Chapter 3

Monocyte functions in multiple sclerosis


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Abstract

CD4<sup>pos</sup> T cells producing T<sub>H1</sub> cytokines such as IFN-γ are believed to be important in the pathogenesis of Multiple Sclerosis (MS), a chronic demyelinating disease of supposed autoimmune origin. Interestingly, peripheral blood T cells of MS patients secrete high amounts of IFN-γ compared to healthy donors. The differentiation of naive T cells towards the T<sub>H1</sub> type is facilitated by interleukin (IL)-12, a cytokine produced by antigen presenting cells (APC). We investigated whether peripheral blood monocytes of MS patients differed from control groups with respect to in vitro production of IL-12. Upon optimal stimulation with endotoxin, MS patients showed a markedly lower secretion of IL-12(p40) compared to normal controls. Also IL-6, IL-10 and TNF-α secretion was lower in MS patients, while IL-8 secretion did not differ between the groups. Markers for monocyte activation in vivo, i.e. IL-12(p40) and soluble CD14 (sCD14) levels in plasma, serum and cerebrospinal fluid (CSF) did not significantly differ between MS patients and control groups. In conclusion, no evidence was obtained that an intrinsic enhanced production of IL-12 by antigen presenting cells plays a role in the immunoregulatory disturbances seen in MS patients. The mechanism and the biological significance of the lower monokine secretion in vitro secretion remain to be defined.

Introduction

Based on cytokine production profile and effector function, distinct types of helper (H) CD4<sup>pos</sup> T cells have been described in humans. T<sub>H1</sub> type cells secrete IFN-γ and tumour necrosis factor (TNF)-β, and are involved in cell-mediated immunity, whereas T<sub>H2</sub> type cells that secrete interleukin 4 and 5 are mainly committed to humoral immune reactions<sup>29,34</sup>. In addition, T<sub>H0</sub> cells can be discriminated that produce a combination of T<sub>H1</sub> and T<sub>H2</sub> cytokines. Under physiological conditions, a balance exists between T<sub>H1</sub> and T<sub>H2</sub> cell subsets, but in pathological conditions, such as autoimmune diseases and allergy, a predominance of either of these two helper T-cell subsets can be found<sup>10,18</sup>. The differentiation of helper T cells is for a considerable degree regulated by antigen presenting cell(APC)-derived cytokines. IL-12, a p70 heterodimer, consisting of p35 and p40 chains, is an important inducer of IFN-γ production by T cells and NK cells<sup>21,25</sup>. P40 is produced in excess over the p35 chain, but thus far no biological function has been assigned to it. Initiation of a T<sub>H2</sub> type response appears to be mediated by IL-4, although there is still some debate about the primary source of this cytokine<sup>13,14,16</sup>. It has been hypothesized that the disease process in MS is propagated by the presence of T<sub>H1</sub> cytokine producing autoreactive T cells in the central nervous system (CNS). Indeed,
some studies reported relatively high amounts of Th1 cells in peripheral blood and increased production of IFN-γ by peripheral T cells of MS patients. A number of authors have suggested that systemic and local APC functions in MS patients are disturbed. Interestingly, it has been reported that serum levels of heterodimeric IL-12 are higher in chronic progressive (CP) MS patients in comparison with control groups. To investigate the IL-12 producing capacity of peripheral blood monocytes of MS patients we used a whole blood culture system. Next to this, we measured IL-12, and soluble CD14 (sCD14), as a general marker of monocyte activation, in cerebrospinal fluid (CSF) and serum from MS patients, to extend our findings to the in vivo situation.

Materials and Methods

Subjects
In the first part of the study monokine secretion in vitro was measured in a group of 27 MS patients (mean age ± SD 45.6 ± 11.1; 12 males). 35 healthy donors (mean age ± SD 36.6 ± 11.2; 15 males) served as controls. Although in the second part of the study we used different groups of individuals for serum analyses, we considered comparison of these findings with those of the first part of the study permissible because we were mainly interested in generalized defects in MS patients. Levels of IL-12 (p40) and sCD14 in cerebrospinal fluid were measured in 32 MS patients (mean age ± SD 35.2 ± 9.7; 10 males), 29 patients with other inflammatory neurological diseases (mean age ± SD 48.5 ± 15.0; 17 males), 65 patients with other neurological diseases (mean age ± SD 40.9 ± 14.1; 26 males), and 32 healthy controls (mean age ± SD 41.0 ± 13.9; 14 males). In 20 MS patients, 11 OIND patients, 32 OND patients, and 20 healthy controls from these groups levels of IL-12 (p40) and soluble CD14 (sCD14) were also measured in serum.

Reagents
IL-8, TNF-α, IL-6, and IL-12 (p40) ELISA's were developed at the Department of Autoimmune Diseases, CLB, The Netherlands. IL-10 ELISA was kindly provided by DNAX Research Institute (Palo Alto, CA). Soluble(s) CD14 was measured in ELISA as described.

In vitro cytokine secretion by peripheral blood monocytes
Venous blood was collected in evacuated blood collection tubes (Vacutainer, Becton Dickinson, Meylan, France) containing sodium heparin (143 USP Units), and was kept at room temperature for 24 hours. Whole blood cultures were performed as described before. Briefly, blood was diluted 1:10 in IMDM, supplemented with 0.1 % FCS, antibiotics, and 50
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IU/ml sodium heparin. Monocytes were stimulated either with or without 100 pg/ml lipo-oligosaccharide (LOS). Supernatants were harvested after 24 hours. IL-8, TNF-α, IL-6, IL-10, and IL-12 (p40) secretion was measured in specific ELISA's.

Statistical analysis

Results are given as median values with resp. 25 and 75 interquartile ranges (IQR). Differences between groups were analyzed using the Mann-Whitney U Test, or in case of multiple group comparisons, the Kruskal-Wallis test. Spearman’s Rho was used as a measure
Results

Lower secretion of IL-12, IL-10, IL-6 and TNF-α but not IL-8 by monocytes from MS patients

With the exception of IL-8, none of the cytokines tested show any spontaneous secretion, i.e. secretion in unstimulated conditions (data not shown) and therefore no significant differences between MS patients and healthy controls were found.

In figure 1 the monokine secretion patterns of both MS patients and healthy controls upon stimulation with 100 pg/ml LOS are given. High amounts of IL-8 are measured in most individuals (figure 1A) and comparison of the two groups shows that the secretion of this cytokine did not differ between MS patients (median (IQR): 22 (13-39) ng/ml) and controls (17 (8-37) ng/ml). For the proinflammatory cytokine TNF-α (figure 1B) a slightly decreased secretion was found in MS patients (controls 1548 (1059-2728), MS 905 (646-2412) pg/ml; p<.05). MS patients secreted less IL-6 (figure 1C; controls 2305 (1117-4461), MS 1347 (509-1887) pg/ml; p<.01), and IL-10 (figure 1C; MS 120 (0-211) pg/ml), compared with healthy controls (239 (176-379) pg/ml; p<.001). Finally, the secretion of the Th1-inducing cytokine IL-12 (p40) was significantly lower in MS patients (figure 1E; controls 353 (127-646), MS 114 (14-300) pg/ml; p<.01).

Table I. Correlations between production of different monokines (Spearman’s Rho)

<table>
<thead>
<tr>
<th></th>
<th>IL-8</th>
<th>TNF-α</th>
<th>IL-12</th>
<th>IL-10</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>HC</td>
<td>MS</td>
<td>Total</td>
</tr>
<tr>
<td>IL-6</td>
<td>.45***</td>
<td>.71***</td>
<td>.34</td>
<td>.38**</td>
</tr>
<tr>
<td>IL-8</td>
<td>.11</td>
<td>.00</td>
<td>.42*</td>
<td>.44***</td>
</tr>
<tr>
<td>TNF-α</td>
<td>.41***</td>
<td>.27</td>
<td>.37</td>
<td>.42***</td>
</tr>
<tr>
<td>IL-12</td>
<td>.25</td>
<td>-.07</td>
<td>.24</td>
<td></td>
</tr>
</tbody>
</table>

* p<.05; ** p<.01; *** p<.001

To analyze whether there are qualitative differences in monokine secretion patterns
between individuals or that in vitro secretion of these cytokines is predominantly the
reflection of the responsiveness of the monocyte, we also calculated correlation coefficients
(Spearman's Rho) between the stimulated secretion of the various cytokines. The results in
table I show that in most cases cytokine secretion patterns are positively correlated, but that
only the correlation between IL-6 and IL-12 secretion is relatively high and also significantly
correlated in both the control group (R=.69; p<.0001) and the MS group (R=.57; p<.001), and
in the total group of individuals (R=.73; p<.0001). Furthermore, in the control group but not
in the group of MS patients, IL-8 secretion is positively correlated to both IL-6 and IL-12
secretion (R=.71; p<.0001 in both cases).

Table II. Serum levels of IL-12 (p40) (pg/ml)

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>OND</th>
<th>OIND</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>serum</td>
<td>34.0 (29.3-67.0)</td>
<td>36.5 (23.5-54.5)</td>
<td>40.0 (26.0-57.0)</td>
<td>32.5 (21.0-37.5)</td>
</tr>
<tr>
<td>CSF</td>
<td>0.0 (0.0-6.8)</td>
<td>0.0 (0.0-7.5)</td>
<td>0.0 (0.0-14.0)</td>
<td>0.0 (0.5-12.8)</td>
</tr>
</tbody>
</table>

Table III. Serum levels of soluble CD14 (U/ml)

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>OND</th>
<th>OIND</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>serum</td>
<td>164.0 (110.5-202.5)</td>
<td>142.5 (118.3-168.8)</td>
<td>144.0 (125.0-162.0)</td>
<td>130.0 (110.0-143.8)</td>
</tr>
<tr>
<td>CSF</td>
<td>11.5 (8.3-16.0)</td>
<td>11.0 (8.5-17.0)</td>
<td>15.0 (6.0-33.0)</td>
<td>11.0 (8.0-18.8)</td>
</tr>
</tbody>
</table>

Serum levels of IL-12 and soluble CD14 in MS patients and healthy controls
We measured the concentration of IL-12 in serum and cerebrospinal fluid (CSF). Patients with
other inflammatory neurological diseases, other neurological diseases and healthy donors
served as control groups in this part of the study. The results in table II show that all groups
show similar distribution patterns (median concentrations (IQR) HC 34 (29-67), OND 37 (24-55),
OIND 40 (26-57), MS 33 (21-38) pg/ml). In CSF IL-12 was barely detectable, and as a
result no significant differences were found between MS patients and control groups (median
concentrations (IQR) HC 0 (0-7), OND 0 (0-8), OIND 0 (0-14), MS 1 (0-13) pg/ml). As a
confirmation of the validity of our assay, occasionally a patient with an inflammatory neurological disease (neuroborreliosis, meningitis) was found that showed high or very high levels of IL-12 in CSF (53 and 444 pg/ml, respectively, data not shown). In none of the groups a correlation was found between IL-12 concentrations in serum and in CSF (not shown).

To investigate whether monocyte activation in MS patients in general may be disturbed, we also measured soluble CD14 levels in the groups mentioned above. As can be seen in table III, the concentration of sCD14 in CSF was lower than in serum. Similar to what was found for IL-12, sCD14 levels in serum (median concentrations (IQR) HC 164 (111-203), OND 143 (118-169), OIND 144 (125-162), MS 130 (110-144) U/ml) as well as in CSF (median concentrations (IQR) HC 12 (8-16), OND 11 (9-17), OIND 15 (6-3), MS 11 (8-19) U/ml) did not differ significantly between the groups.

Table IV. Correlations between concentrations of IL-12 and sCD14 in serum and in cerebrospinal fluid (CSF)

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>HC</th>
<th>OND</th>
<th>OIND</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>serum</td>
<td>.24*</td>
<td>.43</td>
<td>.37*</td>
<td>-.05</td>
<td>.00</td>
</tr>
<tr>
<td>CSF</td>
<td>.19*</td>
<td>-.13</td>
<td>.06</td>
<td>.41*</td>
<td>.41*</td>
</tr>
</tbody>
</table>

* p<.05

Also for sCD14, in none of the groups serum levels and levels in CSF were significantly correlated. Finally, we analyzed whether IL-12 concentration were correlated to sCD14 concentration both in serum and in CSF. Table IV shows mostly low but in some cases significant correlation coefficients.

Discussion
In this study no evidence was found that in MS an aberrant monokine secretion pattern might be involved in directing T helper cells into the Th1 differentiation pathway. While in vitro IL-8 secretion did not differ between MS patients and healthy controls, secretion of other, Th2-associated cytokines such as IL-6 and IL-10 were lower in MS patients. However, also the pro-inflammatory cytokines TNF-α and IL-12 (p40) were secreted in significantly lower amounts by peripheral blood monocytes from MS patients compared with healthy controls. We contemplated that one possible explanation for this phenomenon could be that monocytes from MS patients have been primed in vivo to produce IL-12 and possibly other cytokines,
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and therefore are refractory to stimulation in vitro. As a consequence, MS patients in this situation should have higher levels of IL-12 in serum. However, in our study no differences were found in this parameter when comparing MS patients with patients suffering from OIND or OND and healthy controls. In addition, sCD14 levels, which we used as a general marker of monocyte activation, also did not differ between these groups. The low IL-12 levels in MS seem to be in contrast with findings of others, who showed higher serum levels of the heterodimeric form of IL-12 in chronic progressive (CPMS) patients than in patients with OND and in healthy controls. Although these authors measured the bioactive form of IL-12, which may give a more valid indication for the functional consequences, it should be stressed that heterodimeric IL-12 is hardly detectable in serum, and hence may be not a very reliable parameter.

Other possible explanations for our finding that MS patients secrete lower amounts of all cytokines studied except for IL-8 could be related to the culture conditions. It has been demonstrated that human monocytes/macrophages express constitutively nitrogen oxide synthase (cNOS) and upon appropriate activation inducible (i) NOS\textsuperscript{20}. The inflammatory mediator nitrogen oxide (NO) produced by these cells enhances IL-8 secretion while not affecting IL-6 secretion\textsuperscript{5}. Several investigators have presented evidence for enhanced NO production in brains of MS patients\textsuperscript{2,4,9,12,17}. Therefore, we investigated (in cooperation with dr. C.A. de Groot, dept. of Pathology, Free University Hospital Amsterdam, The Netherlands) in a pilot study whether MS patients are more prone to produce NO in our system compared to healthy controls. In agreement with what was found by others, in none of the individuals NO could be measured in culture supernatant, indicating that NO probably does not play a role.

In one other study it has been shown that, irrespective of disease state, monocytes from MS patients secreted the same amount of TNF-\textgreek{c} and interleukin (IL)-1\textgreek{b} upon stimulation with LPS compared to controls\textsuperscript{27}. However, using a similar system, Imamura and coworkers\textsuperscript{23} demonstrated that secretion of these cytokines, and also IL-6 is elevated in MS patients. For patients with active disease the difference was significant compared to control groups. In contrast to the studies mentioned above, we used a system in which monocytes were not isolated, and culture media were kept free of endotoxins, to prevent preactivation of monocytes. This could be confirmed by the fact that little or no spontaneous secretion of monokines was seen in our study (data not shown), while in the study of Rudick and coworkers\textsuperscript{27} considerable amounts of cytokines were spontaneously secreted. Irrespective of these differing results, bacterial lipo-polysaccharides can not be considered to be physiological stimuli in case of autoimmune diseases. Using activated CD\textgreek{d}{4}pos T cells as a stimulus it was shown that in a CD40 ligand-dependent manner monocytes from progressive but not from relapsing-remitting MS patients secrete more IL-12 than controls\textsuperscript{1}.
In other autoimmune diseases, similar observations of *in vitro* cytokine secretion patterns of monocytes have been reported. For instance, systemic lupus erythematosus (SLE) patients also secreted less IL-6 than healthy controls, while secretion of TNF-α did not differ between these groups. The same results, and also a low secretion of IL-12, have been found in rheumatoid arthritis (RA) patients (Van der Pouw Kraan et al., unpublished observations). In septic patients proinflammatory cytokine production (IL-1β, IL-6 and TNF-α) in general seems to be downregulated. Also in HIV-1 infected individuals, that apparently have disturbed function of TH1 type cells, a low secretion of IL-12 by monocytes was demonstrated, but probably this is not specific for IL-12. In conclusion, it seems that systemic defects in immune function as were shown for all of the above mentioned diseases are also reflected in disturbed secretion of monokines in systems using bacterial lipo-polysaccharides, irrespective of whether the disease is generally associated with either a TH1 type response or a TH2 type response. Several studies in which other markers of monocyte function were used, underline this conclusion. Decreased expression of HLA-DR was found on monocytes from MS patients, which was associated with impaired suppressor cell function. However, this seemed to be related to disease activity. A lowered cytotoxic function of monocytes from MS patients was related to higher levels of intracellular cAMP. Because, as mentioned above, considerable evidence has been presented that APCs in CNS of MS patients produce high amounts of inflammatory mediators, decreased *in vitro* peripheral blood monocyte function might be caused by leakage of these factors into the circulation.

In summary, although disturbances were documented in the function of circulating accessory cells, the results of our study do not support the hypothesis that enhanced production of IL-12 by APCs might be responsible for a preferential differentiation into the TH1 pathway in MS. The mechanisms that might be involved in the lower capacity of MS monocytes to produce IL-12 *in vitro* remain to be further investigated.

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References


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