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Lymphomononuclear cells from multiple sclerosis patients spontaneously produce high levels of oncostatin M, tumor necrosis factors α and β, and interferon γ

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Proinflammatory cytokines are deemed to play a pivotal role in the pathogenesis of multiple sclerosis (MS). They provide signals for T-cell activation and inflammatory cell recruitment in the brain and might directly alter neuroglial and neuronal cell survival and function. We found that peripheral blood mononuclear cells (PBMCs) from MS patients spontaneously produce high levels of TNFα, TNFβ, IFNγ, and oncostatin M (oncM), a proinflammatory cytokine acting on cells of neural, vascular, hematopoietic, and lymphoid origin. Spontaneous production of these cytokines was significantly higher (p<0.01) in PBMC short-term culture supernatants from MS patients than in blood donors (HC). On average, lectin-induced production of these cytokines by PBMC was higher in MS patients than in HC, significantly so only for TNFα (p=0.013). Determination of TNFα, TNFβ, IFNγ, and oncM in corresponding sera showed that, on average, oncM levels were higher in MS patients than in HC, though the results were not statistically significant, whereas levels of TNFα, TNFβ, and IFNγ were below the assay threshold in most patients. The finding that MS PBMCs are primed in vivo to produce and release high levels of proinflammatory cytokines suggests the presence of a basal activation of the immune system which, in turn, may play a role in the complex circuitry of molecular and cellular interactions responsible for neurologic damage in MS.
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Introduction

Multiple sclerosis (MS) is a human chronic inflammatory demyelinating disease that often leads to progressive physical impairment.[1,2] Pathologically, MS is characterized by multifocal inflammatory lesions constituted by activated T-lymphocytes and macrophages throughout the central nervous system (CNS).[3,4] At advanced stages of disease progression, demyelinating plaques and oligodendrocyte loss become the dominant feature.[4,5] Although the cause of MS is unknown, the pathogenesis is deemed to be autoimmune, mediated by autoreactive, HLA class II-restricted CD4 T-cells.[3,6] Lesion formation requires CNS influx of nonspecific inflammatory cells[7,8] and is achieved by either direct or indirect (activation of accessory glial cells) mechanisms, which rely upon the production of soluble proinflammatory mediators such as IFNγ, TNFα, and TNFβ and different chemokines by antigen-specific $CD4^+$ cells.[7,9–12] This leads to an increased production of inflammatory mediators in the parenchyma which, in turn, appear responsible for demyelination and the resulting disturbance of neural transmission.[13–15] Recent lines of evidence also suggest that diffusable factors present in cerebrospinal fluids of MS patients can promote neuronal in addition to oligodendrocyte damage.[16,17]

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In addition to TNFα, TNFβ, and IFNγ, several cytokines are produced by lymphomononuclear cells upon activation and may play a pathogenetic role in MS by both supporting cell recruitment and immune-mediated inflammation within the brain and directly altering neuroglial and neuronal cell survival and function.

Oncostatin M (oncM), a cytokine whose pleiotropic activities suggest a role in inflammatory processes,[25,31] and extrathymic T-cell development[32] can exert multiple activities in the CNS including immunoregulatory functions on brain endothelial cells[21] and damaging effects on nerve cells.[18,19] Since oncM is potently induced upon stimulation of lymphomononuclear cells,[19] it might be released at inflammatory sites and may thus contribute to MS lesion formation by both direct and indirect mechanisms.

In the present study, the levels of both spontaneous and activation-dependent production of oncM, TNFα, TNFβ, and IFNγ were examined in lymphomononuclear cells short-term culture supernatants from MS patients in conjunction with the assessment of the same cytokines in the corresponding serum samples.

Patients and methods

Patients

Twelve clinically definite MS patients[22] and 10 age- and sex-matched, healthy individuals were investigated. The

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course was relapsing–remitting in all; median age was 27 years (range 17–43). The median elapsed time from MS diagnosis was 4.6 years (range 2–9). Nine individuals were treated with interferon beta 1a (IFNβ1a, Rebif; Serono Pharma, Rome, Italy; 22 μg, three times weekly) with a treatment duration ranging from 6 to 18 months, mean 9 months. Mean disease duration was 7 years, range 3–11 years; mean EDSS was 3.5, range 2.0–4.5. Two of the treated patients had clinical signs and symptoms of a relapse at the time of peripheral blood collection. Three patients were untreated and stable, with a mean disease duration of 6 years and a mean EDSS of 2.0. In addition to immunological, hematological, and biochemical assessment, all patients underwent neurologic evaluation, independently from clinical signs and symptoms of disease activity. Since MS patients may have persistent MRI activity even in the absence of clinical manifestations, patient's stability has been defined according to the clinical status which was assessed both at the time of blood collection and during the following 60 days of clinical observation. Ten blood donors served as healthy controls (HC).

Peripheral blood mononuclear cells (PBMCs) short-term cultures from MS patients and normal blood donors

To determine the levels of cytokines produced by MS lymphomonocytes, PBMCs from MS patients or HC were isolated from freshly collected blood samples and cultured as previously described.[19] In MS patients as well as in HC, blood was collected in endotoxin-free tubes containing sodium citrate as anti-coagulant. PBMCs were isolated from whole blood by Ficoll–Hypaque gradient centrifugation, counted and cultured at $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 atmosphere at 37°C. The same lot of FBS was used in all experiments.

Conditioned media (CM) preparation

CM were prepared from either unstimulated or activated PBMC from MS patients and HC, as previously described.[19] Briefly, 1×10^6 cells were cultured in the absence of stimuli to verify cytokine spontaneous production. Alternatively, PBMCs were activated by incubation with PHA (1 μg/ml). CM were collected after 72 h from unstimulated cells and after 48 h from PHA-activated PBMC, centrifuged at 3000 rpm at 4°C for 30 min, and tested for cytokines. To avoid loss of cytokines, all samples were handled in plasticware precoated with 0.1% BSA in PBS.

Serum samples from MS patients and HC

To compare the levels of oncM, TNFα, TNFβ, and INFγ in PBMC supernatants to their concentration in sera, these cytokines were also assessed by enzyme-linked immunosorbent assay (ELISA) in serum samples obtained at the time of PBMC specimen collection.

Measurements of oncM, IFNγ TNFα, and TNFβ in PBMC supernatants and serum samples

PBMC supernatants or sera from MS individuals or HC were collected as described above. CM and sera were then tested for oncM, IFNγ, TNFα, and TNFβ content by ELISA according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The lower limits of detection of the different ELISA assays were as follows: oncM, range 15.6–1000 pg/ml; modified to reach a detection of 1.9 pg/ml (used for CM and sera). TNFα, range 15.6–1000 pg/ml (used for CM); high sensitive TNFα kit range 0.5–32 pg/ml (used for sera). TNFβ, range 31.2–2000 pg/ml; modified to reach a detection of 1.9 pg/ml (used for CM and sera). IFNγ, range 15.6–1000 pg/ml; modified to reach a detection of 1.9 pg/ml (used for CM and sera).

Statistics

All statistical evaluations were performed by ANOVA. Statistical significance level was set at 0.05.

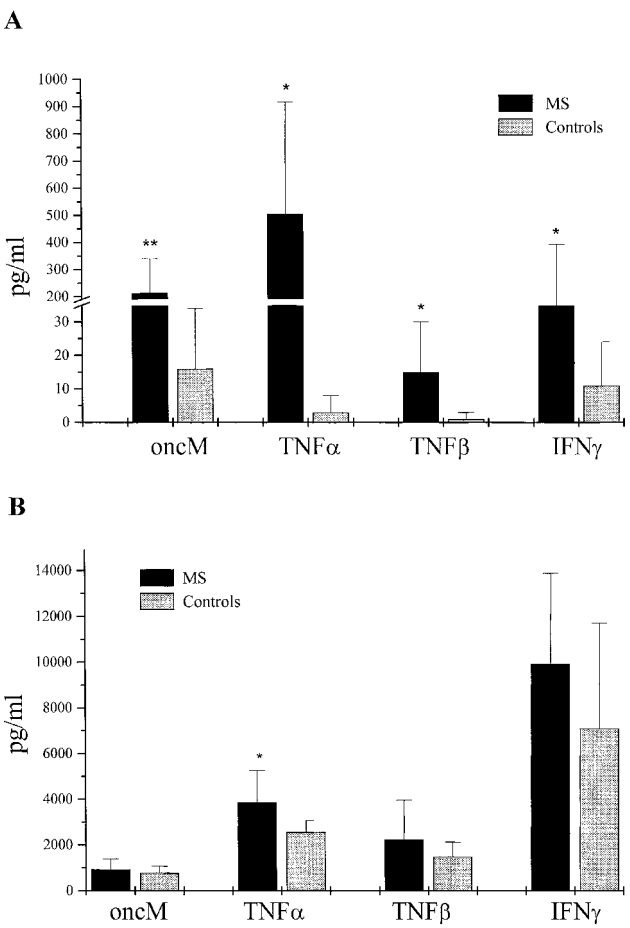

Figure 1 *PBMCs from MS patients spontaneously produce high levels of oncM, TNFα, TNFβ, and IFNγ. Panel A: Unstimulated PBMCs from MS patients produce significantly higher levels of oncM ($p<0.001$), TNFα ($p=0.001$), TNFβ ($p=0.008$), and IFNγ ($p=0.009$) as compared to HC. The asterisk marks statistically significant results (**$p<0.001$; *$p<0.01$). Panel B: Upon PHA stimulation, the production of oncM, TNFα, TNFβ, and INFγ by PBMC from MS patients reached levels higher than those determined in controls. However, the difference was statistically significant only for TNFα ($p=0.01$). The asterisk marks statistically significant results (*$p<0.01$)*

Results

PBMC from MS patients spontaneously produce high levels of oncM, TNFα, TNFβ, and IFNγ

The results of these experiments, summarized in Figure 1A and B and detailed in Table 1, indicate that unstimulated, short-term cultured PBMCs from all MS patients investigated produce significantly higher levels of oncM ($p<0.001$), TNFα ($p=0.001$), TNFβ ($p=0.008$), and IFNγ ($p=0.009$) when compared to HC. In our small group of patients, this appears independent from clinical status (relapse or remission), as well as from the administration of IFNβ therapy (Table 1 and Figure 1A). Upon PHA-induced activation, the production of oncM, TNFα, TNFβ, and INFγ by PBMC reached, on average, levels higher than those determined in HC. However, the difference was statistically significant only for TNFα ($p=0.01$) (Table 1 and Figure 1B).

Serum samples from MS patients contain relatively high concentrations of oncM, while serum TNFα, TNFβ, and INFγ are generally below the assay detection limits

Concentrations of oncM in sera from all MS patients examined (Table 2) showed levels higher than those found in HC. Again, this was independent from the clinical status, as well as from the administration of IFNβ therapy (Table 2). On the other hand, serum TNFα, TNFβ, and INFγ were below the assay detection limits with most patients' samples examined, although they were generally within measurable limits in normal sera (Table 2). Thus, the simultaneous determination of these cytokines in sera and unstimulated PBMC supernatants provided further evidence that their concentration in serum samples may not reflect the levels of production by PBMC.

Table 1 Spontaneous and PHA-induced production of oncM, TNFα,TNFβ, and IFNγ by PBMC from MS patients[a]

	oncM		*TNFα*		*TNFβ*		*IFNγ*	
Patients	*CTR*	*PHA*	*CTR*	*PHA*	*CTR*	*PHA*	*CTR*	*PHA*
1[b]	407.4	941.2	880.5	3522	8.3	572.4	82.6	8645
2[b]	129.5	578	352.3	4272	9	736.4	296.3	15057
3[b]	65.2	752.8	35.8	3554	29	3794	71.1	4963
4[b,c]	350.7	1020.4	736.5	2834	2.8	952.4	308.3	9005
5[b]	154.7	823.6	648.5	3074	16.6	856.4	656.5	15701
6[b,c]	173.2	974	155.1	2514	36.8	1457.2	452.3	12032
7[b]	160.2	1843	163.1	4262.6	32.3	2946	74.6	10260
8[d]	161.6	493.2	436.3	3122	0	1785.2	9.9	6085
9[b]	109.6	572.4	704.5	3282	0	1793.2	5.6	6805
10[b]	102.8	2053.2	30.5	7506.1	38.8	6628.4	185.1	11433
11[d]	336.4	755.6	1450.4	5429.2	5.5	2962	141.1	14724
12[d]	414.3	684.4	478.3	3026	2.8	2386	0.6	4503
Mean	214	941	506	3867	15	2239	190	9934
SD	126	463	411	1393	15	1715	204	3937
HC	16	795	3	2559	1	1495	6	7100
SD	18	275	5	517	2	634	13	4605

Results are expressed as picograms per milliliters. CTR=unstimulated cultures; PHA=PHA-stimulated cultures. [a]Short-term cultured, unstimulated PBMCs from all MS patients investigated produce significantly higher levels of oncM ($p<0.001$), TNFα ($p=0.001$), TNFβ ($p=0.008$), and IFNγ ($p=0.009$) as compared to HC. This appears independent from clinical status (relapse or remission), as well as from the administration of IFNβ therapy. Upon PHA stimulation, the production of oncM, TNFα, TNFβ, and INFγ reached, on average, levels higher than those determined in controls. However, the difference was statistically significant only for TNFα ($p=0.01$). [b]IFNβ-1a-treated. [c]Relapse at time of blood collection. [d]No therapy.

Table 2 Serum samples from MS patients contain detectable oncM concentrations, while serum TNFα, TNFβ, and INFγ are generally below the assay detection limits[a]

Patients	*oncM*	*TNFα*	*TNFβ*	*IFNγ*
1[b]	8	1.7	0	0.6
2[b]	5.2	0	n.d.	0
3[b]	n.d.	n.d.	n.d.	n.d.
4[b,c]	3.6	3.5	0	0
5[b]	3.6	0	0	0
6[b,c]	3.6	0	0	0
7[b]	n.d.	n.d.	n.d.	n.d.
8[d]	6.8	0	0	1.9
9[b]	15.2	1.7	0	0
10[b]	n.d.	n.d.	n.d.	n.d.
11[d]	12	0	0	0
12[d]	15.6	0	0	0
Mean	8.2	0.8	0	0.3
SD	4.9	1.3	0	0.6
HC	5	1.9	6.9	4.2
SD	5	2.1	19	7.4

Results are expressed as picograms per milliliter. n.d.=not done. [a]Concentrations of oncM were detected in sera from all MS patients examined, at levels higher than those found in normals, though the difference was not statistically significant. Serum TNFα, TNFβ, and INFγ were below the assay detection limits with most samples examined. This was independent from the clinical status, as well as from the administration of IFNβ (Rebif, 22 μg) therapy. [b]INFβ-1a-treated. [c]Relapse at time of blood collection. [d]No therapy.

Discussion

Our results indicate that PBMCs from MS patients are primed *in vivo* and readily release *in vitro* relatively high levels of proinflammatory cytokines, i.e., TNFα, TNFβ, IFNγ, and oncM. The enhanced cytokine production by unstimulated PBMC, which is consistent with previous observations on IL-4 and IFNγ production by CD4 and CD8 T-cells from MS patients,[23] reveals a persistent activation of the immune system.[24] Upon activation, the levels of oncM, TNFα, TNFβ, and IFNγ increased in MS PBMC, reaching, on average, concentrations higher than those observed in HC, although only for TNFα we found statistically significant differences. Determination of the same cytokines in serum samples gave contrasting results. In fact, only oncM was consistently detected in sera from MS patients, whereas TNFα, TNFβ, and IFNγ, which were generally within measurable limits in normal sera, were below the assay detection limits in most MS patients. Although these data were not statistically significant and thus remain an isolate – though intriguing – observation, they suggest that the levels of

inflammatory cytokines detectable in the blood stream or produced by PBMC upon polyclonal activation might not reflect the levels of cytokines produced by lymphomonocytes and have the potential to act within sites of inflammation. These data suggest the presence of a basal activation of the immune system in MS, which can go undetected by the analysis of the same cytokines in serum samples or activated PBMC supernatants.

OncM is a pleiotropic cytokine that belongs to a family of structurally and functionally related factors such as CNTF, LIF, IL-6, IL-11, and G-CSF that utilizes the gp130 or gp130-related receptor subunits on target cells.[25–27] These cytokines exhibit differential effects on a variety of cell types including cells of neural, hematopoietic, lymphopoietic, and vascular origin.[27–30] OncM is potently induced by functional activation of lymphocytes and monocyte–macrophages[19,31] or upon viral infection;[19,28] thus, it represents a component of the cytokine milieu at sites of lymphocyte and monocyte infiltration in immune-mediated inflammation.[30,31] In mice transgenic for the oncM gene, or injected with the recombinant protein, extrathymic T-cell differentiation is enhanced in lymph nodes and it generates a broad TCR Vβ repertoire of functional T-cells,[32] indicating that oncM may contribute to thymus-independent T-cell lymphopoiesis. The exact role of oncM in inflammatory processes is still unclear. By regulating endothelial cells functions and directly inducing monocyte/macrophages chemoattractant proteins[33,34] and integrins expression,[35] oncM is likely to be involved in events controlling leukocyte–endothelial cell interactions and, as a consequence, lymphoid/monocytic cell infiltration at inflammatory sites. This may favour the influx of activated immune cells into the CNS in MS. Recent immunopathological findings suggest that oncM might act on human endothelial cells favouring blood–brain barrier breakdown.[21] In fact, upon oncM binding, human endothelial cells express ICAM-1, but not VCAM-1, and up-regulate IL-6 and MCP-1, but not IL-8.[21] Indeed, oncM can either positively or negatively regulate the production and/or the biological effects of different proinflammatory cytokines such as IL-1, IL-8, GM-CSF, G-CSF, and IL-6.[31,33,36,37] By acting in concert with TNFα and TGFβ, oncM can increase the effects of these cytokines on target cells.[19,20] On the other hand, studies on murine models indicate that oncM inhibits LPS-induced TNFα production and may play a part in suppressing EAE.[20] This confirms the pleiotropic properties of the cytokine and suggests that, by playing multiple roles in regulating the inflammatory response, oncM may participate in the circuitry of interactions capable of mediating neurologic damage in MS. The latter notion is reinforced by the observation that in transgenic animals, oncM appears detrimental to normal brain development[18] and that native oncM can directly alter neuronal development and survival by inducing apoptotic cell death.[19,29] Thus, a persistent production of oncM in the CNS may exert damaging effects on neural tissues. This further emphasizes the concept that the inappropriate production of inflammatory cytokines such as TNFα, TNFβ, INFγ, and oncM, which have the potential to exert both direct and indirect CNS injury, can play a major role in the pathogenesis of MS by acting through different mechanisms on multiple cell types. In addition, they can potentially cooperate, thus amplifying their effects on responsive targets.

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