Interleukin 23 in Acute Inflammatory Demyelination of the Peripheral Nerve

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Background: Interleukin (IL) 23, a newly identified heterodimeric proinflammatory cytokine and a novel IL-12 family member comprising the p40 subunit of IL-12 but a different p19 subunit, has been reported to preferentially act on memory T cells and play an important role during cellular immune responses. Recent evidence suggests that IL-23 rather than IL-12 is critically involved in the pathogenesis of various immune-mediated disorders.

Objective: To determine the role of IL-23p19 during the course of acute immune-mediated demyelinating diseases of the peripheral nervous system.

Design: The sequential RNA expression of IL-23p19 in sciatic nerves from rats with experimental autoimmune neuritis, an animal model of the human Guillain-Barré syndrome (GBS), was analyzed by semiquantitative reverse transcriptase–polymerase chain reaction. Expression and distribution patterns of IL-23p19 protein were studied in sural nerve biopsies and cerebrospinal fluid samples from 5 patients with classical Guillain-Barré syndrome and 5 controls with noninflammatory neuropathies using immunohistochemistry and immunoblotting, respectively.

Results: We found IL-23p19 RNA to be up-regulated prior to the onset of first clinical symptoms with peak expression levels preceding maximum disease severity during experimental autoimmune neuritis. In patients, IL-23p19 protein was detectable in cerebrospinal fluid samples from patients with Guillain-Barré syndrome, and endoneurial macrophages were identified as the cellular source of IL-23p19 in sural nerve biopsies.

Conclusion: Our present data indicate that IL-23 may play an important role during the early effector phase in immune-mediated demyelination of the peripheral nerve.

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IL-12p40 subunit. The sharing of the p40 subunit between IL-12 and IL-23 raises the question whether the effects previously ascribed to IL-12 may be the sum effect of both cytokines or, in part, related to IL-23 only. Thus, a reassessment of the individual cytokines may be warranted. To distinguish the pathogenetic contribution between members of the IL-12 family in the inflamed PNS, the present study focused on the expression of IL-23 by studying its p19 subunit to gain further insights into the immunopathogenesis of immune-mediated demyelination of the PNS.

**METHODS**

**INDUCTION OF EAN AND CLINICAL ASSESSMENT**

All experiments were conducted according to the local regulations for animal experimentation and approved by the responsible authorities. We used female Lewis rats aged 7 to 8 weeks old and weighing 145 to 160 g (Charles Rivers, Sulzfeld, Germany). Experimental autoimmune neuritis was induced by injection of 4.5 mg of myelin per animal as described before. Rats injected with adjuvant served as controls. Animals were weighed and clinically scored daily on a 10-point score as described before. After 11, 13, 15, 17, and 29 days postimmunization (PI), groups of 5 animals each were killed and 1-cm-long segments of the sciatic nerves just proximal to the sciatic notch were dissected, flash-frozen in liquid nitrogen, and stored at −80°C.

**HUMAN NERVE BIOPSIES**

Sural nerve biopsies were obtained with informed consent from patients as published before. Two groups of patients were studied. The first (n=5) was diagnosed as classical GBS (acute inflammatory demyelinating polyradiculoneuropathy) according to the criteria of Asbury and Cornblath. The second group (n=5) served as controls and included noninflammatory neuropathies. None of the patients studied had received any immunomodulatory or immunosuppressive treatment within 3 months before biopsy.

**CEREBROSPINAL FLUID SAMPLES**

To detect the expression of IL-23p19 protein in cerebrospinal fluid (CSF), matched samples were obtained with informed consent from patients diagnosed with GBS (n=5) at the Düsseldorf Department of Neurology. All samples were obtained within 5 days of the onset of first clinical symptoms, and none of the patients had received any immunomodulatory therapy before sampling. Cerebrospinal fluid from patients with noninflammatory neurological disorders (n=5) served as a control. After lumbar puncture, CSF samples (10-15 mL per patient) were immediately centrifuged at 3000 g for 10 minutes; supernatants were aliquoted and stored at −80°C.

**ANTIBODIES**

The following antibodies were used for immunohistochemistry: antihuman IL-23p19 as published before, antihuman IL-23p19 (R&D Systems, Minneapolis, Minn), antihuman CD3, antihuman CD68 (DAKO, Hamburg, Germany), antihuman CD3 phycocerythrin (Beckman Coulter, Fullerton, Calif), antihuman CD68 phycocerythrin (BD Biosciences, San Jose, Calif), and swine antirabbit IgG fluorescein isothiocyanate (DAKO). For Western blot analysis, the following antibodies were applied: antihuman IL-23p19 (R&D Systems) and biotinylated antigoat IgG (Vector Laboratories, Peterborough, England). Further details are given in the Table.

**SEMIQUANTITATIVE REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION**

The presence of IL-23p19 RNA in sciatic nerves from the inflamed PNS during the clinical course of EAN was determined using a semi quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). The sequences of the synthetic oligonucleotide primers for rat IL-23p19 were sense, 5’-GCACAC-TAGCCTGGAGTGCA-3’, and antisense, 5’-GTTCGGAGTC-CAGTAGGTGCT-3’; and for rat GAPDH, serving as a housekeeping gene, sense, 5’-CCACCCATGGCAAATTC-CATGGA-3’, and antisense, 5’-TCTAGAGGCGGTAC- GTCCACC-3’. All primers were synthesized by Gibco/Invitrogen (Karlsruhe, Germany). Total cellular RNA was extracted from frozen nerves according to standard proto-
**IL-23p19 RNA as a Fraction of GAPDH**

Figure 1. Interleukin (IL) 23p19 RNA expression levels during active experimental autoimmune neuritis (EAN). A, Disease course of EAN induced with myelin. Mean clinical scores are given as mean±SD. B, Expression of IL-23 RNA as a fraction of GAPDH measured at 5 different points after disease induction. Maximum expression levels can be detected at day 13 postimmunization, prior to peak clinical disease severity. Values for each day represent RNA data from 5 animals each (1 sciatic nerve per animal), given as mean±SD. BD indicates below detection limit.

**RESULTS**

**CLINICAL COURSE OF EAN**

Weight loss and first clinical signs of the disease were noted 13 days after the injection of myelin. Clinical disease severity peaked at day 15 with an average score of 7.7, clinically presenting as severe paraparesis. At day 17 PI, the first signs of clinical improvement became apparent (Figure 1A). Control rats injected with adjuvant exhibited no signs of disease throughout the experiment.

**IL-23p19 RNA EXPRESSION DURING EAN**

At defined points during the clinical course of EAN, IL-23p19 expression was monitored at the RNA level by semiquantitative RT-PCR. An up-regulation of RNA was noticed at day 11 PI with peak expression levels at day 13 PI, just prior to peak clinical disease severity. Afterwards, RNA levels declined by day 15 PI and persisted at lower levels up to day 17 PI. Interleukin 23p19 RNA was not discernable at day 29 PI (Figure 1B). In contrast, no IL-23p19 RNA expression could be detected in the control group at any given point studied.

**IMMUNOHISTOCHEMISTRY**

Formalin-fixed and paraffin-embedded sural nerve biopsy specimens were cut at 10 µm; sections were deparaffinized in xylene, dehydrated through graded ethanol, and pretreated with protease XXIV (Sigma Chemical Co, St Louis, Mo). All primary antibodies were incubated at 4°C overnight after blocking the sections with 10% bovine serum albumin for 30 minutes. Endogenous peroxidase was blocked with 30% hydrogen peroxide in methanol. Biotinylated secondary antibodies and avidin-biotin-horseradish peroxidase complex (DAKO) were applied with 3,3′-diaminobenidine as peroxidase substrate ac-
the endoneurium (Figure 2A and B). In the noninflammatory neuropathies control group, however, only a few macrophages and T cells could be detected in the epineurium and endoneurium (Figure 2D and E).

**EXPRESSION AND CELLULAR LOCALIZATION OF IL-23p19 IN THE HUMAN PNS**

In GBS, IL-23p19 immunoreactivity was localized to mononuclear cells and was found to be highly expressed within the endoneurium (Figure 2C). The distribution of the positive IL-23p19 immunoreactivity was similar to the pattern obtained with the macrophage marker anti-CD68. Double immunofluorescence localized IL-23p19 immunoreactivity to CD68-positive macrophages (Figure 2F-H), whereas the staining pattern for CD3, indicative of T lymphocytes, did not match with the pattern for IL-23p19 (Figure 2I). In the noninflammatory neuropathies control group, no positive stain for IL-23p19 was observable.

**Figure 2.** Inflammatory infiltrates and detection of interleukin (IL) 23p19 in a sural nerve biopsy specimen from a patient with Guillain-Barré syndrome (GBS). A, CD3-positive T lymphocytes can be localized to perivascular infiltrates in the endoneurium and epineurium (arrows). B, CD68-positive cells, indicative of macrophages, are found in the endoneurium and in perivascular cuffs in the epineurium (arrows). C, IL-23p19 immunoreactivity can be localized to mononuclear cells, primarily within the endoneurium. Inflammatory infiltrates in noninflammatory controls: D, CD68-positive cells are depictable within the endoneurium, indicative of tissue resident endoneurial macrophages (arrows). E, CD3-positive T cells are rarely found in cases of noninflammatory neuropathies (arrow). Immunofluorescence in a sural nerve biopsy specimen from a patient with GBS: F, Positive immunoreactivity against IL-23p19 is depictable within the endoneurium. G, Similarly, macrophages (CD68) can be found in the same compartment, and H can be identified as the cellular source of IL-23p19 in the inflamed peripheral nervous system by double immunofluorescence. I, A colocalization with the T-cell marker CD3 can be excluded.
IL-23p19 PROTEIN EXPRESSION IN CSF OF AFFECTED PATIENTS

To examine whether increased expression of IL-23p19 in the inflamed PNS is mirrored in the CSF of affected patients, we studied the protein expression of this cytokine in CSF samples from patients with GBS and non-inflamatory controls by immunoblotting. In CSF from all GBS samples investigated (5 of 5), a positive signal for the IL-23p19 protein was observable, whereas in the control group, immunoreactivity was absent in all of the CSF samples studied (Figure 3). Control experiments after omission of the primary antibody revealed only background staining. Preabsorbance of the antihuman IL-23p19 antibody with recombinant human IL-23 selectively affected IFN-γ–positive T cells to be present in active EAN before overt clinical signs and demyelination and to disappear at later stages when most of the axons were demyelinated. Thus, IFN-γ induction in early EAN seems to occur in the absence of active IL-12. However, this discrepant expression of IFN-γ and IL-12 RNAs have been substantially examined in EAN using semiquantitative RT-PCR. Whereas IL-12 RNA was found to be up-regulated during the recovery phase of EAN, IFN-γ RNA levels did not parallel the expression of IL-12 with maximum levels being observed during the onset and the acute phase of clinical disease in EAN. Furthermore, immunohistochemistry revealed abundant IFN-γ–positive T cells to be present in active EAN before overt clinical signs and demyelination and to disappear at later stages when most of the axons were demyelinated. This may be due to a lack of sensitivity or other technical restraints. Recent data suggest that IL-23 induces a specific cytokine pattern, distinct from IL-12, in activated human naïve T lymphocytes. Interleukin 23 could induce IFN-γ production in resting naïve T cells within short time. These results strengthen the concept of a critical role of IL-23 during the early phase of the disease, where naïve T cells are likely to be more abundant than memory cells. A different study suggested that IL-23 promotes a T-cell population characterized by the production of IL-17, tumor necrosis factor α, and IL-6 and that this IL-23–dependent T-cell subset invades and promotes the development of organ-specific autoimmune inflammation. This pathway appears independent of IFN-γ, and the authors proposed

Figure 3. Western blot analysis of the cerebrospinal fluid (CSF) from patients with Guillain-Barré syndrome (GBS) and noninflammatory neurological controls. In CSF from all GBS samples investigated (5 of 5), a positive signal at the level of 59 kd, indicative of the IL-23 subunit p19, was detectable. In none of the CSF samples from the control group studied (0 of 5) could we discern immunoreactivity.
an interplay between IL-23 and IL-17, potentially regulated via an IL-12-induced IFN-γ pathway. It is obvious that further studies are needed to address this issue in greater detail and to elucidate the functional interaction between IFN-γ and IL-23.

Experimental autoimmune neuritis is an acute inflammatory demyelinating disease of the PNS. The close clinical, histopathological, and electrophysiological similarities between EAN and human GBS make this disorder a suitable model to offer insights into the pathophysiological features of an acute demyelinating disease in humans. To see whether our observations in the model disease also pertain to the human disorder, we further investigated the protein expression of IL-23p19 in sural nerve biopsy specimens obtained from patients diagnosed with GBS. Using immunohistochemistry, we were able to detect positive staining of IL-23p19 in the acute inflamed PNS and localize it to macrophages, primarily discerned within the endoneurium by immunofluorescence. As such, macrophages appear as the main cellular source producing IL-23p19 during acute inflammatory demyelination of the PNS. Our observation finds support in recent studies demonstrating that IL-23 is produced by microglia and macrophages in the inflamed central nervous system as shown in EAE. Macrophages within the PNS may be derived from circulating monocytes that invade the PNS, or they may already reside in the PNS as resident endoneurial macrophages, which might be critical in triggering an autoimmune process.

Because we observed an up-regulation of IL-23p19 during the initial phase of EAN, when resident endoneurial macrophages represent the predominant group in the PNS, it appears most likely that tissue resident rather than invading macrophages are mainly responsible for the expression of IL-23 in the acute inflamed PNS. Because of technical limitations, we were not able to localize IL-23p19 protein within the PNS during EAN. Nevertheless, our present findings underline the common concept that macrophages propagate the immune response within the inflamed peripheral nerve by secreting various proinflammatory cytokines, such as IL-1, IL-6, and tumor necrosis factor α. Based on our findings, IL-23 could be added to this list.

By Western blotting, IL-23p19 protein could be detected in all CSF samples studied. Because all samples from patients with GBS were obtained within days after clinical disease onset, this observation strengthens our findings in the animal model where IL-23 was up-regulated early in the course of the disease as well. To what extent IL-23 could be a useful biomarker for GBS cannot be judged by our observation and needs further evaluation in a larger number of patients.

**CONCLUSIONS**

Our present data suggest that the new member of the cytokines, IL-23, primarily expressed by macrophages, plays an important role during the early effector phase of EAN and therefore appears to be critically involved in the initiation of an immune response in acute immunemediated demyelination of the peripheral nerve. Because the perpetuation of a local immune response by proinflammatory cytokines is considered to be of paramount importance in the pathogenesis of autoimmune inflammatory demyelination, such an action would assign IL-23 a strategic role in this process. It is hoped that further knowledge will help design specific inhibitors, which may enlarge our therapeutic armamentarium for neuroinflammatory diseases.

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**REFERENCES**


