

Theiler's virus induces the MIP-2 chemokine (CXCL2) in astrocytes from genetically susceptible but not from resistant mouse strains

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Abstract

The murine encephalomyelitis virus of Theiler (TMEV) induces demyelination in susceptible strains of mice by a CD4⁺ Th1 T cell mediated immunopathologic process. We focused on the production of one chemokine, the macrophage inflammatory protein-2 (MIP-2 or CXCL2), by cultured mouse astrocytes infected with the BeAn strain of TMEV. Analysis of a murine genome DNA hybridized with cRNA from mock- and TMEV-infected astrocytes, revealed up-regulation of three sequences encoding MIP-2. Northern blot analysis indicated increased MIP-2 mRNA expression. Levels of MIP-2 in the supernatants of infected cells as detected by ELISA, varied directly with the multiplicity of infection used. This secreted CXCL2 was biologically active inducing chemoattraction of neutrophils but not of lymphocytes. CXCL2 was specifically induced by TMEV infection, since induction was inhibited by anti TMEV antibodies. The inflammatory cytokines, IL-1 α and TNF- α , which are also induced in astrocytes by TMEV, were very potent inducers of CXCL2. Nevertheless, both mechanisms of induction follows different pathways as antibodies to both cytokines fails to inhibit TMEV-induced CXCL2 up-regulation. Sera from TMEV-infected SJL/J mice with chronic demyelination, but not from BALB/c TMEV-resistant mice, revealed CXCL2 at the peak of clinical disease. Our main novel finding is the strain-dependent differences in CXCL2 expression both in vitro and in vivo. This suggest an role for this chemokine in attracting immune cells within the CNS, which in turn, might trigger demyelination in this experimental model of MS.

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1. Introduction

Viral infections stimulate the molecular machinery of an eukaryotic cell within the first minutes to hours of infection. This early response involves an initial wave of change in cellular gene expression that does not require any new protein synthesis. After virus immediate-early proteins are translated, some act as transcriptional regulators and trigger a secondary wave of gene expression changes that control further processes such as synthesis of cytokines and chemokines [1].

Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus that persistently infects the murine central nervous system (CNS) [2]. After intracerebral (i.c.) inoculation of the low-neurovirulence BeAn strain in susceptible strains of mice, myelin breakdown reminiscent of human multiple sclerosis (MS), takes place. This demyelination is primarily mediated by the immune system rather than as a consequence of viral infection of oligodendrocytes, the myelin forming cells [3–7]. In the model proposed to explain the immune response that destroy myelin in TMEV infection [5] chemokine production precedes infiltration of mononuclear cells within the CNS.

Chemokines are a group of 6–14 kDa, heparin binding cytokines that attract inflammatory response cells and act

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in the regulation of leukocyte trafficking. Chemokines exert its biological role via a group of transmembrane, G protein coupled-receptors [8,9]. The mouse macrophage inflammatory protein-2 or MIP-2 was originally identified as a 6 kDa protein secreted by an LPS-stimulated mouse macrophage cell line [10]. A cDNA clone encoding the protein was isolated and characterized [11]. Based on its protein and DNA sequences, mouse MIP-2 (CXC chemokine ligand 2 or CXCL2) was classified as a member of the α (CXC; C = cysteine) chemokine family, in which both conserved cysteines are separated by any other amino acid [12]. The protein sequence of mouse MIP-2 shares 63% identity to that of mouse KC, another mouse α chemokine, while neither shares significant protein sequence homology with human IL-8, a potent CXC chemokine chemoattractant for neutrophils that has not been identified in mice. MIP-2 and KC are considered functional homologues of human IL-8 in mice [12]. A putative mouse homolog of the human IL-8 receptor β (IL-8 R β) has also been cloned, showing 71% identity with it. Both mouse KC and MIP-2 bind mouse IL-8 R β with high affinity [13]. Like human IL-8, mouse MIP-2 exhibits a potent neutrophil chemotactic activity and may be a key mediator of recruitment and influx of immune cells in inflammation and infection [14,15].

Chemokines have been associated with the trafficking of immune cells in connection with inflammatory cell recruitment in different diseases [16]. Those diseases includes CNS demyelinating diseases as MS, but little is known about the actual neuropathological roles of chemokines. The rationale of this article is based on the demonstrated fact that astrocytes are an early component of the immune response to TMEV [17–20]. The robust expression of MIP-2 mRNA and biologically active protein by astrocytes from TMEV-susceptible mice, suggest a crucial role for this glial population in TMEV-induced demyelination.

2. Materials and methods

2.1. Astrocyte cultures

Astrocyte cultures were prepared by mechanical dissociation of the cerebral cortex from newborn SJL/J and BALB/cCum mice [21]. The cortex was isolated under a dissecting microscope and cleaned of choroid plexus and meninges. Cell suspensions were filtered through 135- μ m pore size mesh into Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) and penicillin–streptomycin (Gibco BRL, Paisley, Scotland). After centrifugation, cells were filtered through a 40 μ m nylon cell strainer (Falcon-Becton–Dickinson, Le Pont De Claix, France) and cultured in 75-cm² tissue culture flasks (Costar, Cambridge, MA) at 37 °C. The medium was changed after 4 days in culture and subsequently 2 time a week for the entire culture period. Cultures were enriched for astrocytes by the removal of less adherent microglia and oligodendrocytes by shaking overnight at 37 °C at 250 rpm in a table top shaker (Thermo Forma, Marietta, OH). Cellular con-

fluence was observed 10 days after plating, producing around 1×10^7 cells per flask and showing a polygonal flat morphology. A mean of 98% astrocytes was confirmed by indirect immunofluorescence staining of methanol-fixed cultures using rabbit anti-glial fibrillar acidic protein (GFAP) antiserum (Dakopatts, Glostrup, Denmark). The lack of noticeable mature oligodendrocytes or microglial/macrophage cells was determined using a guinea pig anti-myelin basic protein (MBP) antiserum prepared as described elsewhere [22] and monoclonal anti-Mac-1 antibody (Serotec, Oxford, UK). Secondary fluorescein-labeled antibodies were purchased from Sigma Chemical Co (St. Louis, MO).

2.2. Viruses and infection

For these studies, a strain of TMEV isolated in 1957 from a feral mouse in Belem, Brazil, called BeAn 8386, was used. Baby hamster kidney cells (BHK-21) were grown at 37 °C in DMEM containing 10% FCS and penicillin–streptomycin. BHK-21 cell cultures were infected for 48 h at 33 °C, sonicated and centrifuged in the cold to remove cell debris. Purified astrocytes in 75-cm² tissue culture flasks were infected with BeAn virus at several multiplicities of infection (m.o.i) in a volume of 10 ml of DMEM containing 0.1% BSA, at room temperature for 1 h. After infection, cells were washed, 10 ml of DMEM plus 10% FCS was added and flasks were incubated at 37 °C for different periods of time. No changes in cell viability nor cytopathic effect after infection with BeAn virus, even at a m.o.i of 10, were detected [21]. Cells used for mock infections were incubated with a virus-free BHK-21 cell lysate. For UV-light virus inactivation, viral samples were irradiated at 560 μ W/cm² for 15 min. These conditions produced inactivated virus stocks devoid of infectious virus, as determined by plaque assay (<10 PFU/ml).

2.3. cRNA target preparation and hybridization

Three different cultures replicates of SJL/J astrocytes infected at a m.o.i of 10 were harvested 24 h post-infection, washed with phosphate-buffered saline, and total RNA was isolated by using the TRIzol reagent (Gibco BRL), followed by a further purification with RNeasy Mini Kit (Qiagen, Valencia, CA). Ten micrograms of RNA from each culture were converted to cDNA by using the kit SuperScript Choice System (Gibco BRL). Second-strand synthesis was performed using T4 DNA polymerase, and cDNA was isolated by phenol–chloroform extraction. Isolated cDNA was transcribed using the BioArray High Yield RNA transcript Labeling Kit (Enzo Biochem, New York, NY) with biotin-labeled UTP and CTP to produce biotin-labeled cRNA. Labeled cRNA was isolated using the RNeasy Mini Kit and fragmented in 100 mM potassium acetate–30 mM magnesium acetate–40 mM Tris-acetate (pH 8.1) for 30 min at 94 °C. Hybridization performance was analyzed using Test 2 arrays (Affymetrix, Santa Clara, CA)

and spike and housekeeping controls. Target cRNA was hybridized to the murine genome U74v2 microarray (Affymetrix) according to manufacturer's protocols.

2.4. Data analysis

Each gene on the U74v2 array is represented by 20 different 25-base cDNA oligonucleotides complementary to a cRNA target transcript (perfect match). As a hybridization specificity control, an oligonucleotide containing a single base substitution corresponding to each perfect-match cDNA oligonucleotide (mismatch) is represented on the array. The combination of perfect-match and mismatch cDNA oligonucleotides for each gene is termed a probe set. By using Affymetrix-defined absolute mathematical algorithms describing perfect-match and mismatch intensities, each gene was defined as absent or present and assigned a value. Binding intensity values were scaled to evaluate differential expression following TMEV virus infection. Based on Affymetrix-defined comparison mathematical algorithms, a virus-induced transcript was classified as unchanged, marginally increased, marginally decreased, increased or decreased.

2.5. Northern blot analysis

Confluent monolayers of purified astrocytes were infected with TMEV as previously stated. Total RNA was purified using the RNeasy Mini Purification Kit (Qiagen). Ten micrograms per lane was electrophoresed on a formaldehyde-agarose gel and transferred to nylon membranes (Zeta-Probe, Bio-Rad Laboratories, Hercules, CA) and probed in Rapid-hyb buffer (Amersham Biosciences). The probe used was a cDNA fragment specific for MIP-2 [23]. The probe was labeled with (γ - ^{32}P) ATP (Amersham Biosciences) using Ready-To-Go T4 polynucleotide kinase (Amersham Pharmacia Biotech). Unincorporated radiolabel was removed on Micro-Spin G-50 columns (Amersham Pharmacia Biotech). Blots were exposed to Kodak X-OMAT film with an intensifying screen at -80°C . Hybridization to a human β -actin probe (Clontech, Palo Alto, CA) served as an internal control for loading and was labeled with (^{32}P) dCTP using the random-prime method (Boehringer Mannheim, Germany). Unincorporated radiolabel was removed on S-300 Micro-Spin columns.

2.6. Chemokine assay

MIP-2 concentrations in cell culture supernatants and serum were determined using the Quantiquine^R M mouse MIP-2 ELISA (R&D Systems, Minneapolis, MN). This kit has a sensitivity of 1.5 pg/ml, and its cross-reactivity with the mouse chemokine KC is only 0.03%.

2.7. Migration assays

Peripheral blood from anesthetized SJL/J mice was collected into EDTA-containing tubes by retroorbital punc-

ture. After red blood cell lysis in 150 mM Tris, 20 mM ammonium chloride, pH 7.2, cells were washed twice in medium. Cell viability was assessed by trypan blue exclusion and was >98%. Chemotactic assays were conducted in the upper chamber of Transwell chambers of 3- μm pore size membranes (Costar, Cambridge, MA). Briefly, each upper chamber were loaded with 25,000 cells in a total volume of 500 μl of DMEM plus 0.5% BSA medium. Six hundred microliters of the different dilutions of TMEV-infected astrocyte cultures medium was added to the lower chamber and transwells were incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for 3 h. Cells were collected from the bottom compartment, centrifuged, resuspended and quantified using a flow cytometer (Coulter EPICS-XL). As controls the lower chamber contained 100 ng/ml of stromal cell-derived factor-1 α (SDF-1 α) (Calbiochem, La Jolla, CA), a potent chemoattractant routinely used as positive control, or DMEM plus 0.5% BSA medium. Cell characterization was assessed by flow cytometry using staining with anti-CD3 antibody for lymphocyte staining and anti CD11b for neutrophil staining, purchased from BD Biosciences-Pharmingen, San Diego, CA. An irrelevant P3X63 mAb was used for negative control staining.

2.8. Viral neutralization by antibodies

Anti-TMEV antiserum was produced in New Zealand White rabbits by subcutaneous and intramuscular injections at multiple sites. Four injections containing 300 μg of CsCl gradient-purified BeAn virus emulsified with complete Freund's adjuvant (Difco) were given at 2-week intervals. Blood was obtained 10 days after the last booster and antiserum was frozen at -20°C until used. Specificity of the antibody response was checked by Western immunoblotting using purified TMEV virion proteins separated by SDS-PAGE and shows that the antiserum contains antibodies against VP1 and VP2 capsid proteins. BeAn virus samples were incubated with increasing dilutions of antisera for 30 min at 37°C . Thereafter, the incubation mixtures were used for infection of astrocyte cultures. Antiserum against CsCl-purified adenovirus Ad. β Gal was used as a negative control for neutralization [24].

2.9. Proinflammatory cytokine treatments

Cells were treated for 48 h with 10 ng/ml of the following mouse recombinant proinflammatory cytokines: mouse recombinant IL-1 α purchased from Genzyme, Cambridge, MA; rIFN- γ of murine origin from Holland Biotechnology, Leiden, The Netherlands; recombinant murine tumor necrosis factor- α (TNF- α) from Innogenetics, Antwerp, Belgium and rIL-6 from Boehringer Mannheim, Germany. To test antibodies against IL-1 α and TNF- α , astrocytes were trypsinized from 75 cm^2 flasks to 6-well multiwell plates (Costar) and infected for 1 h at room temperature with TMEV at a m.o.i of 10. After washing, cultures were incubated for 24 or 48 h in 2 ml of complete medium per

well containing 1, 10, or 100 μg of either neutralizing IgG fraction of goat antiserum against mouse Interleukin-1 α or goat affinity isolated antibodies against TNF- α , both from Sigma. After this periods of time, centrifugated supernatants were tested for the presence of MIP-2 by ELISA.

2.10. Intracerebral inoculations of mice

Six-week-old SJL/J and BALB/cCum mice were purchased from The Jackson Laboratory, Bar Harbor, ME and maintained on standard feed and water ad libitum at the Instituto Cajal. Mice were anesthetized with fluorothane and injections made using a 25- μl Hamilton syringe in the right cerebral hemisphere. Twenty microliters of a suspension of BeAn virus (2×10^6 PFU) were injected per animal. The blood was obtained at different times after inoculation and sera were frozen at -20°C until used. At the same time, the number of animals showing clinical signs of demyelination was also scored [4].

3. Results

3.1. TMEV-induced alterations of gene expression

Previous studies have demonstrated, using RNase protection and reverse transcriptase coupled PCR assays, that several chemokine genes are up-regulated in mouse astrocytes after TMEV infection [17,18]. In this work we have used the pioneer DNA hybridization microchip array technique and, despite the possible presence of other chemokines, focused on the most prominent MIP-2. The novel aspects of our work are the study of the mouse strain differences but also the ELISA characterization of the protein product and the demonstration of its biological activity by chemotaxis.

Our Affimetrix GeneChip SE437 and SE438 microarray mouse gene expression analysis reveals that a total number of 533 sequences were up-regulated in TMEV-infected cells, with a maximal fold change increase of 18.8, and that 1440 were down-regulated, with a maximal fold change decrease of -13.3 . Three sequences with PFAM (Proteins families) database hit description as “IL-8” or “small cytokines / intercrine/chemokine,” above the fluorescence background of the chip (56.84), hybridize with TMEV-infected astrocytes RNA but not with mock-infected astrocytes RNA.

Table 1
SCOP described “macrophage inflammatory protein” up-regulated sequences in the TMEV-induced expression of the IL-8 family gene in astrocytic cells

Affimetrix sequence	Mock-infected astrocyte signal	TMEV-infected astrocyte signal	Signal log ratio	Fold change	Chromosome location	PFAM ^a description	UniGene title	SCOP ^b description
94146 at	6.0	255.5	5.1	10.2	#11	IL-8	Small inducible cytokine A4	Macrophage inflammatory protein
98772 at	13.6	116.2	2.8	5.6	#5	IL-8	Small inducible cytokine B5	Macrophage inflammatory protein-2
102424 at	20.5	106.8	1.8	3.6	#11	IL-8	Small inducible cytokine A3	Macrophage inflammatory protein

^a PFAM, “Protein FAMILY” database.

^b SCOP, “Structural Classification Of Proteins” database.

Two of them are located in the mouse chromosome number 11 and one in chromosome number 5. The characteristics of this three up-regulated sequences, having a SCOP (Structural Classification Of Proteins) database description of “Macrophage inflammatory protein” and MIP-2, were explained in Table 1. Its GO (Gene Ontology) database cellular component notation indicated that all are extracellular, involved in chemotaxis and inflammatory response.

3.2. Induction of MIP-2 mRNA in astrocytes by TMEV

Northern blot analysis of total RNA isolated from SJL/J astrocytes cultured for 10–12 days and infected at different m.o.i.s for 24 h, revealed a dose-dependent increase in mRNA levels, peaking at a m.o.i of 10 (Fig. 1). Transcripts above background were detected to a limit of a m.o.i of 0.1. Densitometric quantitation of the relative amount of mRNA with a Model 300A computing densitometer (Molecular Dynamics Inc., Sevenoaks, Kent, UK), indicated 12- and 7-fold increases in band intensities at a m.o.i of 10 and 1, respectively, as compared to background levels (Fig. 1). Hybridization to a human house keeping β -actin probe (Clontech) served as an internal control for loading (Fig. 1, lower panel). The probing of BALB/c astrocytes infected in the same conditions were consistently found negative (not shown).

3.3. ELISA determination of MIP-2

Supernatants of astrocytic cell cultures derived from SJL/J infected at different m.o.i showed high MIP-2

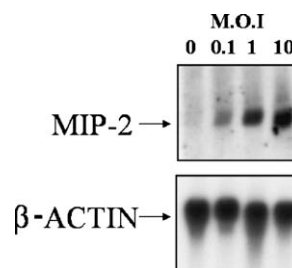


Fig. 1. Abundance of MIP-2 mRNA in SJL/J astrocytes mock-infected (0) or TMEV-infected at m.o.i.s of 0.1, 1, or 10, for 24 h, as detected by Northern blot analysis of 10 μg of purified total RNA. Hybridization to the housekeeping gene β -actin was used as a control for equal RNA loading. The figure is a representative autoradiograph from three separate experiments.

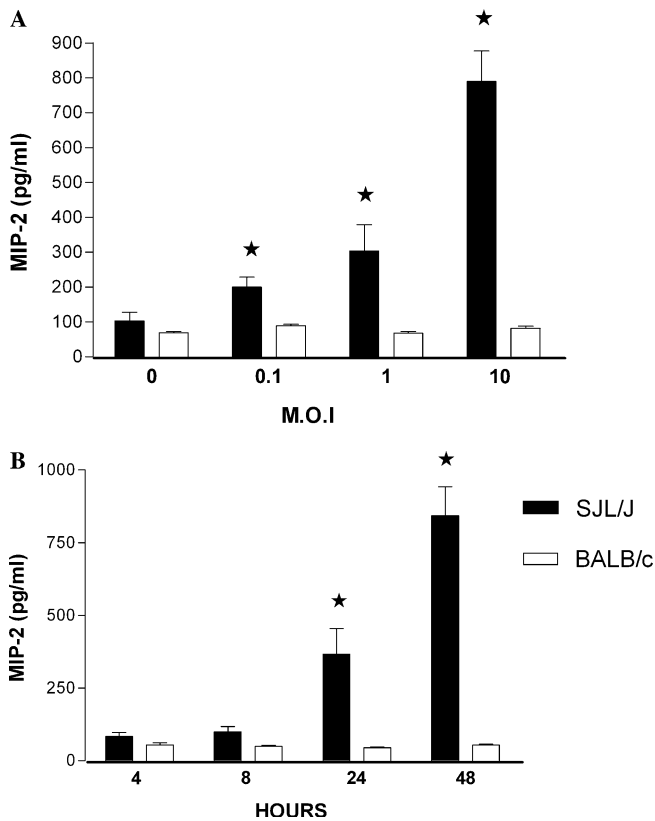


Fig. 2. Analysis of MIP-2 levels in TMEV-infected astrocyte cultures supernatants as quantified by ELISA. (A) Centrifuged supernatants from astrocytes mock-infected (0) or infected at m.o.i.s of 0.1–10, were tested 48 h after infection. (B) The kinetics of MIP-2 production after infection at a m.o.i. of 10 was measured from 4 to 48 h post-infection. Results represent mean values \pm SD of triplicate samples. *Significant differences with the untreated control group determined by the Student's *t*-test ($P < 0.01$).

production in ELISA as determined by using the Quantiquine^R M mouse MIP-2 kit (Fig. 2A), whereas supernatants from mock-infected cultures had not MIP-2 activity. Interestingly, astrocytes from BALB/c mice, a strain resistant to TMEV demyelination, produce almost undetectable levels of MIP-2 (15 pg/ml to <7 pg/ml) when infected at the same m.o.i as SJL/J astrocytes. BALB/c astrocytes are susceptible to infection in the same extent as SJL/J, producing 24 h post-infection titers of $5\text{--}30 \times 10^5$ PFU/ml as determined by titration of supernatants on BHK-21 cells [21]. MIP-2 levels were maximal at a m.o.i of 10. Analysis of the kinetics of MIP-2 production by cells infected at such m.o.i indicate maximal release into the supernatant after 48 h, decreasing later (Fig. 2B). Some supernatant samples revealed concentrations higher than 1000 pg/ml and were diluted to fit the standard curve. Supernatants of BALB/c astrocytes infected at m.o.i of 10 produced no detectable MIP-2 at any time after infection. To rule out that the BALB/c strain is a poor producer of MIP-2, we stimulated cultures of peritoneal exudate cells (10^6 cells) with 1 μ g/ml of LPS for 24 h, that induced similar levels of MIP-2 to those produced by SJL/J peritoneal cell cultures (not shown).

3.4. Chemotaxis induced by MIP-2-containing supernatants

Our results showed that undiluted supernatants, containing 1 ng/ml of MIP-2 based on ELISA readings, and possibly other chemokines, had almost the same chemotactic activity as the positive control on mouse peripheral blood mononuclear cells. This biological activity decreases in a dose response manner (Fig. 3A). At a 1:5 dilution (200 pg/ml), the migration induced was equal to the negative medium control. The mock control supernatant had no chemoattractant activity (not shown). The positive control was based on the chemotaxis induced by the chemokine stromal cell-derived factor 1 α (SDF-1 α). This potent chemokine attracts several cell types including T lymphocytes and progenitors for hematopoietic cells, B-cells and megakaryocytes [25]. The nonspecific relative migration of the cells towards culture medium in the lower compartment of the transwell chamber was considered as 1 (Fig. 3A). When we added different amounts of neutralizing goat polyclonal antibodies anti MIP-2 (R&D Systems) to the chemotactic supernatants, we obtained only a maximum 50% inhibition, probably due to the presence of another functionally redundant chemokines (Fig. 3B).

3.5. Flow cytometry antigenic characterization of the chemoattracted population

Because the MIP-2 chemoattracted cells showed significant difference in size, we evaluated phenotypic differences among them by using flow cytometry focusing on CD3 expression, indicative of T lymphocytes and the adhesion molecule CD11b commonly used to identify neutrophils [26]. In the SDF-1 α chemoattracted population (B population in Fig. 4) 85.8% of the cells were CD3-positive T lymphocytes whereas 97.6% of cells chemoattracted by TMEV-infected astrocyte supernatants (A population in Fig. 4) was CD11b-positive but CD3-negative. Thus, the later population does not contain lymphocytes and was composed of neutrophils and monocytes stained by anti-CD11b antibodies.

3.6. Inhibition of MIP-2 production by anti TMEV antibodies

To exclude the possibility that factors in the BHK-21 cellular extract used as a source of TMEV virions induced the observed MIP-2 activity, rabbit anti-TMEV antibodies primarily directed to the VP-1 and VP-2 capsid proteins [27], were tested for their ability to block MIP-2 expression. Incubation with antiserum dilutions ranging from 10^{-2} to 10^{-6} completely abrogated the induction of MIP-2 supernatant activity as detected by ELISA (Fig. 5). Presumably, antibody binding to the virus capsid sterically inhibits BeAn binding to its receptor(s) on astrocytes, blocking MIP-2 production. A rabbit antiserum against an unrelated adenovirus had no inhibitory effect on the MIP-2 inducing capacity of

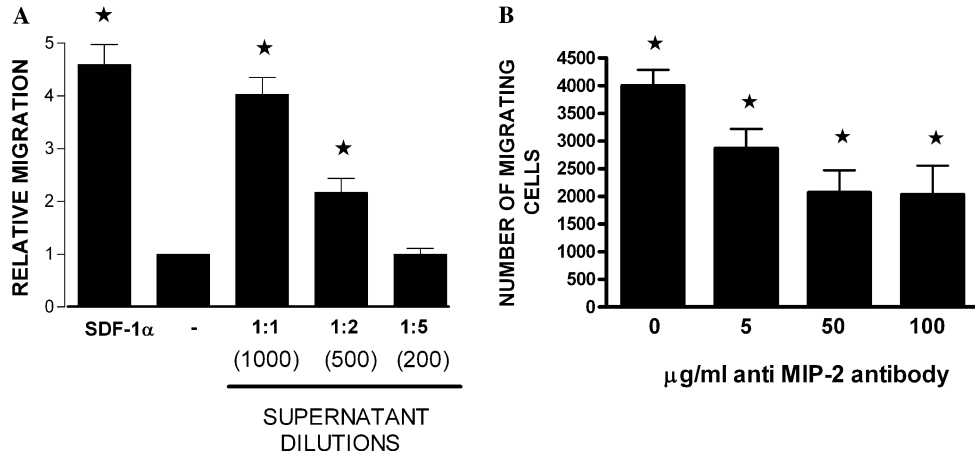


Fig. 3. Chemotaxis of SJL/J mice blood cell induced by MIP-2-containing astrocyte supernatants. (A) The positive migration control was induced by stromal cell derived factor-1α (SDF-1α) at a concentration of 100 ng/ml. The negative control (–), obtained with culture medium, was assigned a relative migration of 1. Supernatants of TMEV-infected astrocytes were tested undiluted or diluted 1:2 or 1:5. The numbers in parenthesis indicated the concentrations of MIP-2 in pg/ml as determined by ELISA. The number of chemoattracted cells was determined by flow cytometry and relative migration was calculated (mean ± SD). (B) Different amounts of antibody anti MIP-2 were studied for its capacity to inhibit migration. Data represent the mean ± SD of triplicate samples. *Significant differences with the unspecific migration control group was determined by the Student's *t*-test.

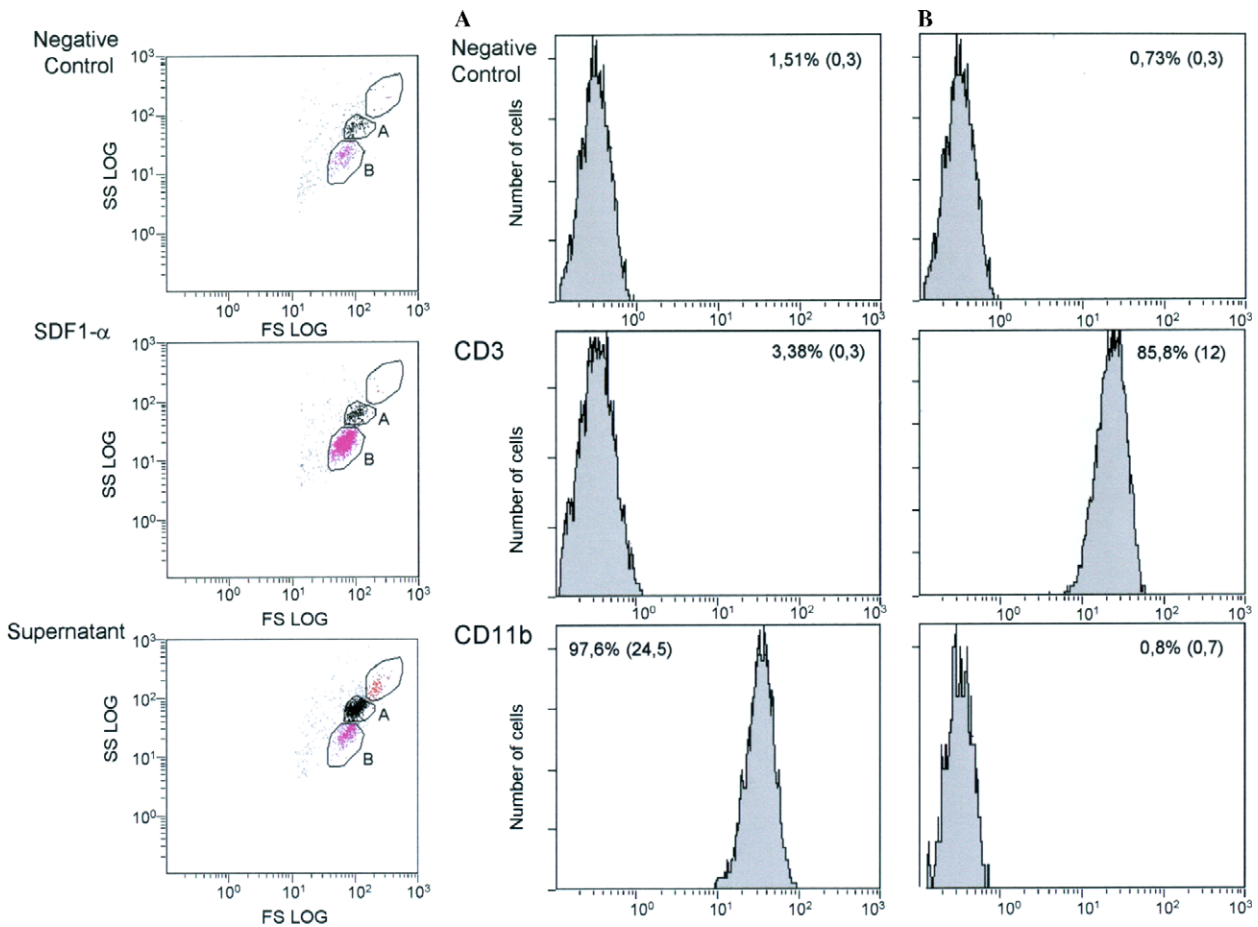


Fig. 4. Cytofluorometric analysis of the cells chemoattracted by astrocyte supernatants. The CD3 and CD11b profile of the two significant migrating cell populations (A and B) was studied. The A and B populations were induced by astrocytic supernatant and SDF-1α, respectively. The relative percent of stained cells were indicated in the upper right corner of each quadrant, followed by the mean fluorescence intensity (in parentheses). Negative controls were stained with an irrelevant antibody (P3X63 mAb). The figure shows a representative result of three separate experiments.

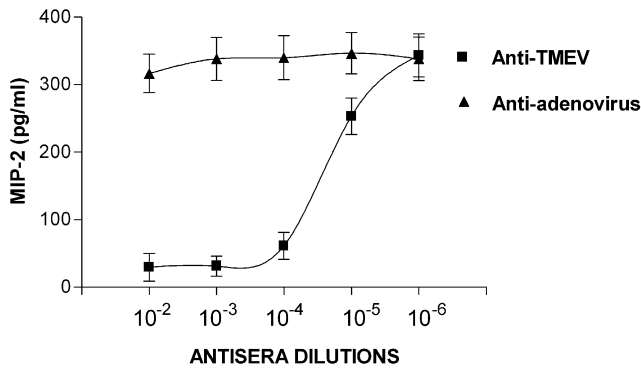


Fig. 5. Inhibition of the TMEV-induced expression of MIP-2 by anti-TMEV antibodies. TMEV samples calculated to produce a m.o.i. of 1, were incubated for 30 min at 37 °C with different dilutions of anti-TMEV or anti-adenovirus Ad. β Gal antisera and added to the astrocyte cultures. After 48 h, the presence of MIP-2 in the supernatants was monitored by ELISA. Data represent the mean \pm SD of triplicate samples. Results representative of two experiments are shown.

TMEV, when tested at the same dilutions (Fig. 5). Furthermore, inactivated, UV-light-irradiated Theiler's virus induced no detectable MIP-2 in the supernatants of infected cells (not shown).

3.7. MIP-2 induction by inflammatory cytokines

Four recombinant inflammatory cytokines (IL-1 α , IL-6, IFN- γ , and TNF- α) were tested for a possible role in the up-regulation of MIP-2 in astrocytes. Whereas neither IL-6 nor IFN- γ exert any up-regulatory effect in MIP-2 production by astrocytes, IL-1 α and TNF- α increases the release of MIP-2 into the cultures supernatants by 14- (1400 \pm 90 pg/ml) and 13-fold (1320 \pm 70 pg/ml) as compared with untreated cultures (Fig. 6A). Palma and Kim [17] did not observe MIP-2 up-regulation by TNF- α treatment of astrocytes. This difference could be explained by the fact that these authors measure MIP-2 mRNA after 24 h and we measure active MIP-2 protein after 48 h of treatment. MIP-2 levels were even higher than those obtained after 48 h of TMEV infection (1000 \pm 80 pg/ml). The Quantiquine^R M mouse MIP-2 kit has no significant cross-reactivity with any of the four inflammatory cytokines studied, even at concentrations of 50 ng/ml. Gene Chip microarray gene analysis (Table 2), reveals that IL-6 was overexpressed (signal log ratio of 4.0) while IFN- γ transcripts were absent of both sham-infected or virus-infected cultures. Moreover, transcripts encoding IL-1 α and TNF- α , high MIP-2 inducers, were also overexpressed (signal log ratios of 2.3 and 1.2, respectively) after infection. As both IL-1 α and TNF- α could be important stimulus for MIP-2 autocrine expression in vitro, we tested neutralizing antibodies against both cytokines on TMEV-induced up-regulation 24 and 48 h post-infection (Figs. 6B and C). No significant reduction of the levels of MIP-2 in supernatants were detected even adding concentrations of 100 μ g/ml of both purified, efficient neutralizing antibodies to the culture medium. The same negative results were obtained when both antibodies were added

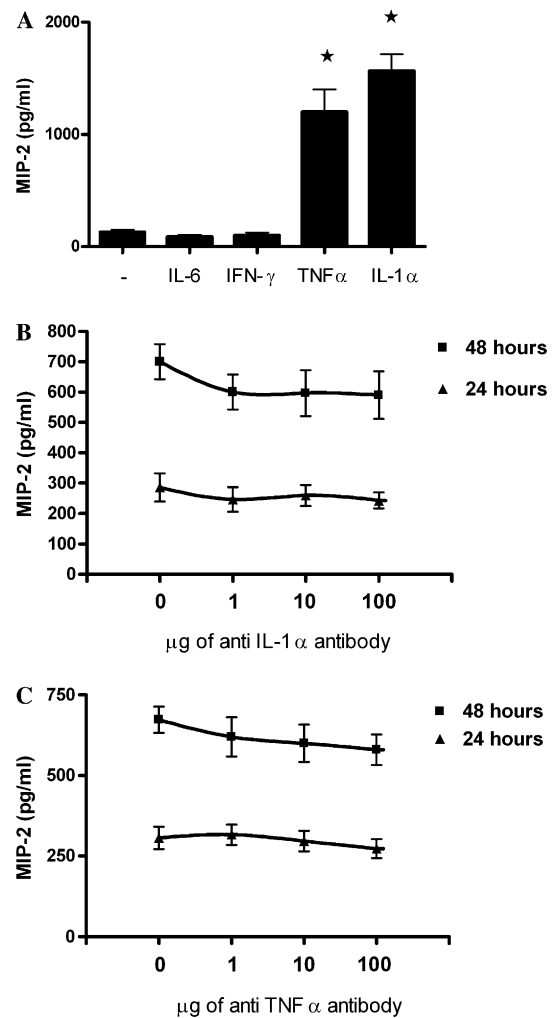


Fig. 6. Induction of MIP-2 in astrocytes by inflammatory cytokines. In panel A, SJL/J astrocytic cultures were untreated (–) or treated with 10 ng/ml each of recombinant IL-1 α , IL-6, IFN- γ and TNF- α for 48 h. Supernatants were then centrifuged and tested for the presence of MIP-2 by ELISA. (B and C) Astrocytes in 6-well multiwell plates were infected at a m.o.i. of 10 for 1 h at room temperature, washed and incubated for 24 or 48 h in complete medium containing different amounts of anti IL-1 α or anti TNF- α purified neutralizing antibodies. Supernatants were then tested for MIP-2 by ELISA. Data represent the mean \pm SD of triplicate samples. *Significant differences with the untreated control group (–) determined by the Student's *t*-test ($P < 0.01$).

together to the cultures. Therefore, both chemokine and cytokine induction by TMEV seems to follow independent pathways, with distinct temporal patterns.

3.8. MIP-2 induction in serum of TMEV-infected mice

We attempted to determine the levels of MIP-2 within the CNS compartment by using cerebrospinal fluid but we were not able to collect enough fluid per individual animal to perform the ELISA test followed by a correct statistical analysis of the results. Therefore, we investigated the pathological relevance of our findings for demyelinating disease using ELISA to monitor changes of serum MIP-2 in SJL/J and BALB/cCum mice inoculated i.c. with the BeAn strain

Table 2
Overexpression of transcripts coding for inflammatory cytokines

Uni Gen title	Affymetrix sequence	Mock-infected astrocytes signal	TMEV-infected astrocytes signal	Signal log ratio	Fold change	Chromosome location
Interleukin 6 IL-6	102218 at	9.9	200.4	4.0	8.0	# 5
Interleukin 1 α IL-1α	94755 at	7.6	39.0	2.3	4.6	# 2
LPS-induced TNF- α TNF-α	93753 at	70.4	132.7	1.2	2.4	# 16

Transcripts for IFN- γ were absent from both mock- or TMEV-infected astrocytes.

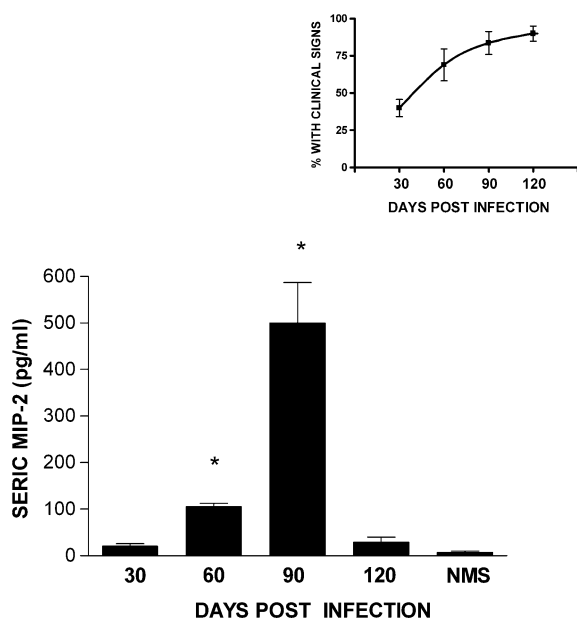


Fig. 7. Serum levels of MIP-2 in SJL/J mice infected intracranially with TMEV (2×10^6 PFU). Levels of MIP-2 in normal SJL/J mouse serum were also shown (NMS). Data represent the mean \pm SD of three individual serum samples. The inset represents the increase with time in the percent of animals with clinical signs of demyelination ($n = 20$). *Significant differences determined by the Student's *t*-test ($P < 0.01$).

and displaying a range of clinical signs. Serum levels of this chemokine were significantly increased ($p < 0.01$) in infected SJL/J individuals in a time course manner (Fig. 7), as compared with the mean \pm SD of 18.70 ± 11.10 pg/ml calculated for 10 individual mouse sera from uninfected 2 month-old SJL/J. This increase was time-dependent with peak levels reaching 490 ± 20 pg/ml at day 90 post-infection, decreasing thereafter to normal levels by day 120 (Fig. 7). The kinetics of MIP-2 production precedes and paralleled the increase in the percent of TMEV-infected animals showing clinical signs of demyelination (Fig. 7, inset), including gait abnormality, limb spasticity, decreased activity, and urinary incontinence [4,28]. Ten individual sera from uninfected or for infected (30, 60, 90, and 120 days post-infection, three animals per time point) healthy TMEV-resistant BALB/cCum mice, were consistently in the range of 15–20 pg/ml (not shown).

4. Discussion

The demyelinating process associated with i.c. inoculation of low-neurovirulence BeAn virus is currently used as

an experimental model for MS. Clatch et al. [5] proposed that cytokines and chemokines released in the site of TMEV infection by virus-specific CD4⁺ T cells, and possibly astrocytes, lead to recruitment and accumulation of immune cells in the CNS, triggering demyelination by a nonspecific bystander response that leads to stripping of myelin lamellae. In contrast to the previously held views regarding the immune-privileged status of the CNS, it is now clear that circulating immune cells, migrate across the blood brain barrier in pathological conditions. It has also been shown that, in the presence of foreign antigens in the CNS, such as viral pathogens like TMEV, specific immune cells accumulate in the brain parenchyma. Such cells are increasingly implicated in chronic CNS inflammatory disorders as MS.

In the present study, we detected the overexpression of IL-8-like genes in TMEV-infected murine astrocytes by hybridization to the U74v2 DNA microarray from Affymetrix. Because MIP-2 is the most convincing mouse chemokine homologous to human IL-8, we studied in this article its specific production by astroglial cells infected with the BeAn strain of TMEV. MIP-2 detected and quantified by ELISA is biologically active as cell chemoattractant.

MIP-2 transcripts has been previously detected in the brains of mouse hepatitis virus-infected mice at 3–7 days post-infection [29]. In addition, primary cultures of astrocytes infected with the same virus also express MIP-2 transcripts [30]. Furthermore, IP-10, RANTES and MCP-1 chemokines mRNA were detected in brains and spinal cords of mice infected with another low neurovirulence strain of TMEV, the Daniel's virus [31]. The detection of several chemokines in the CNS of mice with experimental allergic encephalomyelitis (EAE), another T-cell mediated model for MS, as well as in the brain of patients affected by MS itself has been reviewed [16]. Our DNA hybridization results indicating expression of the CXCL2 chemokine by TMEV-infected astrocytes are consistent with previous reports of the detection of increases in several chemokine mRNAs, including RANTES, IP-10 and MIP-2, by RNase protection assay or RT-PCR techniques [17,18].

Recruitment of immune cells from the blood to the site of tissue injury is thought to depend on the production of chemokines and the establishment of chemoattractant gradients. Thus, we proposed that MIP-2 produced intracranially by astrocytes, in addition to other cell types as monocyte-macrophages were TMEV persist, could be one of the chemokines used for the recruitment and influx into

the CNS of polymorphonuclear leukocytes, prominent in the TMEV-induced chronic bystander response resulting in destruction of myelin [32]. In addition to the pathological changes (Fig. 7), two immunological important parameters as TMEV-specific delayed-type hypersensitivity and splenic cells proliferation in vitro, peaks at the same period of time (80–90 days) and with the same strain specificity after infection [5]. The presence MIP-2 in sera of SJL/J but not in resistant TMEV-infected BALB/c mice at the time of demyelinating disease suggest a direct role for MIP-2 in the chemotactic attraction of neutrophils contributing, in addition to the majoritary monocytemacrophage and T cells, to the characteristic infiltrates in areas of demyelination. The possible explanation for this strain difference must be found in the proved fact that the SJL strain of mouse express a variety of immunological abnormalities [33].

MIP-2 expression was also induced in astrocytes treated by some, but not all, inflammatory cytokines (Fig. 6A). Such cytokines are usually involved in immune-mediated inflammatory processes [34–36]. The results on induction of MIP-2 by recombinant cytokines are coherent with our previous demonstration that BeAn virus induces TNF- α and IL-1 α in SJL/J astrocytes but not in BALB/c astrocytes [19,20], and are further reconfirmed here using the DNA microarray gene expression analysis (Table 2). Peak production of TNF- α was reached at 24 h [20], that of IL-1 α at 12 h [19] and that of MIP-2 48 h post-infection (Fig. 2), indicating that the induction of the two cytokines precedes in the cascade of events related to TMEV infection. Other authors have reported the differential expression of TNF- α as well as IFN- γ , IL-4 and IL-10 mRNAs in the CNS of TMEV-infected SJL/J mice [37]. Nevertheless, neutralizing antibodies against IL-1 α and TNF- α fail to reduce TMEV-induced MIP-2 production in vitro (Fig. 6, B and C), indicating that the induction of chemokines and cytokines follows independent pathways with distinct temporal patterns.

Based on our gene chip analysis there are at least two different IL-8-like proteins up-regulated by TMEV infection; one is coded by a gene located in chromosome number 5 and one or two from gene(s) in chromosome 11 (Table 1). Another possibility is that the sequences encode domains from the same IL-8-like protein. Multiple unlinked genes are involved in mouse strain susceptibility to TMEV-induced clinical disease. One is located the H-2D region [38], but others map to chromosomes numbers 6 and 3 [39,40]. Interestingly, a locus on chromosome number 11 has been reported also to affect TMEV-induced demyelinating disease [41]. In addition to the genes identified thus far, the MIP-2 gene located at chromosome 11 might represent an important susceptibility gene as it is overexpressed in astrocytes (up to 10.2 times, Table 1) and its protein product is present in sera from the susceptible SJL/J but not from the resistant BALB/c strains of mice, in the later phase of this biphasic disease. The novelty of the strain-dependent expression

of MIP-2 reported is therefore the central focus of interest in this article.

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