Increased IL-23p19 expression in multiple sclerosis lesions and its induction in microglia

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IL-12 has long been considered important in the pathogenesis of multiple sclerosis. However, evidence from recent studies strongly supports the critical role of IL-12-related proinflammatory cytokine IL-23, but not IL-12, in the development of experimental autoimmune encephalomyelitis (EAE), an animal model of this disease. The role of IL-23 in the CNS immunity of multiple sclerosis patients has not been elucidated; nor is it known whether human microglia produce this cytokine. In this study we investigated the expression of IL-23p19 and p40, with its key subunit p19 as the focus, in histologically characterized CNS specimens from multiple sclerosis and control cases using in situ hybridization and immunohistochemistry. A significant increase in mRNA expression and protein production of both subunits of IL-23 was found in lesion tissues compared with non-lesion tissues. Double staining showed that activated macrophages/microglia were an important source of IL-23p19 in active and chronic active multiple sclerosis lesions. We also detected IL-23p19 expression in mature dendritic cells which were preferentially located in the perivascular cuff of active lesions. The finding that human microglia produce IL-23 was further confirmed by the inducible production of IL-23p19 and p40 in cultured human microglia in vitro upon different Toll-like receptor stimulations. Taken together, these findings on the expression of IL-23p19 in multiple sclerosis lesions may lead to a better understanding of the events culminating in human multiple sclerosis.

Keywords: multiple sclerosis; CNS; microglia; IL-23

Abbreviations: APC = antigen presenting cell; DCs = dendritic cells; EAE = experimental autoimmune encephalomyelitis; ISH = in situ hybridization; LC = lesion centre; LE = lesion edge; LPS = lipopolysaccharide; NAWM = normal-appearing white matter; TNF-α = tumour necrosis factor-α


Introduction

Multiple sclerosis is a chronic inflammatory disease of the CNS whose pathogenic process is thought to be sustained by autoreactive T cells and auto-antibodies against myelin proteins (Hemmer et al., 2002). Lesions are characterized by loss of myelin, oligodendrocytes and axons associated with a mononuclear infiltrate and a reactive gliosis. Inflammatory immune responses have been widely implicated in the development of the illness (Zamvil and Steinman, 2003; Sospedra and Martin, 2005). The lesions express most immunological molecules that are associated with inflammatory response, such as adhesion molecules and co-stimulatory molecules, as well as proinflammatory cytokines including tumour necrosis factor-α (TNF-α) and IL-12 (Sospedra and Martin, 2005).

It has been suggested that, during pathogenesis, macrophage/microglia are active participants in myelin breakdown. Microglia, as resident antigen presenting cells (APCs), make up 10–20% of all glial cells in the CNS (Benveniste, 1997). In multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), microglia are activated, express high levels of MHC class II and function as APCs (Carson, 2002; Ambrosini and Aloisi, 2004). Microglia can be activated to express a broad range of cytokines, such as IL-1, TNF-α, IL-6 and IL-12, most of which have proinflammatory properties and have been involved in inflammation and demyelination within the CNS (Carson, 2002; Ambrosini and Aloisi, 2004). Dendritic cells (DCs), a main type of professional APC in the
IL-23p19 in MS lesions

Becher et al. indicated that IL-23 is a proinflammatory cytokine and that IL-23p19 in multiple sclerosis patients using in situ hybridization (ISH) and immunohistochemistry. As the p40 subunit of IL-12/IL-23 has been well-studied in such brain tissues (Windhagen et al., 1995; van Boxel-Dezaire et al., 1999), we have focused on the p19 subunit. Fourteen plaques from 10 multiple sclerosis brains were compared with normal-appearing white matter (NAWM) from the same tissues and with normal control tissues. We found that both subunits of IL-23, p19 and p40, expressed in the centre and edges of active and chronic active lesions. Cell morphology and surface markers indicated activated microglia/macrophages, and also mature DCs as the main cellular sources of IL-23p19. The capacity of human microglia to produce bioactive IL-23p19 was further confirmed in an in vitro culture system.

Table 1: Clinical data on the 10 multiple sclerosis patients and 5 control subjects

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex/age</th>
<th>Lesion type (lesions examined)</th>
<th>Neuropathology/clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F/43</td>
<td>Active (2)</td>
<td>Multiple sclerosis (primary progressive)</td>
</tr>
<tr>
<td>2</td>
<td>M/62</td>
<td>Active (2)</td>
<td>Multiple sclerosis, tuberous sclerosis, lung</td>
</tr>
<tr>
<td>3</td>
<td>M/75</td>
<td>Chronic active (2)</td>
<td>Multiple sclerosis, depression, hypertension, osteoporosis</td>
</tr>
<tr>
<td>4</td>
<td>M/55</td>
<td>Chronic active (1)</td>
<td>Multiple sclerosis (primary progressive)</td>
</tr>
<tr>
<td>5</td>
<td>F/49</td>
<td>Chronic active (2)</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>6</td>
<td>F/51</td>
<td>Chronic active (1)</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>7</td>
<td>M/84</td>
<td>Chronic inactive (1)</td>
<td>Multiple sclerosis (primary progressive), hypertension</td>
</tr>
<tr>
<td>8</td>
<td>M/60</td>
<td>Chronic inactive (1)</td>
<td>Multiple sclerosis, hypertension</td>
</tr>
<tr>
<td>9</td>
<td>F/59</td>
<td>Chronic inactive (1)</td>
<td>Multiple sclerosis, depression, hypercholesteroslaemia</td>
</tr>
<tr>
<td>10</td>
<td>M/60</td>
<td>Chronic inactive (1)</td>
<td>Multiple sclerosis (secondary progressive)</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td></td>
<td>No evidence of neuropathology, renal hypertension</td>
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<tr>
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<td>F</td>
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<td></td>
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<td>F</td>
<td></td>
<td>No evidence of neuropathology, chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td></td>
<td>No evidence of neuropathology, chronic obstructive pulmonary disease</td>
</tr>
</tbody>
</table>
**In situ hybridization (ISH)**

ISH for IL-23p19 was performed using digoxigenin-labeled riboprobes. Sections were cut serially at 10 μm. Briefly, a 764 bp IL-23p19 cDNA fragment was cloned from inflamed human brain and the PCR products were sequenced to ensure identity to GenBank NM_016584. The DIG-labelled cRNA probe was prepared by in vitro transcription using SP6 polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA). All prehybridization procedures were performed under RNase-free conditions at room temperature. Hybridization was carried out for ~12 h at 58°C in hybridization buffer. After being washed, slides were incubated overnight at 4°C with anti-DIG antibody conjugated with alkaline phosphatase (1:2000; Roche). Signals were incubated overnight at 4°C in hybridization buffer and were then washed, incubated with biotinylated secondary antibody at room temperature and resuspended after trypsin–EDTA detachment (Invitrogen, CA, USA). Purity was observed by flow cytometry (FACSAria; BD Bioscience, CA, USA). Cultured microglia were stained with FITC-label Ricinus Communis Agglutinin-I (1:50 RCA-I; Vector Laboratories) for 1 h at room temperature and resuspended after trypsin–EDTA detachment (Invitrogen, CA, USA). Purity was observed by flow cytometry (FACSAria; BD Bioscience, CA, USA). Concentration of IL-12p40 was measured with ELISA kits (BD OptEIA; BD Bioscience). The ELISA kit for IL-23 was purchased from Bender MedSystems (Burlingame, CA, USA), in which the capture Ab specifically recognizes IL-23p19 subunit and the detection Ab is anti-IL-12p40 mAb.

**Assay of microglia purity by FACS and IL-23 production by ELISA**

Cultured microglia were stained with FITC-label Ricinus Communis Agglutinin-I (1:50 RCA-I; Vector Laboratories) for 1 h at room temperature and resuspended after trypsin–EDTA detachment (Invitrogen, CA, USA). Purity was observed by flow cytometry (FACSAria; BD Bioscience, CA, USA). Concentration of IL-12p40 was measured with ELISA kits (BD OptEIA; BD Bioscience). The ELISA kit for IL-23 was purchased from Bender MedSystems (Burlingame, CA, USA), in which the capture Ab specifically recognizes IL-23p19 subunit and the detection Ab is anti-IL-12p40 mAb.

**Quantification of gene expression level by real-time PCR**

Real-time PCR was performed to quantify the p40, p35 and p19 mRNA level in microglia. The TaqMan primers for genes of the three subunits and two endogenous controls, 18S RNA and β-actin, were designed by and purchased from Applied Biosystems (Foster City, CA, USA). PCR was performed in 96-well microtiter plates under the following conditions: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The PCR product was detected as an increase in fluorescence with the ABI PRISM 7000 instrument. Quantification of gene expression was performed using standard curves and comparative Ct methods recommended by the manufacturer and by another laboratory (Pennequin et al., 2004).

**Induced IL-17 production by supernatant of activated microglia**

Supernatants of activated human DCs containing high levels of IL-23 have been reported to strongly induce IL-17 production in naive murine splenocytes, but not in human T cells (Schnurr et al., 2005). Similar results were found in our preliminary study (data not
showed). Murine splenocytes were thus used in this study as readout for analysing the level of bioactive IL-23 in culture supernatants of the human microglia cell line, as suggested in human DC culture (Schnurr et al., 2005). Briefly, splenocytes were isolated from naïve female C57/BL6 mice, which are susceptible to EAE. These cells were then activated by anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) mAb and continued to culture for 5 days with RPMI 1640/10% FCS supplemented with 100 U/ml IL-2. Different ratios (vol/vol) of activated microglia-derived supernatant (2:1; 1:1) were added to the cultures. As controls, recombinant mouse IL-23 and blocking mAb against IL-12/23 p40 were added in separate wells. Supernatants were harvested five days after culture and assayed using an IL-17 ELISA kit according to the manufacturer’s recommendations (BD OptEIA).

Statistical analysis
Total cell counts (cell nuclei) and p19-immunoreactive cells were made using a 20x objective and a standard square grid within defined plaque zones, namely, parenchymal (main body of the plaque) and plaque border, and also in surrounding non-plaque tissue. A minimum of 10 grids were used in each area and the mean count calculated. The relative degree of p19-immunoreactivity was determined by the number of p19-immunoreactive cells with respect to the number of stained nuclei. The results are presented as mean ± SD. Statistical significance was calculated using Student’s t-test.

Results
IL-23p19 gene expression in active multiple sclerosis lesions
We analysed the presence and distribution of IL-23p19 mRNA in brain sections of multiple sclerosis patients and normal control by ISH. LFB and HE stainings were performed to characterize the lesions (Table 1), which revealed the lesion centre (LC) of demyelination and abundant perivascular inflammation; the parenchymal area consisted of the main body of the lesion, the hypercellular lesion edge (LE), and the white matter surrounding the demyelinated lesion (Fig. 1A and B). In some lesions, there is myelin debris surrounded by phagocytic macrophages, indicating an active lesion (Fig. 1C).

Sections were hybridized with an anti-IL-23p19 mRNA probe to detect its expression in brain sections. Strong, dark purple hybridization signals were seen in active lesions. A p19 mRNA-positive signal was detected in LC, more prominently around blood vessels (Fig. 1D and E). p19 mRNA expression was also present in the LE with widely segregated distribution (Fig. 1D and F). There was no hybridization signal in the white matter surrounding lesions (Fig. 1D). In contrast, hybridization signals of IL-23p19 mRNA were occasionally detected in the LC of inactive plaques (data not shown). Sections from NAWM and normal controls had undetectable hybridized signals (not shown). Sections hybridized with sense probe or buffer alone were devoid of signals (Fig. 1G).

IL-23p19 and p40 immunoreactivity in multiple sclerosis lesions
To define the distribution of IL-23p19-immunoreactive cells in lesions, sections were analysed with IL-23p19 and CD11b mAb by immunohistochemistry. Two populations of CD11b+ cells were observed in active lesion (Fig. 2A): perivascular CD11b+ cells had the appearance of activated monocytes with prominent granular cytoplasm mainly distributed in LC (Fig. 2B), whereas parenchymal CD11b+ cells exhibited the morphology of activated process-bearing microglia (Fig. 2C) mainly distributed in LE. Fig. 2D-G shows IL-23p19 immunoreactivity in one chronic active lesion. Limited IL-23p19 immunoreactivity could be detected within LC and LE (Fig. 2D). A sharp decline of IL-23p19 expression was observed in the white matter surrounding lesions (data not shown). IL-23p19+ cells within LC were either perivascular or dispersed in the parenchyma with a foamy, macrophage-like appearance (Fig. 2E). Mainly IL-23p19 immunoreactive cells were scattered within LE (Fig. 2F) and exhibited predominantly the morphology of activated process-bearing microglia (Fig. 2G). There was no p19-immunoreactivity in serial slides that omitted IL-23p19 mAb (Fig. 2I).

We have analysed the distribution of p19-immunoreactivity in various multiple sclerosis lesions. As shown in Fig. 2K, cells positive for p19 have readily been identified in perivascular or LEs of active and chronic active lesions. In contrast, no p19 expression was detected throughout the white matter in either control or NAWM CNS tissue. In active lesions, p19+ cells were primarily clustered in perivascular activated macrophage-like cells. In chronic active lesions, primary immunoreactivity was detected in activated microglia-like cells at LEs. In chronic inactive lesions, there was no p19-immunoreactivity in the lesion (2 h), only in a few instances occasional p19-immunoreactivity was found in the blood vessel. Quantitative immunohistochemical analysis of IL-23p19 expression in multiple sclerosis lesions revealed a significant difference in the percentage of p19+ cells among these lesions (42 ± 12% in active lesions; 28 ± 7% in chronic active lesion; 6 ± 2% in inactive lesions, respectively) (Fig. 2K). These data indicate that IL-23p19 expression correlates with lesion activity.

Although the expression of p40, a subunit for both IL-12 and IL-23, has been reported in multiple sclerosis lesions (Windhagen et al., 1995) and thus is not a focus in our current study, the immunoreactivity of this subunit was nonetheless determined in the same tissues in our p19 study for the entire component of IL-23. Indeed, p40 expression has also been revealed in lesions. As shown in Fig. 2J, p40+ cells were found in perivascular cuff and the parenchyma around blood vessels in active lesions, a distribution pattern similar to p19+ cells. p40 expression in CD11b+ cells was shown in Fig. 2J (insertion). These results for both p19 and p40 indicate the production of IL-23 protein in active lesions.
and imply the involvement of IL-23 in the pathogenesis of multiple sclerosis.

**Expression of IL-23p19 in macrophages/microglia in multiple sclerosis lesions**

To confirm macrophages and microglia as a possible cellular source of IL-23p19 in lesions, we examined the colocalization of IL-23p19 and CD11b, a marker of macrophages and microglia. Double immunofluorescence staining revealed that IL-23p19 was highly expressed in the perivascular area and the parenchyma of active and chronic active lesions. Most round, foamy CD11b+ cells, a feature of activated macrophages, are IL-23p19 positive (Fig. 3A, arrow and in insertion). We further determined the activation status of these IL-23p19-producing cells by co-staining tissues with anti-p19 and anti-CD68, a surface marker for activated macrophage/microglia (Liu et al., 2001). The majority of CD68+ cells produced IL-23p19, with morphology of both activated microglia and macrophages (Fig. 3C). In contrast, IL-23p19 negative CD11b+ cells appeared to have a resting morphology (shown by arrowhead in Fig. 3A). Quantitative analysis of IL-23p19 immunoreactivity in CD11b+ or CD68+ cells revealed that most of these monocytes produce IL-23p19 in lesions (IL-23p19+/CD11b+: 44 ± 14% in chronic active and 52 ± 18% in active lesions; IL-23p19+/CD68+: 50 ± 15% in chronic active and 63 ± 10% in active lesions, respectively) (Fig. 3B and D). We therefore concluded that activated microglia/macrophages produce IL-23p19 in both active and chronic active lesions.
Fig. 2 Distribution and morphology of IL-23p19+ and CD11b+ cells in active lesions. Immunohistochemistry for CD11b and IL-23p19 was performed on serial sections. Additionally, dual-label immunofluorescence histochemistry for CD11b and IL-23p40 was performed. BV refers to blood vessels, LC to lesion centre and LE to lesion edge. Two populations of CD11b+ cells were found in active lesions: perivascular cells with the appearance of monocytes within LC (B, arrows) and parenchymal cells exhibiting the morphology of activated microglia within LE (C, arrowheads). (D-G) Immunohistochemistry for IL-23p19 located p19+ cells within LC (E) and LE (F and G). p19+ cells within LC were either in perivascular area (E, arrows) or dispersed in the parenchyma with foamy, macrophage-like appearance (E, arrowhead). p19+ cells within LE exhibited the morphology of activated process-bearing microglia (F and G; arrowheads). There was no p19-immunoreactivity in inactive chronic lesions (H). Absence of IL-23p19 mAb is used as negative control and no immunoreactivity (I). (J) Immunoreactive p40 subunit of IL-12/IL-23 was detected in MS lesions (arrows) and its expression (green) in CD11b+ cells (red) was colocalized by double-fluorescence staining (upper-left insert, OM ×20) (A and D, OM ×4; B, C, E and J, OM ×20; F and H, OM ×10; G and I, OM ×40). (K) Quantitative analysis of IL-23p19 immunoreactivity among MS lesions. *, comparison versus NAWM; **P < 0.01; #, comparison versus inactive lesions; #P < 0.05; ##P < 0.01.
Mature perivascular DCs express IL-23p19 in multiple sclerosis lesions

Mature DCs in the periphery have been reported to secrete IL-23 (Oppmann et al., 2000). DCs were also found to accumulate in the CSF and be present in multiple sclerosis and EAE lesions (Serafini et al., 2000; Greter et al., 2005). We further determined IL-23p19 expression in DCs using DC-SIGN for whole DCs and CD83 for mature DCs (Serafini et al., 2006). By immunoperoxidase labelling, DC-SIGN+ cells were mostly detected in the perivascular cuff with an irregular, ramified morphology (Fig. 4A). Some DC-SIGN+ cells were scattered in the parenchyma surrounding the perivascular cuff (Fig. 4B). Blood vessels surrounded by DC-SIGN+ cells were localized in the centre of active lesions as well as at the edge of chronic active lesions (data not shown). CD83+ cells were present only around the perivascular cuff and their numbers were dramatically reduced compared with DC-SIGN+ cells. Occasionally, a few CD83+ cells were detected in the parenchyma (data not shown). In the perivascular cuff in all multiple sclerosis tissue sections examined, CD83+ cells were only detected in active lesions. No DC-SIGN+ and CD83+ cells were detected in normal brain.

Double immunofluorescence staining with anti-IL-23p19 mAb and anti-DC-SIGN mAb (Fig. 4D) showed that in active and chronic active lesions, only few perivascular DC-SIGN+ cells were IL-23p19-positive (Fig. 4D). However, mature DCs represented by CD83+ were stained positively for IL-23p19 in the perivascular cuff and the parenchyma close to blood vessels in three out of four active lesions (Fig. 4E). A high percentage of IL-23p19 immunoreactivity in CD83+ cells (62 ± 12%) was observed in active lesions and few IL-23p19+CD83+ cells (9 ± 9%) were observed in chronic active lesions (Fig. 4F). These findings suggest that, in addition to activated microglia/macrophages, mature DCs are also an important source of IL-23p19 in active lesions.
Inducible expression of p19 in human microglia

To confirm our finding that human microglia are a potential source of IL-23 in the CNS, we determined the capacity of producing IL-23 by microglia upon culturing with different stimuli in vitro. The purity of cultured microglia was in excess of 98% (Fig. 5A), as determined by flow cytometry with FITC-conjugated RCA-1, a specific histochemical marker for human microglia (Mannoji et al., 1986). With a specific human IL-23p19 probe, activated microglia stimulated by LPS express a substantial amount of IL-23p19 mRNA, as detected by ISH, consistent with ISH results in multiple...
sclerosis lesions (Fig. 5B). Expression of IL-23p19 is undetectable in non-activated microglia (Fig. 5C).

Microglia were then examined by real-time PCR to determine the expression patterns of IL-23p19, IL-12p40 and IL-12p35 following stimulation individually by CD40L and various Toll-like receptor (TLR) ligands, for TLR2, −3, −4 and −9. These TLR ligands were chosen because engagement of these bacteria products by APCs can result in expression of innate immune cytokines that play a role in mediating the adaptive immune response. Microglia were cultured with Pam3Cys (TLR2 ligand), poly I:C (TLR3 ligand), LPS (TLR4 ligand), CpG ODN 2006 (TLR9 ligand) or CD40L. As shown in Fig. 5D, unstimulated microglia did not constitutively express significant mRNA levels for IL-23p19, IL-12p40 and IL-12p35. Microglia were strongly induced to express IL-23p19 following PamCys or LPS stimuli. Stimulation by CD40L or CpG ODN induced a low level of p19 expression. In contrast, microglia significantly increased IL-12p35 mRNA expression after stimulation by CD40L, LPS or CpG ODN. Poly I:C was the weakest inducer of mRNA expression. All these stimuli induced significant expression of IL-12p40 mRNA. Together, our data indicate inducible expression of p19 and different regulation of IL-12p35 and IL-23p19.

We also determined the p19 expression in response to soluble cytokines including IFN-γ, IL-17, IL-12 and IL-23. These cytokines were chosen since they are produced by CNS-infiltrating leukocytes (IL-12 and IL-23 by APCs and IL-17 and IFN-γ by T cells) and can influence the function of microglia. As illustrated in Fig. 5E, low levels of p19 were detected in microglia in the presence of these cytokines alone. High expression was observed in microglia induced by LPS with or without cytokines, and there is no significant difference among these LPS-treated microglia groups.

Activated microglia produce bioactive IL-23

We then set up an ELISA assay to measure IL-12p40 and IL-23 secretion in the supernatant of activated microglia. For IL-23 ELISA, p19 subunit specific mAb and anti-p40 antibody were used as capture and detection antibodies, respectively. Resting microglia produced very low or undetectable levels of p40 subunit and IL-23. Yet after 24 h of stimulation with LPS, the secretion of IL-23 and p40 subunit was clearly demonstrable (Fig. 6A).

To determine whether IL-23 produced by activated microglia in vitro has biological significance, the capacity of IL-23 to induce IL-17 production was tested in murine splenocytes. These cells rather than human T cells were chosen due to their strong response to IL-23-enriched human DC supernatants and to recombinant human IL-23 (Schnurr et al., 2005). As shown in Fig. 6B, supernatants of unstimulated microglia did not induce a detectable level of IL-17. In contrast, the supernatant from activated microglia induced high levels of IL-17. The secretion of bioactive IL-23
Differences were compared with CD4
IL-17 production in culture supernatants was measured by
IL-12/23 p40 was used at 10
antibodies. Blocking mAb against
unstimulated and LPS-stimulated microglia in the presence of
(see separate experiments. * with anti-IL-12p40 mAb. Data represent mean
resting microglia and activated microglia. * and IL-23 production.
were harvested at 6 h of culture, and assayed for IL-12p40
supernatants from unstimulated and LPS-stimulated microglia
of IL-17, impaired IL-17 production in the presence of this
activity and found impaired IL-17 production in microglia
culture. Although p40 neutralizing may block the activity of
both IL-12 and IL-23, as IL-23, but not IL-12, is an inducer
of IL-17, impaired IL-17 production in the presence of this
antibody must be attributed to a blocking of IL-23 activity in
the culture. Taken together, these data provide evidence that
human activated microglia produce high levels of bioactive
IL-23.

Discussion
Results presented in this study provide evidence for the first
time that proinflammatory cytokine IL-23 is highly
expressed in active and chronic active multiple sclerosis
lesions, and we have identified activated macrophages/
microglia and mature DCs as cellular sources of IL-23p19 in
lesions. The capacity of human microglia to express IL-
23p19 mRNA and produce bioactive IL-23 protein was
confirmed in an in vitro system upon various TLR
stimulations.

Multiple sclerosis is considered to be an autoimmune-
mediated inflammatory disorder affecting the CNS. It is
generally believed that inflammation is an obligatory and
possibly primary feature of demyelination in multiple
sclerosis (Lassmann et al., 1998). Autoreactive T cells enter
the CNS and induce an inflammatory cascade that results in
the injury of normal neural tissue. Inflammatory infiltrates
comprising predominantly T cells and macrophages occur
throughout early, active lesions and are instrumental in
disease progression (Zamvil and Steinman, 2003). IL-12 has
long been considered important in the pathogenesis of the
disease (Windhagen et al., 1995). However, accumulated
evidences in mice support the critical role of IL-23, instead
of IL-12, in the development of EAE (Becher et al., 2003;
Cua et al., 2003; Zhang et al., 2003). Whether this cytokine is
also involved in immunopathogenesis is not yet known. In
this study we demonstrate that the p19 subunit of IL-23 is
produced by activated macrophage/microglia in multiple
sclerosis lesions. This finding, combined with the production
of p40 subunit in these lesions shown by us and others
(Windhagen et al., 1995), as well as IL-23 production in
activated microglia in vitro, strongly corroborates the
hypothesis that microglia produce bioactive IL-23 in
pathogenesis. Furthermore, our current study clearly
demonstrates highly expressed p19 mRNA and protein in
macrophages/microglia in active and chronic active lesions,
but low or even undetectable expression in silent lesions,
NAWM and normal brain sections. Correlation of
inflammatory and demyelinating lesions with IL-23 expres-
sion indicates that IL-23 is likely to be important for the
recruitment and activation of a range of inflammatory cells
that are required for the induction of chronic inflammation,
thus playing an important role in the immunopathogenesis
of multiple sclerosis.

Our studies also showed that mature CD83+ DCs
producing IL-23p19 were preferentially located around the
perivascular cuff in active lesions. DCs, as highly specialized
APCs, have been found to accumulate in CSF and lesions
(Pashenkov et al., 2001; Greter et al., 2005). The presence of
mature DCs in the perivascular cuff raised the possibility
that these cells could be retained in the CNS to stimulate
autoactive T cells locally. The concept has been demon-
strated in the EAE model where CD11c+ DCs alone are
sufficient to present antigen in vivo to primed myelin-
reactive T cells, mediating CNS inflammation and clinical
disease development (Greter et al., 2005). Recent studies
have shown that reactivation of infiltrating T cells by local
APCs/DCs within the CNS is required for encephalitogenic
epitope spreading in relapsing EAE (McMahon et al., 2005).
These observations, combined with the finding of therapeutic inhibition of EAE by anti-IL-23p19 (Chen et al., 2006), indicate that IL-23-producing DCs are probably involved in the pathogenesis of relapsing multiple sclerosis and suggest that anti-IL-23p19 has a therapeutic effect.

Identification of activated microglia as one source of IL-23 led us to investigate its regulation within the CNS. TLR play a central role in the initiation of cellular innate immune responses and serve as pathogen-associated molecular pattern receptors that bind microbial molecular motifs with high specificity (Akira et al., 2001; Iwasaki and Medzhitov, 2004). Microglia activation via TLR signalling triggers rapid proinflammatory cytokine expression (including TNF-α, IL-1β and IL-6) that contributes to the initiation and maintenance of local immune response (Lehnardt et al., 2003). Our data indicate that signalling through TLR2 and TLR4 can induce strong p19 expression of microglia, while IL-12p35 expression is more sensitive to signalling by CD40L and TLR4. Unlike IL-12, which requires IFN-γ synergized with LPS for its production (Verreck et al., 2004), expression of p19 appears less dependent on IFN-γ. Together, our studies indicate differential regulators in IL-12 and IL-23 expression and suggest unique roles for both cytokines in adoptive immunity. Early events in a pathogen encounter can therefore potentially shape the subsequent immune response through IL-12 and IL-23 expression (McKenzie et al., 2005).

It has been suggested that microglia are the major APCs in the CNS and play an important role in the reactivation of infiltrating T cells (Aloisi et al., 1999; Zamvil and Steinman, 2003). Microglia-T cell interaction via IL-23 may have a critical impact on local inflammatory response in multiple sclerosis brain. Recent studies have demonstrated that, while TGF-β in the context of an inflammatory cytokine milieu induces Th17 cell differentiation, IL-23 primarily contributes to the maintenance and expansion of this proinflammatory T cell population (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). In a passive transfer EAE model, IL-23-driven IL-17 producing T cells are highly potent inducers of CNS immune pathology (Langrish et al., 2005). IL-17-producing T cells are distinct from the classical Th1 and Th2 lineages with different gene expression and are thus named Th17 (Harrington et al., 2005; Langrish et al., 2005; Park et al., 2005). IL-17, as a potent proinflammatory cytokine produced by activated CD4+ memory T cells, primarily acts on stromal endothelial cells and a subset of monocytes to induce secretion of proinflammatory mediators such as IL-8, CXC ligand 1, TNF and granulocyte macrophage colony-stimulating factor, which promote rapid neutrophil recruitment. Dysregulated production of IL-17 and TNF has been found in the target organs of several human autoimmune diseases, including multiple sclerosis, rheumatoid arthritis and psoriasis (Kotake et al., 1999; Li et al., 2004; McKenzie et al., 2005). Here we show that IL-23 produced by human microglia is able to specifically induce IL-17 in activated T cells. This finding, combined with our observation that activated microglia produce IL-23, implies the existence of an IL-23/IL-17 axis in multiple sclerosis lesions. IL-23-producing microglia might play a critical role in Th17 cell expansion/maintenance in the CNS, thus promoting the pathogenesis of multiple sclerosis. Targeting this IL-23/IL-17 axis in the CNS could be of therapeutic benefit for multiple sclerosis patients.

In summary, we described the distribution of IL-23 in multiple sclerosis brain tissue sections and identified the cellular sources of IL-23p19. We further demonstrated that bioactive IL-23 can be produced by cultured human microglia upon different stimuli in vitro. Our results provide insights into the significance of IL-23 in the pathogenesis of this disease.

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