both groups (Table I). Moreover, there was no difference in onset of disease between LIF−/− mice and WT control mice. In the early phase of disease (day 17–20 p.i.), WT and LIF−/− mice suffered from mild paraparesis. Yet, in the late phase of MOG-EAE, LIF−/− mice displayed a significantly milder disease course with only tail weakness while disability in the WT mice remained unchanged (Fig. 1A, p < 0.05).

LIF deficiency does not influence the disease course of adoptive transfer EAE

To shed further light on the role of endogenous LIF in the initiation phase compared with the effector phase of MOG-EAE, we used a newly generated encephalitogenic MOG 35–55-specific T cell line from WT C57BL/6 mice in adoptive transfer experiments. MOG specificity of cultured blasts was shown in vitro by Ag-specific proliferation (data not shown). After adoptive transfer, MOG 35–55-specific T cells led to a chronic course of disease with prominent gait ataxia as well as tail tremor and—in severe cases—also spasticity, but no tail weakness. The clinical course of adoptive transfer EAE in LIF−/− vs WT mice in a total of two experiments (n = 8 vs 7; Fig. 1B) was without significant difference. Histologic analysis revealed predominantly meningeal and, in severe cases, also parenchymal infiltrates (data not shown). These data speak for a role of endogenous LIF already in the initiation phase of the disease impacting on the further course of active MOG-EAE.

LIFRβ expression on resting and activated immune cells

We next wanted to dissect whether endogenous LIF may be able to directly act on T cells or APC. To that end, we investigated LIFRβ expression in a RT-PCR analysis of T cells, macrophages, and DCs (Fig. 2). LIFRβ mRNA was easily detected in naive DCs as well as peritoneal macrophages without further up- or down-regulation after adherence or stimulation with TNF-α or LPS. In contrast, LIFRβ mRNA was neither found in naive nor mitogen-stimulated T cell cultures. Yet, MOG-specific T cell blasts displayed a clear LIFRβ message. Thus, LIFRβ is present on several relevant immune cell subsets. In particular, Ag-specific T cell activation leads to LIFRβ up-regulation thus rendering these cells responsive to LIF.

Altered composition of the inflammatory infiltrate in LIF−/− mice early and late during active MOG-EAE

The results from active in comparison to passive MOG-EAE in LIF−/− mice speak for a role of endogenous LIF in the initiation phase of the disease. We wondered why LIF−/− mice nevertheless develop a clinical disability similar to WT controls in the early

<table>
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<tr>
<th></th>
<th>WT 13/14 p.i.</th>
<th>LIF−/− 13/14 p.i.</th>
<th>WT 60 p.i.</th>
<th>LIF−/− 60 p.i.</th>
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<tbody>
<tr>
<td>Mac-3-positive cells</td>
<td>881 ± 92</td>
<td>813 ± 171</td>
<td>46 ± 22.5</td>
<td>25 ± 22*</td>
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<tr>
<td>CD-3-positive cells</td>
<td>592 ± 247</td>
<td>644 ± 343</td>
<td>234 ± 171</td>
<td>144 ± 121</td>
</tr>
<tr>
<td>7/4 Ag-positive cells</td>
<td>228 ± 49</td>
<td>590 ± 23*</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Demyelination score (LFB)</td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.4</td>
<td>2.8 ± 0.7</td>
<td>1.6 ± 0.6**</td>
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</table>

* LIF−/− mice display increased numbers of 7/4 Ag-positive cells on day 13 p.i. and less Mac-3-positive cells and demyelination on day 60 p.i.

† Demyelination was assessed according to Storch et al. (7); cell numbers are given per mm². *p < 0.05; **p < 0.01. Data are pooled from at least two independent experiments per time point and displayed as mean ± SD.
phase of MOG-EAE. Therefore, we performed immunohistochemistry to investigate the composition of the inflammatory infiltrate in situ. Early in the course of disease (day 13 p.i.), 7/4 Ag-positive cells were abundant in the inflammatory infiltrate in LIF\(^{-/-}\) mice (Fig. 3, A and B, Table II). The identity of 7/4-positive cells as neutrophilic granulocytes was confirmed by positive histochemistry for chloroacetate-esterase (see inset Fig. 3B) as well as a polymorphonuclear appearance in an H&E staining in situ and ex vivo after MACS isolation from spinal cord (data not shown). On consecutive sections, 7/4 Ag-positive cells were not positive for Mac-3. On day 13 p.i., numbers of Mac-3-positive macrophages/ microglia and T cells were not different in comparison to WT controls. In the intermediate as well as late phase of MOG-EAE (days 27 and 60 p.i., respectively), 7/4-positive cells could not be found in the lesions in LIF\(^{-/-}\) or WT mice.

We also investigated the infiltrate composition in the later stages of the disease (intermediate phase, day 27 p.i. and late phase, day 60 p.i., respectively). Immunohistochemical analysis of spinal cord cross-sections revealed a significant reduction of Mac-3-positive macrophages and microglia in LIF\(^{-/-}\) mice in the intermediate as well as late phase of MOG-EAE (Fig. 3, C and D; Table II) as well as a reduction in T cell infiltration. To investigate whether fewer macrophages in the lesions of LIF\(^{-/-}\) mice also have a functional impact on the target tissue, Luxol Fast Blue staining was performed to analyze a parameter of tissue destruction. The extent of demyelination was not different in the early phase of disease on day 13 p.i., but clearly reduced in LIF\(^{-/-}\) mice on day 27 as well as day 60 p.i. (Fig. 3, E and F, Table II).

Ag-specific T cell proliferation is impaired after immunization of LIF\(^{-/-}\) mice

To gain more insight into the mechanisms governing T cell function in LIF\(^{-/-}\) mice, we investigated the proliferative capacity of LIF-deficient T lymphocytes in primary culture of lymph node cells. After immunizing LIF\(^{-/-}\) and WT control mice with MOG 35–55 and CFA, draining lymph nodes were prepared 10 days later and proliferation was assessed by [\(^{3}\)H]thymidine incorporation. Unspecific polyclonal activation with the mitogens phytohemagglutinin (data not shown) or Con A revealed a small, but significant, increase in T cell proliferation in LIF\(^{-/-}\) mice as compared with WT controls (Fig. 4A, \(p < 0.05\)). Yet, in response to purified protein derivative (a component of CFA), there was only few and in response to MOG there was hardly any [\(^{3}\)H]thymidine incorporation detectable in LIF\(^{-/-}\) mice 72 h after restimulation while WT mice displayed a clear Ag-specific response (Fig. 4A, \(p < 0.01\)). Similar results were seen at an earlier time point, 24 h after recall with MOG 35–55 and a similar trend was observed after stimulation with OVA as another protein Ag (data not shown). Yet, addition of exogenous LIF to MOG recall assays or MOG-specific T cell lines did not influence WT T cell responses in vitro (data not shown).

To investigate the cellular target for LIF, we next measured T cell proliferation in MOG recall assays where WT T cells were cocultured with LIF-deficient APC and vice versa. Although LIF deficiency of only T cells or APC did not significantly alter T cell proliferation, LIF deficiency of both T cells and APC resulted in a significant impairment of T cell proliferation (Fig. 4B, \(p < 0.05\)).

In a next step, we investigated the role of neutrophilic granulocytes in the regulation of T cell responses. To that end, Ly6G-positive cells were isolated from spleen and spinal cord by MACS. In the immunocytological analysis, these cells were to 93% 7/4 Ag positive and in the morphological analysis 96% had a polymorphonuclear appearance, thus proving that the majority were neutrophilic granulocytes. When they were added to several stable,
long-term MOG-specific T cell lines in the presence of MOG Ag or Con A, neutrophils significantly increased T cell proliferation in a dose-dependent manner, both after Ag-specific and unspecific stimulation (Fig. 4C). Addition of neutrophils in MOG recall assay cultures yielded similar results, while neutrophilic granulocytes alone did not proliferate (data not shown).
The proliferation defect in \textit{LIF}^{-/-} mice might be paralleled by a lack of cytokines which stimulate T cell proliferation or exert T cell effector functions. Therefore, supernatants from primary lymph node tissue culture were assessed for IL-2 and IL-6, two cytokines implicated in T cell proliferation. Production of IL-2, and IL-6 was not different between \textit{LIF}^{-/-} mice and WT controls (data not shown). In a next step, we tried to restore the proliferative capacity in LIF-deficient primary lymph node cell culture by addition of cytokines in vitro. Addition of exogenous IL-2 at different concentrations (0.1–10 ng/ml) did not lead to an increase in thymidine incorporation neither in \textit{LIF}^{-/-} nor in WT cultures. Moreover, neither the addition of LIF itself nor addition of the related cytokine IL-6 (0.1–10 ng/ml) was able to reconstitute proliferation in \textit{LIF}^{-/-} cultures (data not shown). Next, we investigated the expression of IFN-\gamma, a critical T cell effector cytokine, in supernatants from primary lymph node tissue culture by ELISA. Three days after recall with MOG 35–55, levels of IFN-\gamma were significantly reduced in \textit{LIF}^{-/-} cultures after addition of MOG 35–55 (Fig. 4D). In a RT-PCR analysis, levels of IFN-\gamma mRNA were reduced in the spinal cord of \textit{LIF}^{-/-} mice on day 27 p.i., but not on day 13 p.i. IFN-\gamma expression in the brain as well as IL-17 expression in the CNS was not altered in comparison to WT control mice (Fig. 4E). The production of the “Th2” cytokine IL-5 and production of IL-12p70 and IL-12/IL-23p40, the latter implicated in IFN-\gamma production, were not different in supernatants from primary lymph node tissue culture from \textit{LIF}^{-/-} mice and WT controls (data not shown).

\textbf{LIF deficiency does not alter DC migration or APC function}

To analyze the impact of endogenous LIF on APC, the ability of LIF-deficient APC for Ag presentation was assessed in vitro. To this end, newly generated MOG-specific T cell lines were restimulated in culture using irradiated spleen cells from \textit{LIF}^{-/-} or syngeneic WT mice. There was no difference in the amount of \[^{3}H\]thymidine incorporation after Ag-specific stimulation with MOG peptide 35–55 or whole MOG protein in the presence of LIF-deficient or WT APC (Fig. 5A, data are shown for a representative MOG-specific T cell line, MOG.6). Experiments with two further MOG-specific T cell lines (MOG.2 and MOG.10) yielded similar results. Therefore, LIF deficiency does not influence Ag-processing and presentation and costimulatory function of APC.

In a next step, we evaluated migration of DCs to exclude impaired migration of APC causing insufficient T cell priming in \textit{LIF}^{-/-} mice. Mature bmDC displayed only a mild migratory response toward LIF which was neither dose dependent nor statistically significant in vitro (data not shown). Therefore, we investigated the migratory capacity of bmDC directly in vivo. To this end, CFSE-labeled mature WT bmDC were injected s.c. in the hind pads of \textit{LIF}^{-/-} and WT control mice. Three days after simultaneous immunization with a low dose of MOG 35–55, draining lymph nodes were harvested and investigated for the presence of labeled cells. FACS analysis revealed a similar percentage of CFSE-positive DC in popliteal lymph nodes of \textit{LIF}^{-/-} mice and WT control mice. Thus, in vivo migration of WT bmDC was not different in \textit{LIF}^{-/-} and WT mice (Fig. 5B).

\textbf{LIF deficiency does not lead to altered T cell subsets}

Because no differences were observed in APC function of \textit{LIF}^{-/-} mice, we next investigated the T cell compartment. First, immune organs of immunized \textit{LIF}^{-/-} mice and WT mice were analyzed. Cell counts of peripheral blood and lymph nodes revealed similar cell numbers in \textit{LIF}^{-/-} WT blood and lymph nodes, respectively (47.5 million cells \pm 0.5 in WT vs 46.2 million cells \pm 1.47 in \textit{LIF}^{-/-} mice for pooled mesenteric, inguinal, axillary, and cervical lymph nodes). H&E staining was performed to investigate the morphology of immune organs. Lymph node architecture as well as the structure of spleen and thymus was not altered in \textit{LIF}^{-/-} mice (data not shown). To investigate T cell development and commitment, FACS analysis of thymus and lymph nodes was performed and confirmed the normal distribution of single-positive, double-positive, and double-negative cells in the thymus as well as normal CD4$^{+}$ and CD8$^{+}$ compartments in the periphery in \textit{LIF}^{-/-} mice as well as a similar percentage of early activation marker CD69 and CD25 positive T cells compared with WT mice (Table III, data are shown as percentages).

In summary, the priming defect in \textit{LIF}^{-/-} mice can neither be explained by obvious qualitative or quantitative differences in T cell subsets nor by an impaired APC function or DC migration.\n
\textbf{Altered pattern of chemokine production in \textit{LIF}^{-/-} mice}

We were interested in the molecular mechanisms governing the distinct pattern of inflammatory infiltration in \textit{LIF}^{-/-} mice. To
investigate expression patterns of chemokines, isolated mRNA from spinal cord of \( \text{LIF}^{-/-} \) mice and WT controls was investigated by RT-PCR at different time points of MOG-EAE. At the onset of disease (day 13 p.i.), the neutrophil attracting chemokine CXCL1 (KC) was increased in the spinal cord of \( \text{LIF}^{-/-} \) mice (Fig. 6A). At that time point, there was also an increase of GM-CSF, CCL3 (MIP-1\( \alpha \)), and milder also of CCL5 (RANTES) expression in \( \text{LIF}^{-/-} \) mice, although these effects were not statistically significant. At day 13 p.i., there were no differences in expression of CCL2 and CXCL10 (IP10) between both groups. In the later phase of MOG-EAE, again patterns of immune cell infiltration were correlated with chemokine production in a RT-PCR analysis. In the early late stage of MOG 35–55-EAE (day 27 p.i.), CXCL1 (KC), CCL2 (MCP-1), CCL3 (MIP-1\( \alpha \)), CXCL10 (IP10), and CCL5 (RANTES) mRNA expression were significantly reduced in the spinal cord of \( \text{LIF}^{-/-} \) mice (Fig. 6B). At that time point, there was also a trend toward a reduced expression of GM-CSF.

In summary, the expression of chemokines at the RNA level correlates with the observed infiltrates of different immune cells in the spinal cords. Neutrophil invasion in \( \text{LIF}^{-/-} \) mice early during MOG-EAE is associated with increased CXCL1 production. The decreased inflammatory infiltration in the later phase of the disease is paralleled by a decreased expression of several chemokines including CCL2, CCL3, and CXCL10.

**Discussion**

In this study, we show that a deficiency of the neuroprotective cytokine LIF affects the immune response in autoimmune demyelination. Although the early phase of active MOG 35–55 EAE in \( \text{LIF}^{-/-} \) mice is similar to WT controls, the late phase is characterized by an alleviated disease course. In search of the explanation for this phenotype, we found an Ag-specific T cell priming defect. Importantly, the inflammatory infiltrate in the LIF-deficient mice is dominated by massive infiltration of neutrophilic granulocytes in the early phase of the disease. On the molecular level, this is paralleled by a distinct pattern of chemokine expression with an increase of CXCL1 (KC). In the later phases, a reduced macrophage infiltration accompanied by decreased levels of CCL2, CCL3, and CXCL10 were observed. In extension of previous studies by Escary et al. (12), \( \text{LIF}^{-/-} \) mice display an impaired T cell priming. We could exclude structural abnormalities in the immune system of \( \text{LIF}^{-/-} \) mice as a possible explanation. This is in contrast to LIF-overexpressing mice which are characterized by interconversion of thymic and lymph node morphologies (13). LIFR\( \beta \) is present in Ag-activated T cell blasts and LIF is produced by monocytes, macrophages, and also T cells (30, 31), thus opening the possibility of paracrine actions. Yet, similar to previous studies (9, 32), exogenous addition of LIF to MOG-primed T cell cultures does not have any additional effect on T cell proliferation and adoptive transfer of WT T cells in \( \text{LIF}^{-/-} \) mice does not change the course of EAE. In conclusion, we could not delineate a direct impact of LIF on the control of T cell function although indirect effects are certainly conceivable.

In the early phase of MOG-EAE, infiltrates in \( \text{LIF}^{-/-} \) mice are characterized by an abundance of 7/4 Ag-positive cells. Expression of the 7/4 Ag is confined to neutrophilic granulocytes and monocytes, but not macrophages (22, 33). Most of the 7/4 Ag-positive

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**Table III. Distribution of CD4-, CD8-, CD25-, and CD69-positive cells in thymus and lymph nodes of WT and LIF\(^{-/-}\) mice\(^{a}\)**

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<tr>
<th></th>
<th>Thymus</th>
<th>Lymph Node</th>
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<tbody>
<tr>
<td></td>
<td>CD4(^+)</td>
<td>CD8(^+)</td>
</tr>
<tr>
<td>WT</td>
<td>85.6 ± 4.5</td>
<td>9.7 ± 3.3</td>
</tr>
<tr>
<td>LIF(^{-/-})</td>
<td>88.4 ± 0.7</td>
<td>6.4 ± 0.7</td>
</tr>
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\(^{a}\) There were no differences in the distribution of different T cell subsets in the thymus and lymph nodes between LIF\(^{-/-}\) and WT mice. Data are shown as percentages and are summarized from a FACS analysis of three mice per group as mean ± SD.
cells in EAE lesions of LIF−/− mice are also reactive for chloroacetate esterase and display a typical polymorphonuclear configuration. Thus, we characterize these 7/4 Ag-positive cells as neutrophilic granulocytes which usually represent a minor cell type in C57BL/6 MOG-EAE. Well in line with the results on neutrophils enhancing T cell proliferation, neutrophilic granulocytes were shown earlier to orchestrate inflammation and tissue damage leading to clinical symptoms in the early phase of EAE in CCR2 knockout mice (34). Yet, these cells can also regulate T cell responses (35), probably depending on the activation status of cells, type of T cell, and the milieu. Interestingly, EAE in mice deficient for the CCL2 receptor, CCR2, is also characterized by neutrophil invasion (34). Moreover, reports describe neutrophil infiltration in EAE lesions of IFN-γ knockout mice (35, 36). LIF-deficient mice are characterized by a decreased expression of both CCL2 and IFN-γ. In view of these data, it is tempting to speculate that a decrease in expression of some cytokines and chemokines (or their receptors) may lead to counterregulatory up-regulation of other chemotactants which result in a qualitatively different composition of the inflammatory infiltrate. Indeed, an up-regulation of CXCL1 in LIF−/− mice may lead to granulocyte attraction. This concept is further sustained by recent studies investigating LIF or the LIF-related cytokine IL-6 in peritoneal inflammation and in endotoxic shock. Reminiscent to MOG-EAE in LIF−/− mice, IL-6 knockout mice display increased levels of CXCL1 expression and higher numbers of infiltrating neutrophils while LIF−/− mice are characterized by an increased neutrophil sequestration (37, 38).

In the late phase of MOG-EAE, LIF deficiency leads to impaired macrophage recruitment in EAE lesions. In good agreement with previous studies using radioidinated LIF (39), we confirm expression of LIFRβ in these cells. Previous in vitro studies revealed that mouse peritoneal macrophages respond to LIF in a microchamber assay, thus demonstrating a chemotactic action of this cytokine (14). Analysis of LIF−/− mice in neurotrauma models has also provided evidence for a role of LIF in macrophage chemotaxis in vivo (16, 40). In these paradigms, it might well be possible that LIF acts indirectly on macrophage migration via induction of other chemokines. In our model, a role of endogenous LIF for macrophage recruitment was exclusively observed in the later phases of active MOG-EAE. In contrast, macrophage recruitment in the early phase of active MOG-EAE and in adoptive transfer EAE is not different between LIF−/− and WT mice. These results argue against a direct effect of LIF on macrophage recruitment in neuroinflammation. Rather, LIF deficiency may influence inflammatory infiltration by indirect mechanisms like the regulation of chemokine expression (15). Previous studies revealed that CCL2, CCL3, and also GM-CSF play a pivotal role in local macrophage chemotaxis and thus finally a milder disease course.

Although some previous studies mainly point at proinflammatory actions of LIF (16), others argue for an anti-inflammatory role of this cytokine (17, 18). Our data present evidence for an inverse impact of LIF on macrophage and neutrophil recruitment. These results point to an additional immunomodulatory role rather than a purely pro- or anti-inflammatory function. The role of LIF and CNTF in EAE were also investigated in therapeutic approaches.

Although LIF was shown to prevent oligodendrocyte loss (9), the administration of CNTF also interfered with the immune system, and inhibits inflammatory infiltration into the CNS (10). In good correlation with the data on LIF treatment, cuprizone-induced demyelination in LIF−/− mice resulted in a more pronounced oligodendrocyte loss (43). Moreover, Ab-mediated neutralization of LIF doubled the extent of oligodendrocyte loss in an EAE model (19). In extension of these previous studies, we show here that besides its protective role, LIF can also act as an immunomodu- lator. Our data do not challenge the value of LIF for oligodendrocyte protection in neuroinflammation, but bring in another level of complexity by revealing that endogenous LIF is also an immunologically active molecule. The profound interaction of LIF with the immune system will make it difficult to predict results of possible treatment trials.

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Disclosures

The authors have no financial conflict of interest.

References


