Neurobiology

Role of the Macrophage Inflammatory Protein-1α/CC Chemokine Receptor 5 Signaling Pathway in the Neuroinflammatory Response and Cognitive Deficits Induced by β-Amyloid Peptide

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The hallmarks of Alzheimer’s disease include the deposition of β-amyloid (Aβ), neuroinflammation, and cognitive deficits. The accumulation of activated glial cells in cognitive-related areas is critical for these alterations, although little is known about the mechanisms driving this event. Herein we used macrophage inflammatory protein-1α (MIP-1α) or CC-chemokine receptor 5 (CCR5)-deficient mice to address the role played by chemokines in molecular and behavioral alterations induced by Aβ1–40. Aβ1–40 induced a time-dependent increase of MIP-1α mRNA followed by accumulation of activated glial cells in the hippocampus of wild-type mice. MIP-1α−/− and CCR5−/− mice displayed reduced astrocytosis and microgliosis in the hippocampus after Aβ1–40 administration that was associated with decreased expression of cyclooxygenase-2 and inducible nitric oxide synthase, as well as reduced activation of nuclear factor-κB, activator protein-1 and cyclic AMP response element-binding protein. Furthermore, MIP-1α−/− and CCR5−/− macrophages showed impaired chemotaxis in vitro, although cytokine production in response to Aβ1–40 was unaffected. Notably, the cognitive deficits and synaptic dysfunction induced by Aβ1–40 were also attenuated in MIP-1α−/− and CCR5−/− mice. Collectively, these results indicate that the MIP-1α/CCR5 signaling pathway is critical for the accumulation of activated glial cells in the hippocampus and, therefore, for the inflammation and cognitive failure induced by Aβ1–40. Our data suggest MIP-1α and CCR5 as potential therapeutic targets for Alzheimer’s disease treatment. (Am J Pathol 2009, 175:1586–1597; DOI: 10.2353/ajpath.2009.081113)

Alzheimer’s disease (AD) is the most prevalent cause of dementia in humans, and the symptoms are commonly manifested after the seventh decade of life. Numerous pathological changes have been described in the post-mortem brains of AD patients, including senile plaques, tangles, neuroinflammation, synapse loss, and neuronal death.1 Activated glial cells surrounding senile plaques seem to be responsible for the ongoing neuroinflammatory process in the disease through the release of cytokines and other toxic products, including reactive oxygen species, nitric oxide, and excitatory amino acids.2 However, little is known about the identity of the agent(s) responsible for glial cell accumulation and activation in the AD brain.

Chemokines belong to a family of chemoattractant cytokines that were initially identified according to their ability to regulate leukocyte trafficking during inflammatory responses.3,4 More recently, in addition to their chemotactic activity, chemokines have been implicated in the modulation of cell adhesion, phagocytosis, cytokine secretion, proliferation, apoptosis, angiogenesis, and vi-
The role of chemokines in neuroinflammation. In the central nervous system (CNS), these proteins regulate leukocyte migration across the brain endothelium as well as the activation and movement of cells within the brain parenchyma. There is growing evidence to support the view that resident CNS cells have the capacity to express chemokines and their receptors during a variety of neuroinflammatory and degenerative conditions. Notably, recent evidence has indicated that chemokines and their receptors are up-regulated in the AD brain and that they may play a critical role in controlling the recruitment and accumulation of glial cells at β-amyloid (Aβ) sites in senile plaques.

Macrophage inflammatory protein (MIP)-1α (CCL3) is a member of the β-chemokine subfamily, which also includes MIP-1β (CCL4) and regulated upon activation, normal T-cell expressed and secreted (RANTES, CCL5). These molecules exert their effects through activation of CC-chemokine receptor 5 (CCR5). CCR5 is expressed at low levels in the normal brain, but it can be induced to play important roles in various injuries or infections, including AD. In this context, immunohistochemical analyses have revealed the expression of CCR5, together with its ligands, on the microglia of both normal and AD brains, with increased expression on some reactive microglia in AD. Nevertheless, the precise role of CCR5 and its ligand MIP-1α in AD is poorly understood so far.

In the current study we have investigated the molecular and behavioral alterations induced by a single intracerebroventricular (i.c.v.) injection of Aβ1–40 peptide in mice lacking MIP-1α or CCR5. Although unable to induce pathological AD hallmarks, the acute injection of Aβ peptides into the rodent brain is a useful experimental model for the characterization of Aβ toxicity, as it induces an inflammatory response associated with deficits of learning and memory. Using this model, we demonstrated that activation of the MIP-1α/CCR5 signaling pathway is one of the earliest events after Aβ1–40 injection in mice, representing an important signal for the accumulation of activated glial cells and, consequently, for inflammatory response, synaptic dysfunction, and cognitive failure. These findings raise the possibility that MIP-1α and CCR5 represent promising targets for AD drug development.

Materials and Methods

Animals

Experiments were conducted using male C57BL/6 CCR5 knockout (CCR5−/−) and MIP-1α knockout (MIP-1α−/−) mice (20 to 30 g). They were kept in controlled room temperature (22 ± 2°C) and humidity (55 to 60%) under a 12 hour light/dark cycle (lights on at 6:00 AM). CCR5−/− and MIP-1α−/− mice are on the C57BL/6 background, constructed as described previously. All procedures used in the present study followed the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23) and were approved by the Animal Ethics Committee of the Universidade Federal de Santa Catarina.

Drug Treatment Protocol

Human Aβ1–40 (Tocris, Ellsville, MO) and the inverse peptide Aβ40–1 (Bachem, Torrance, CA) were prepared as stock solutions at a concentration of 1 mg/ml in sterile 0.1 mol/L PBS (pH 7.4), and aliquots were stored at −20°C. Aβ solutions were aggregated by incubation at 37°C for 4 days before use as described previously. The aggregation and/or oligomerization state of Aβ solutions was confirmed through Western blot analysis (data not shown).

The aggregated form of Aβ fragments (400 pmol/mice) or PBS (vehicle) was administered i.c.v. as described previously. In brief, the animals were anesthetized with isoflurane (2.5%, Abbott Laboratórios do Brasil Ltda., Rio de Janeiro, RJ, Brazil) using a vaporizer system (SurgiVet Inc., Waukesha, WI) and then gently restrained by hand for i.c.v. injections. The injection site was sterilized using gauze embedded in 70% ethanol. Under light anesthesia (i.e., just that necessary for loss of the postural reflex), the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye and 1 mm posterior to a line drawn through the anterior base of the eyes (used as external reference). A volume of 3 μl of Aβ1–40, Aβ40–1, or PBS solution was injected into the lateral ventricle, at the following coordinates from bregma: anteroposterior −0.22 mm, mediolateral 1 mm, and dorsoventral = −3 mm. The accurate placement of the injection site (needle track) was confirmed at the moment of dissection of the animals for molecular biology experiments. The administration site was also confirmed in parallel experiments performed by the same technician in which 2 μl of Evans blue dye 0.5% was injected, and the brains were examined microscopically to verify the staining in the walls of the lateral ventricle (more details in Ref.25). Results from mice presenting misplacement of the cannula or any sign of cerebral hemorrhage were excluded from the statistical analysis (overall, less than 5% of the total animals used).

To confirm the involvement of CCR5 in the glial cell accumulation induced by Aβ1–40 i.c.v. injection, some mice were treated with the CCR5 antagonist d-Ala-peptide T-amide (0.01 mg/kg/day s.c., Tocris) administered 1 hour before Aβ1–40 i.c.v. injection and throughout consecutive days until the day of the experiment.

Tissue Collection

For RT-PCR and Western blot experiments, the animals were sacrificed by decapitation, and the skin was removed from the skull with two forceps. Then, one blade of a pair of fine scissors was introduced into the foramen magnum, and the skull was opened by cutting along its caudal to rostral axis. Two cuts were made perpendicular to the first one, with the scissors pointing toward the left and right ears, respectively. The opening of the skull was carefully enlarged with fine forceps. The brain was removed from the skull by means of a spatula, and the brainstem was separated from the cortex. In the next step, the hippocampuses were rapidly dissected on dry ice and stored at −70°C.
For immunohistochemical studies, mice were anesthetized with chloral hydrate (400 mg/kg i.p.) and perfused transcardially with heparin (1000 U/ml) in physiological saline followed by 4% paraformaldehyde in physiological saline. The brains were rapidly removed and postfixed overnight at 4°C in 4% paraformaldehyde.

RNA Preparation and RT-PCR

Total RNA was extracted from the hippocampus using TRIzol reagent (Invitrogen, São Paulo, SP, Brazil) according to the manufacturer’s instructions. Two micrograms of total RNA was reverse transcribed using oligo(dT) as a primer (0.05 µg), 50 µl of reverse transcriptase (Promega, Madison, WI), dNTP (144 µmol/l, Promega), reaction buffer (10 mmol/L dithiothreitol, 3 mmol/L MgCl2, 75 mmol/L KCl, and 50 mmol/L Tris-HCl, pH 8.3), and 2 µl of RNasin Plus (Promega) in a final volume of 12.5 µl. The cDNA was obtained after incubation of the samples at 70°C for 5 minutes, 4°C for 5 minutes, 37°C for 60 minutes, 70°C for 5 minutes, and 4°C for 5 minutes. Specific primers were used for CCR5 (sense, 5'-GCCAGAGGAG-GTGAGAGACATC-3' ; antisense, 5'-AAAGACAGGCTCA-GAGATGGC-3'), MIP-1α (sense, 5'-ATGAGGTCTCCAC-CACTG-3'; antisense, 5'-GTGACCTGTTCCAGGTCA-3'), and β-actin (sense, 5'-TCCTTCGTTGCCGGTGCCACA-3'; antisense, 5'-CGTCTCGGAGTCCTACACA-3'). β-Actin cDNA was used for standardization of the amount of RNA.

Two microliters of RT aliquots was mixed in a buffer containing 10 mmol/L Tris-HCl, pH 9, 1 mmol/L MgCl2, 200 µmol/l dNTP, 300 mmol/l of each primer, and 5 U of Taq polymerase (Ludwig Biotec, Porto Alegre, RS, Brazil) in a final volume of 30 µl. The PCR cycling protocols were as follows: initial denaturation at 95°C for 4 minutes; cycling at 95°C for 30 seconds, 54°C for 30 seconds (CCR5 and MIP-1α), or 62°C for 30 seconds (β-actin), and 72°C for 1 minute; and a final extension period at 72°C for 5 minutes. Optimal amplification was achieved at 30 cycles. Aliquots of 5 µl of each sample were analyzed by polyacrylamide gel electrophoresis and stained with silver nitrate.

Western Blot

Tissues were homogenized in ice-cold 10 mmol/l HEPES (pH 7.4), containing 1.5 mmol/L MgCl2, 10 mmol/L KCl, 1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml peptatin A, 10 µg/ml aprotinin, 1 mmol/l sodium orthovanadate, 10 mmol/l β-glycerophosphate, 50 mmol/l sodium fluoride, and 0.5 mmol/l dithiothreitol (all from Sigma-Aldrich, St. Louis, MO). The homogenates were chilled on ice for 15 minutes, vigorously shaken for 15 minutes in the presence of 0.1% Triton X-100 and then centrifuged at 10,000 x g for 30 minutes. The supernatant containing the cytosolic fraction was stored at –70°C until use. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and then were transferred to a polyvinylidene fluoride membrane (Immobilon P, Millipore, Danvers, MA). The membranes were saturated by incubation with 10% nonfat dry milk solution and then incubated overnight with inducible nitric oxide synthase (iNOS) (1:1000), cyclooxygenase (COX)-2 (1:1000), or β-actin (1:5000) antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA). After washing, the membranes were incubated with adjusted secondary antibody coupled to horseradish peroxidase. The immunocomplexes were visualized using the an ECL chemiluminescence detection system (GE Healthcare, São Paulo, SP, Brazil). Band density measurements were made using ImageJ 1.36b.

Immunohistochemistry

Immunohistochemical analysis was performed on paraffin-embedded brain tissue sections using CD68 (1:100, Abcam, Cambridge, MA), synaptophysin (1:400, Novocastra, Newcastle, UK), glial fibrillary acid protein (GFAP) (1:600), phospho (p)p65 nuclear factor-κB (NF-κB; 1:100), p-cAMP-response element-binding (CREB; 1:200), p-cJun/activator protein-1 (AP-1) (1:300), or COX-2 (1:200) antibodies (Cell Signaling Technology, Danvers, MA). After quenching of endogenous peroxidase with 1.5% hydrogen peroxide in methanol (v/v) for 20 minutes, high-temperature antigen retrieval was performed by immersion of the slides in a water bath at 95 to 98°C in 10 mmol/l trisodium citrate buffer, pH 6.0, for 45 minutes. The slides were then processed using the Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. After the appropriate biotinylated secondary antibody, sections were developed with 3,3'-diaminobenzidine (Dako Cytomation, Glostrup, Denmark) in chromogen solution for the exact amount of time and counterstained with Harris’s hematoxylin. Control and experimental tissues were placed on the same slide and processed under the same conditions.

The immunostaining was assessed at four levels of the dorsal hippocampus. Specifically, four alternate 3-µm sections of the hippocampus with an individual distance of ~150 µm were obtained between 1.6 and 2.4 mm posterior to the bregma. Images of stained hippocampal CA1, CA2, and CA3 and dentate gyrus subregions were acquired using a Sight DS-5M-L1 digital camera (Nikon, Melville, NY) connected to an Eclipse 50i light microscope (Nikon). A threshold optical density that best discriminated staining from the background was obtained using the ImageJ 1.36b. We captured two images of each hippocampal subregion per section (8 images per section and 32 images per mouse).

For p-CREB or COX-2 burden analyses, data are reported as the percentage of labeled area captured (positive pixels) divided by the full area captured (total pixels), as described previously. For synaptophysin analysis, total pixel intensity was determined, and data were expressed as optical density. The data represent the average value obtained by the analysis of images of the hippocampal CA1, CA2, and CA3 and dentate gyrus subregions.
The numbers of CD68, GFAP, p-p65 NF-κB, or p-c-Jun AP-1 stained-positive cells were examined microscopically at ×40 magnification. The numbers of stained-positive cells within the CA1, CA2, CA3, and dentate gyrus subregions of each of the four 3-μm sections were counted. The mean number of stained-positive cells per section was calculated for each animal group.

All histological assessments were made by an examiner blinded to sample identities. Some inherent weaknesses of the two-dimensional counting and densitometry methods applied in this study have been reviewed elsewhere.24

Immunofluorescence

For the immunofluorescence experiments, brains were sectioned at 40 μm using a Vibratome (Pelco, Redding, CA). Sections were first blocked with 3% normal serum with 2% bovine serum albumin and 0.1% Triton X-100 in Tris-buffered saline and then were incubated with ionized calcium binding adaptor molecule-1 (Iba-1) primary antibody (1:200) (Wako Chemicals, Richmond, VA) in blocking solution overnight at 4°C. Sections were then rinsed and incubated for 1 hour with secondary Alexa Fluor-conjugated antibody (Invitrogen) at room temperature. Sections were then mounted onto gelatin-coated slides in Fluoromount-G (Southern Biotech, Birmingham, AL) and examined under a confocal laser microscope (Olympus, Tokyo, Japan) using Laser Sharp 2000 software (Bio-Rad). The number of Iba-1-positive cells was examined microscopically at ×40 magnification at four levels of the dorsal hippocampus with an individual distance of ~150 μm, obtained between 1.6 and 2.4 mm posterior to the bregma. The numbers of stained-positive cells within the CA1, CA2, CA3, and dentate gyrus subregions of each of the four sections were counted. The mean number of stained-positive cells per section was calculated for each animal group.

Chemotaxis Assay

Resident peritoneal macrophages were harvested from wild-type (C57BL/6), CCR5−/−, or MIP-1α−/− mice. Cells were incubated on 24-well cell culture plates (10⁶ cells/well) in RPMI 1640 containing 5% fetal calf serum, 2 mmol/L L-glutamine, 150 U/ml penicillin, and 150 μg/ml streptomycin (Invitrogen) at 37°C and were stimulated with Aβ1–40 or Aβ40–1 (30 μmol/L), Aβ40–41 (30 μmol/L), Escherichia coli lipopolysaccharide (LPS) (serotype 0111:B4, 100 ng/ml, Sigma-Aldrich), or complement component C5a (10 nmol/L, Sigma-Aldrich) for 24 hours. The levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in supernatants were measured using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN). The cell viability of the stimulated macrophages was determined by mitochrondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) to formazan as described previously.25

Water Maze

The Morris water maze test was performed as described previously.26 The experimental apparatus consisted of a circular tank (diameter 97 cm; height 60 cm) containing water at 22 ± 2°C. The target platform (10 × 10 cm) was submerged 1 cm below the surface and placed at the midpoint of one quadrant. The platform was located in a fixed position, equidistant from the center and the wall of the tank. The tank was located in a test room containing various prominent visual cues. Mice were submitted to a spatial reference memory version of the water maze as described previously.23–22 The acquisition training session was performed 7 days after Aβ1–40 injection and consisted of 10 consecutive trials, during which the animals were left in the tank facing the wall and allowed to swim freely to the escape platform. If an animal did not find the platform within a period of 60 seconds, it was gently guided to it. The animal was allowed to remain on the platform for 10 seconds after escaping to it and was then removed from the tank for 5 minutes before being placed at the next starting point in the tank. This procedure was repeated 10 times, with the starting points varying in a pseudo-randomized manner. The test session was performed 24 hours after the training session (on day 8 postinjection). The test session consisted of a single probe trial in which the platform was removed from
the tank and each mouse was allowed to swim for 60 seconds in the maze. Performance was monitored with an AnyMaze video-tracking system (Stoelting Co., Wood Dale, IL).

Open Field
To evaluate the possible effects of MIP-1α or CCR5 genetic deletion and/or Aβ1-40 administration on locomotor activity, the animals were tested in the open-field paradigm. The apparatus, made of wood covered with impermeable Formica, had a black floor of 30 x 30 cm (divided by white lines into nine squares of 10 x 10 cm) and transparent walls, 15 cm high. Each mouse was placed in the center of the open field, and the total number of squares crossed with the four paws and the rearing behavior were registered for 5 minutes.

Statistical Analysis
All data are expressed as mean ± SEM. Statistical evaluation of the results was performed using appropriate analysis of variance with genotype, treatment, time, or the number of trials (repeated measures) as independent variables. After significant analyses of variance, multiple post hoc comparisons were performed using the Bonferroni test. The accepted level of significance for the tests was P < 0.05. All tests were performed using the Statistica software package (StatSoft Inc., Tulsa, OK).

Results

**Aβ1-40 Induces Up-Regulation of MIP-1α and Glial Cell Activation in Mouse Hippocampus**

The expression of the mRNAs for MIP-1α and CCR5 was assessed in the hippocampi of wild-type mice at different time points after Aβ1-40, i.c.v. treatment by means of RT-PCR. Under basal conditions, very low levels of hippocampal MIP-1α mRNA were observed. Conversely, a rapid onset and time-dependent induction of MIP-1α mRNA was found in Aβ1-40-treated mice (Figure 1, A and C). A significant increase in MIP-1α mRNA levels was detected after 1 hour of Aβ1-40 treatment, reaching a peak at 24 hours, and remaining elevated for up to 8 days. Moreover, the time course analysis revealed that the CCR5 mRNA level was not significantly affected by Aβ1-40 treatment (Figure 1, B and D). In addition, the treatment of animals with the reverse peptide Aβ40–1 had no effect on the number of GFAP- or CCR5 mRNA levels (Supplemental Figure S1, see http://ajp.amipathol.org).

To assess whether this increase in MIP-1α levels was associated with the accumulation of activated astrocytes and microglia, we evaluated the number of GFAP- and CD68-positive cells in the hippocampus, respectively. The results in Figure 1, E and F, demonstrate that a striking elevation in the number of GFAP-positive cells occurred between 6 hours and 8 days after Aβ1-40 treatment compared with the number in naive animals. Likewise, a significant increase in the number of CD68-positive cells was found 1 day after Aβ1-40 administration, reaching a peak at day 8 after treatment (Figure 1, E and G). In addition, treatment of animals with the reverse peptide Aβ40–1 had no effect on the number of GFAP- or CD68-positive cells when evaluated 24 hours after the treatment (Figure 1, E–G).

**Effect of Genetic Deletion of MIP-1α or CCR5 in Aβ1-40-Induced Glial Cell Activation in Mouse Hippocampus**

Glia cell activation is one of the earliest pathological features of AD and may occur in response to the increasing number of degenerating neurons and synapses or to the accumulation of Aβ. The data in Figure 2, A and C

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**Figure 1.** Effect of Aβ1-40, on the expression of MIP-1α and CCR5 and in glial cell activation in mouse hippocampus. Wild-type C57BL/6 mice were left untreated (naive mice, N) or were treated i.c.v. with Aβ1-40 or Aβ40–1 (400 pmol/mouse), and brains were harvested at the time points indicated. Total RNA was isolated from hippocampi for evaluation of MIP-1α and CCR5 mRNA expression, and β-actin mRNA was assessed as an internal control for the amount of RNA in each sample. Representative RT-PCR analysis showing MIP-1α (A) and CCR5 mRNA (B) expression. Densitometric analysis is expressed as the MIP-1α/β-actin (C) and the CCR5/β-actin ratio (D). E, Representative images of GFAP and CD68 immunostaining in the CA1 subregion of the hippocampus. Original magnification, ×100. Graphic representation of the number of GFAP- (F) and CD68-positive cells (G) determined in the CA1, CA2, CA3, and dentate gyrus subregions of the hippocampus. The values represent the mean ± SEM (N = 3 to 5 mice/group). *P < 0.05 and **P < 0.01 compared with the naive group.
significantly reduced the increase in the number of Iba-1-positive cells induced by Aβ1-40 in the hippocampus (Supplemental Figure S2, see http://ajp.amjpathol.org).

MIP-1α and CCR5 Are Required for Aβ1-40−
Induced Inflammatory Response in Mouse Hippocampus

The expression of the enzymes iNOS and COX-2 in the hippocampi of mice was assessed 1 and 8 days after i.c.v. injection of Aβ peptides. The data in Figure 3A indicate that the injection of Aβ1-40, but not of Aβ1-40−, resulted in a marked induction of both iNOS and COX-2 up-regulation in wild-type mice, reaching a peak at 1 day and lasting for up to 8 days after treatment. Western blot analysis revealed that genetic deletion of MIP-1α or CCR5 attenuated Aβ1-40−-induced iNOS up-regulation, when evaluated 1 day after administration (inhibition of 47 and 68%, respectively) (Figure 3, B and C). In addition, COX-2 up-regulation induced by Aβ1-40 was found to be significantly diminished in both MIP-1α−/− and CCR5−/− mice at 1 day compared with that in wild-type Aβ1-40−-treated mice, as indicated by reduced immunostaining for COX-2 (inhibition of 50 and 70%, respectively) (Figure 3D).
3, D and E). Notably, no significant alteration in iNOS and COX-2 expression could be observed in MIP-1\(\beta\)/H9251/H11002 and CCR5/H11002/PBS-treated mice compared with wild-type PBS-treated mice.

**Role of MIP-1\(\beta\) and CCR5 in A\(\beta\)1–40-Induced Transcription Factor Activation**

It has been reported previously that CCR5 is involved in the activation of transcription factors in the hippocampus during inflammation.\(^{27}\) In addition, the production of COX-2 and iNOS proteins is tightly regulated at the transcriptional level.\(^{28,29}\) Thus, we attempted to evaluate the effects of MIP-1\(\beta\) and CCR5 in the A\(\beta\)1–40-induced activation of transcription factors established in the 5′-flanking region of the COX-2 and iNOS gene promoter. Basal activation of CREB, p65 NF-κB, and c-Jun/AP-1 can be found in the hippocampi of wild-type, MIP-1\(\beta\)−/−, and CCR5−/− mice. In accordance with our previous reports,\(^{20}\) A\(\beta\)1–40 i.c.v. injection resulted in a significant activation of these transcription factors in the wild-type mouse hippocampus, as indicated by the increase in the CREB, p65 NF-κB, and c-Jun/AP-1 phosphorylated levels 6 hours after treatment. Conversely, MIP-1\(\beta\) or CCR5 genetic deletion significantly inhibited the increase in A\(\beta\)1–40-induced transcriptional activity (Figure 4, A–D). The phosphorylated levels of CREB, p65 NF-κB, and c-Jun/AP-1 found in the MIP-1\(\beta\)−/− A\(\beta\)1–40-treated mice were reduced by 45, 50, and 43%, respectively, in comparison with those in the wild-type A\(\beta\)1–40-treated mice. Likewise, mice lacking CCR5 showed a striking inhibition of A\(\beta\)1–40-induced CREB, p65 NF-κB, and c-Jun/AP-1 activation, with phosphorylation levels similar to those observed in PBS-treated mice (inhibitions of 94, 80, and 80%, respectively).

**Requirement of MIP-1\(\beta\) and CCR5 to A\(\beta\)1–40-Induced Cellular Responses in Vitro**

To confirm the involvement of MIP-1\(\beta\) and CCR5 in the regulation of cellular migration, as well as in the activation of the inflammatory response induced by A\(\beta\)1–40, we performed a series of in vitro experiments using peritoneal macrophages obtained from wild-type, MIP-1\(\beta\)−/−, or CCR5−/− mice. Initially, a chemotaxis assay was performed using as chemotactic stimulus the supernatant obtained from cultured macrophages isolated from wild-type, MIP-1\(\beta\)−/−, or CCR5−/− mice, stimulated for 24 hours with A\(\beta\)1–40 or with the reverse peptide A\(\beta\)40–1. The results in Figure 5A demonstrate no alteration in the mi-
cells stimulated with Aβ_{1–40} compared with the Aβ_{1–40}-treated wild-type mouse group.

**Figure 5.** MIP-1α and CCR5 are required for cellular migration but not for activation induced by Aβ_{1–40} in vitro. Pentoncel macrophages were isolated from wild-type (WT), MIP-1α−/−, or CCR5−/− mice, cultured on 24-well plates, and incubated with 30 μM/L Aβ_{1–40} or Aβ_{1–40-}1 for 24 hours, and the supernatant was collected and assayed for chemotactic activity. Chemotaxis of wild-type, MIP-1α−/−, and CCR5−/− macrophages in response to the supernatants was assayed using the Neuro Probe transwell assay. Chemotactic index represents the number of cells that migrated in response to supernatant from stimulated macrophages/number of cells that migrated in response to supernatant from unstimulated macrophages. A: MIP-1α−/− macrophages produce reduced chemotactic activity for wild-type, MIP-1α−/−, and CCR5−/− cells when stimulated with Aβ_{1–40} compared with wild-type and CCR5−/− macrophages. Macrophages isolated from CCR5−/− mice show a diminished chemotactic index in response to supernant of Aβ_{1–40} stimulated wild-type, MIP-1α−/−, or CCR5−/− cells. No significant chemotactic activity for macrophages was induced in response to the Aβ_{1–40-}1. B: Macrophage chemotaxis toward C5a (10 mmol/L) was not affected by genetic deletion of MIP-1α or CCR5. IL-1β (C) or TNF-α (D) released into the supernatant of macrophages of wild-type, MIP-1α−/−, and CCR5−/− mice stimulated for 24 hours with 30 μM/L Aβ_{1–40} or Aβ_{1–40-}1, LPS (100 ng/ml), or C5a (10 mmol/L) was measured by enzyme-linked immunosorbent assay. E: Cell viability assessed after stimulation with Aβ_{1–40} or Aβ_{1–40-}1, LPS, or C5a. "Control" refers to unstimulated cells. Values represent the mean ± SEM (n = 3/group). Chemotaxis assays were performed in triplicate, and ELISA experiments in duplicate. **P < 0.01 compared with the control group. *P < 0.05 compared with the Aβ_{1–40-}treated wild-type group. N.D., not determined.

**Figure 6.** Contribution of MIP-1α and CCR5 to cognitive deficits induced by Aβ_{1–40} in mice. The spatial reference memory version of the Morris water maze test was used as a measure of cognition. Training trials were performed on day 7 after a single i.c.v. administration of Aβ_{1–40} (400 pmol/mouse) or vehicle (PBS). Data are presented as means ± SEM latency (seconds) for escape to a hidden platform (n = 8–10 mice/group). The probe test session was performed 24 hours after training trials. Data are presented as means ± SEM of the frequency of time spent in the correct quadrant. MIP-1α−/− (A and B) and CCR5−/− (C and D) mice were significantly more resistant than wild-type (WT) C57Bl/6 mice to the deleterious effect of Aβ_{1–40} in spatial learning (A and C) and spatial retrieval (B and D). *P < 0.05 compared with the PBS-treated wild-type group. **P < 0.01 compared with the Aβ_{1–40-}treated wild-type mouse group.

CCR5−/− mononuclear phagocytes had some defect in their ability to become activated by Aβ_{1–40}, we used enzyme-linked immunosorbent assays to measure inflammatory cytokine production. We found that stimulation of wild-type, MIP-1α−/−, or CCR5−/− macrophages with Aβ_{1–40-}1 was not capable of stimulating the production of detectable levels of IL-1β or TNF-α (Figure 5, C and D). Conversely, MIP-1α−/−, CCR5−/−, and wild-type macrophages produced equivalent amounts of IL-1β or TNF-α in response to Aβ_{1–40} (Figure 5, C and D). A similar effect was observed when macrophages derived from different mouse genotypes were subjected to stimulation with either C5a or LPS. Notably, none of the stimuli used in these experiments reduced cell viability at the concentrations used, as demonstrated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Figure 5E). These results indicate that MIP-1α and CCR5 directly regulate the cellular migration process but not the production of inflammatory mediators.

**Effect of Genetic Deletion of MIP-1α or CCR5 in Aβ_{1–40-}Induced Learning and Memory Impairment**

AD is characterized clinically by a progressive and irreversible decline in learning and memory processes. We examined the ability of mice to acquire (training session) and retrieve (test session) spatial information in the water maze paradigm as indicative of learning and memory functions. Consistent with our previous reports,20–22 the i.c.v. administration of Aβ_{1–40} (400 pmol/mouse) resulted in a marked decline in both learning and memory in wild-type mice, as indicated by longer latencies in finding
the correct quadrant compared with those for wild-type A
or CCR5
/ H11002
deletion of MIP-1
shown) or PBS (Figure 6). As shown in Figure 6A, genetic
alterations in the locomotor parameters (total squares
crossed and rearing) evaluated in the open field test were
observed in Aβ1–40-treated mice (Table 1).

Table 1. Effects of I.C.V. Administration of Aβ1–40 or
Control Solution on Locomotor Activity of Wild-
Type C57BL/6, MIP-1α−/−, or CCR5−/− Mice
Evaluated in the Open Field (for 5 minutes)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mouse strain</th>
<th>Squares crossing</th>
<th>Rearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>Wild-type</td>
<td>101 ± 4</td>
<td>34 ± 2</td>
</tr>
<tr>
<td></td>
<td>MIP-1α−/−</td>
<td>125 ± 6*</td>
<td>30 ± 2</td>
</tr>
<tr>
<td></td>
<td>CCR5−/−</td>
<td>96 ± 4*</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Aβ1–40 (400 pmol/mouse)</td>
<td>Wild-type</td>
<td>102 ± 6</td>
<td>32 ± 2</td>
</tr>
<tr>
<td></td>
<td>MIP-1α−/−</td>
<td>135 ± 7*</td>
<td>35 ± 3</td>
</tr>
<tr>
<td></td>
<td>CCR5−/−</td>
<td>93 ± 4*</td>
<td>36 ± 2</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SEM of the total squares
crossed and rearing. Experiments were performed on day 8 after
treatments.

*P < 0.01 compared to the respective wild-type group.

Prevention of Synaptic Dysfunction by MIP-1α and
CCR5 Genetic Deletion

We had demonstrated previously20 that i.c.v. injection of
Aβ1–40 evoked a significant reduction in the synaptophysin
level when assessed 8 days after injection, suggesting
a decrease in synaptic density. As illustrated in Figure
7, A and B, wild-type mice treated with Aβ1–40 (400
pmol/mouse, 8 days before) exhibited markedly reduced
expression of the protein synaptophysin in the hippocam-
pus. Interestingly, both MIP-1α−/− and CCR5−/− mice
were more resistant to the Aβ1–40-induced reduction in
synaptic density, as demonstrated by the significantly
increased levels of synaptophysin compared with those in
wild-type Aβ1–40-treated mice (inhibition of 50 and 100%,
respectively). Of note, no significant alteration was ob-
served in synaptophysin levels of wild-type, MIP-1α−/−,
or CCR5−/− mice treated with PBS or Aβ40–40.

Discussion

Immune cell recruitment to tissue injury sites is a key step
in the inflammatory process.30 In AD, the initial inflam-
matory stimulus is thought to be the extracellular deposition
of insoluble aggregates of Aβ peptide. The Aβ deposits
lead to accumulation of activated glial cells, which in turn
produce cytokines, chemokines, and neurotoxins, and,
therefore, they may contribute to the neuronal degeneration
observed in AD.15,31 However, the exact mechanisms in-
volved in inflammatory cell accumulation observed in AD,
as well as the consequences of this event, are still a matter of debate. We used an experimental
model in which Aβ1–40 was administered to mice defi-
cient in MIP-1α or CCR5 to evaluate the role of this
chemokine and its receptor in AD. The data obtained
indicate a critical role for both proteins in the accumu-
lation of activated glial cells in the hippocampus and, as
a result, in the development of neuroinflammation and
cognitive deficits induced by Aβ1–40.

MIP-1α is one of the most commonly expressed chem-
okines during CNS inflammation.32,33 It has been re-

Figure 7. Role of MIP-1α and CCR5 in Aβ1–40-
induced synaptic disruption. A: Representative
images of synaptophysin immunostaining in the CA1
subregion of the hippocampus evaluated 8 days
after PBS, Aβ40–40, or Aβ40–40 i.c.v. administration
(400 pmol/mouse). Original magnification, × 40.
B: Graphic representation of the average optical
density (O.D.) of the immunostaining for synap-
tophysin evaluated in the CA1, CA2, CA3, and
dentate gyrus subregions of the hippocampus,
demonstrating that MIP-1α−/− and CCR5−/− mice
were significantly more resistant than wild-type
to Aβ1–40-induced synaptic disruption. The
values represent the mean ± SEM (N = 5 mice/
group). *P < 0.01 compared with the PBS-treated
wild-type mouse group. **P < 0.01 compared
with the Aβ40–40-treated wild-type mouse group.
ported that MIP-1α is expressed by neurons and microglia in AD brains and by microglia, macrophages, astrocytes, and oligodendrocytes stimulated with Aβ in vitro. In addition, it has been demonstrated that CCR5 is expressed by neurons, microglia, and astrocytes in the brain. Corroborating these studies, we found that a single i.c.v. administration of Aβ1-40 induces a rapid onset and sustained rise of MIP-1α mRNA levels in the hippocampus. However, we failed to observe any significant alteration in the expression of CCR5 mRNA in response to Aβ1-40, despite recent research showing increased CCR5 expression in Aβ-treated monocytes in vitro and in reactive microglia of AD brains.

Several lines of evidence suggest a role for chemokines in AD. Besides CCR5, CCR2 and its ligand MCP-1 have also been identified as important regulators of microglial accumulation in AD. Overexpression of MCP-1 in mice expressing mutant amyloid precursor precursor protein results in microglial accumulation in the brain. Nevertheless, these mice exhibit increased Aβ deposition. Conversely, genetic deletion of CCR2 in mice expressing mutant amyloid precursor protein is associated with impaired microglial accumulation, accelerated Aβ deposition, and increased mortality. These discrepancies regarding the role played by chemokines in the progression of AD reinforce the need for additional studies describing the exact mechanisms regulating glial responses during the disease. In this sense, to determine whether the Aβ1-40-induced MIP-1α production resulted in accumulation of activated glial cells, we used immunohistochemical staining for GFAP, CD68, and Iba-1, well established markers for activated astrocytes and microglial cells. The results of the present study corroborate literature data, providing strong molecular evidence concerning the effect of Aβ intracerebral administration on glial cell activation in the CNS. We observed a striking increase in the number of activated astrocytes and microglial cells in response to Aβ1-40. Moreover, our results demonstrate a critical role for MIP-1α and CCR5 in this event, as genetic deletion or pharmacological blockade of the MIP-1α/CCR5 pathway resulted in a marked reduction of GFAP- and CD68-, or Iba-1-positive cells in the hippocampus of Aβ1-40-treated mice. Using a model of neuroinflammation induced by LPS infusion, Rosi et al. also found a marked reduction of activated microglia and astrocytes in the hippocampus of rats treated with a CCR5 antagonist, confirming the important role played by this receptor in glial cell responses during CNS inflammation.

Inflammation in the AD brain has been reported in a number of studies. Interestingly, accumulating research has linked polymorphisms in cytokines and other immune molecules with AD, and epidemiological studies indicate that the use of nonsteroidal anti-inflammatory drugs reduces the risk of AD, thus reinforcing the notion that neuroinflammation can contribute to disease progression. In agreement with this hypothesis, we previously reported a correlation between TNF-α-related signaling pathways and iNOS expression, a crucial event for the decline in learning and memory functions induced by Aβ1-40 in mice. The enzyme iNOS is not commonly expressed in the CNS but can be induced after brain insult in astrocytes, microglia, neurons, and endothelial cells, where it catalyzes the production of high amounts of nitric oxide. Nitric oxide overproduction can in turn exacerbate neurodegeneration through the formation of peroxynitrite and protein nitration, affecting the physical and chemical nature of proteins. Moreover, transgenic mice expressing mutant human APP and presenilin-1 with genetic deletion of the iNOS gene are protected from premature mortality and cerebral plaque formation compared with their iNOS-expressing counterparts. Herein, we demonstrated the involvement of MIP-1α and CCR5 in Aβ1-40-induced iNOS up-regulation, as we found reduced levels of the enzyme in MIP-1α−/- and CCR5−/− mouse hippocampus. In addition to iNOS, one of the main proteins induced during inflammation is COX-2. COX enzymes are responsible for prostaglandin biosynthesis and are the primary target for nonsteroidal anti-inflammatory drugs. Interestingly, studies conducted in AD brains have revealed increased COX-2 mRNA levels in the cortex and hippocampus and increased COX-2 protein expression in neurons and glial cells. Recently, studies conducted with genetically modified mice expressing mutant amyloid precursor protein and presenilin-1 have shown that the deletion of the prostaglandin E2 EP2 receptor reduces oxidative damage and amyloid burden. COX-2 expression can also be induced in cultured microglia or astrocytes in response to Aβ. Corroborating these results, our data demonstrate an inhibition of COX-2 up-regulation in the hippocampi of MIP-1α−/- and CCR5−/− mice, suggesting a role for these molecules in the modulation of COX-2 expression by Aβ1-40. It is now well established that the increased expression of COX-2 and iNOS observed during inflammation is orchestrated by different transcription factors. In fact, we have previously demonstrated a critical role for CREB, NF-κB, and AP-1 in the regulation of COX-2 and iNOS expression induced by Aβ1-40 in mouse hippocampus. Herein, we found that the decrease in the expression of both enzymes in the hippocampi of mice lacking MIP-1α or CCR5 is associated with reduced activation of AP-1, NF-κB, and CREB. Nevertheless, additional studies are necessary to establish the identity of the cells expressing activated transcription factors and COX-2 and/or iNOS proteins after Aβ1-40 treatment. The absence of MIP-1α and CCR5 could lead to decreased accumulation of activated glial cells accompanied by reduced inflammation through several possible mechanisms, including effects of MIP-1α/CCR5 signaling on glial cell migration and/or activation. We therefore investigated each of these possibilities using in vitro assays. Notably, macrophages isolated from CCR5−/− mice exhibited decreased migration in response to the supernatant of cells stimulated with Aβ1-40. Furthermore, when MIP-1α−/- macrophages were stimulated with Aβ1-40, their supernatant exerted reduced chemotactic activity, indicating that the production of MIP-1α and the consequent activation of CCR5 are important events for cell migration induced by Aβ1-40 in mice. Of high interest, we found that Aβ1-40 and other stimuli such as LPS and C5a...
were capable of inducing the production of IL-1β and TNF-α in wild-type, MIP-1α−/−, and CCR5−/− cells in a comparable manner. Taken together, these results support a role for glial cells in neuroinflammation observed in this model of AD-like disease. However, despite the requirement of MIP-1α and CCR5 for cell migration, these proteins seem not to be directly involved in the expression of inflammatory mediators induced by Aβ$_{1-40}$.

To evaluate whether the reduced glial cell accumulation and neuroinflammation observed in the absence of MIP-1α and CCR5 were associated with decreased cognitive deficits in our model, we subjected animals to the MIP-1α−/− and CCR5−/− mice are substantially protected against Aβ$_{1-40}$-induced learning and memory impairment. In addition, these effects seem to be related to an inhibition of the synaptic degeneration promoted by Aβ$_{1-40}$, because we found in MIP-1α−/− and CCR5−/− mice synaptophysin levels comparable with those observed in PBS-treated mice. To our knowledge, this is the first in vivo evidence demonstrating a correlation between MIP-1α/CCR5 signaling and the establishment of Aβ cognitive effects. In addition, probably by inhibiting the entry of HIV into microglia, the pharmacological blockade of CCR5 was associated with cognitive improvement in patients with AIDS, a disease that is also characterized by glial activation and extensive brain inflammation.

The relationship between chemokines and their receptors is complex, because individual chemokines can often bind to several different receptors, and a single chemokine receptor can be activated by multiple chemokines. Whereas CCR5 is responsible for some of the effects of MIP-1α, MIP-1β, and RANTES, MIP-1α can also exert its actions through CCR1. This promiscuity may account for the phenotypic differences between MIP-1α−/− and CCR5−/− mice observed in our study, because MIP-1β or RANTES could be activating CCR5 as well. However, additional studies are necessary to address the possible role played by these chemokines in Aβ$_{1-40}$-induced molecular and behavioral responses. Despite that the current results, together with data in the literature, provide clear functional and molecular evidence indicating that the chemokine system has a critical role in modulating the effects of Aβ, a process that could be of great relevance to the pathophysiology of AD.

References

Role of Chemokines in Neuroinflammation


