HIV-1 Nef upregulates CCL2/MCP-1 expression in astrocytes in a myristoylation- and calmodulin-dependent manner

Michael H. Lehmann[1,2,*], Sabine Masanetz[1,2], Susanne Kramer[1] and Volker Erfle[1,2]

[1]Institute of Molecular Virology, GSF-National Research Center for Environment and Health, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany
[2]Institute of Virology, Technical University of Munich, Schneckenburgerstr. 8, 81675 Munich, Germany
*Author for correspondence (e-mail: orlataler@web.de)

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Summary

HIV-associated dementia (HAD) correlates with infiltration of monocytes into the brain. The accessory HIV-1 negative factor (Nef) protein, which modulates several signaling pathways, is constitutively present in persistently infected astroctyes. We demonstrated that monocytes responded with chemotaxis when subjected to cell culture supernatants of *nef*-expressing astrocytic U251MG cells. Using a protein array, we identified CC chemokine ligand 2/monocyte chemotactic protein-1 (CCL2/MCP-1) as a potential chemotactic factor mediating this phenomenon. CCL2/MCP-1 upregulation by Nef was further confirmed by ribonuclease protection assay, RT-PCR and ELISA. By applying neutralizing antibodies against CCL2/MCP-1 and using CCR2-deficient monocytes, we confirmed CCL2/MCP-1 as the exclusive factor secreted by *nef*-expressing astrocytes capable of attracting monocytes. Additionally, we showed that Nef-induced CCL2/MCP-1 expression depends on the myristoylation moiety of Nef and requires functional calmodulin. In summary, we suggest that Nef-induced CCL2/MCP-1 expression in astrocytes contributes to infiltration of monocytes into the brain, and thereby to progression of HAD.

Key words: AIDS, Calmodulin, Chemotaxis, Dementia, Myristoylation

Introduction

HIV-associated dementia (HAD) is a severe complication of viral infection clinically characterized by motor and behavioral dysfunctions leading to seizures, coma and finally death (Navia et al., 1986). Despite highly active antiretroviral therapy (HAART), HAD is still a frequent neurological disorder in HIV-1-infected individuals. Indeed, although the incidence of HAD decreased rapidly after introduction of HAART in the mid-1990s, its prevalence is on the increase, and a more subtle form named minor cognitive motor disorder (MCMD) has became important (McArthur et al., 2003). HAD is characterized as HIV encephalitis (HIVE) with astrogliosis, and is typified histologically by the presence of multinucleated giant cells, microglial nodule formation and neuronal injury (Price et al., 1988).

HIV-1 infects perivascular macrophages, microglia and astrocytes but not neurons, suggesting that the neurotoxic effects caused by HIV might be indirect (Gonzalez-Scarano and Martin-Garcia, 2005). The severity of HAD/HIVE correlates better with the presence of macrophages and microglia than with the presence and amount of HIV-infected cells in the brain (Glass et al., 1995). $CD14^+CD16^+$ peripheral-blood-derived monocytes, as well as $CD4^+$ and $CD8^+$ T lymphocytes, accumulate in the brains of AIDS patients with HIVE (Fischer-Smith et al., 2001; Petito et al., 2003). Monocytes enter the brain by transendothelial migration (Maslin et al., 2005), and it has been suggested that monocytes/macrophages contribute to the destruction of neurons through production of neurotoxic agents such as inflammatory cytokines, free radicals, platelet-activating factor, nitric oxide and eicosanoids (Gonzalez-Scarano and Martin-Garcia, 2005; Kaul et al., 2001). However, there is an ongoing discussion as to the means of monocyte recruitment and activation, as well as their ability to infiltrate the brain (Gartner, 2000; Williams and Hickey, 2002). We hypothesized that a certain brain cell type susceptible to infection with HIV-1, together with an HIV-1 protein with the capacity to direct gene expression in the host cell, induces the production of a soluble factor that mediates infiltration of monocytes into the brain.

Astrocytes, the most abundant cell type in the brain, play a central role in HAD (Blumberg et al., 1994). HIV-1-infected astrocytes remain mainly in a non-productive, chronically infected state, and express marginal viral structural proteins and high amounts of the HIV-1 Nef protein, suggesting a prominent role for Nef in the pathogenesis of HAD (Kohleisen et al., 1992; Kramer-Hammerle et al., 2005b; Ranki et al., 1995; Saito et al., 1994; Tornatore et al., 1994).

Nef, a 27 kDa accessory HIV and simian immunodeficiency virus (SIV) protein necessary for efficient virus replication and high viral load, has for several years been considered as a progression factor to AIDS (Greene and Peterlin, 2002). According to several excellent reviews, a variety of diverse functions including downregulation of cell-surface molecules have been assigned to the protein (Das and Jameel, 2005; Piguet and Trono, 1999; Roeth and Collins, 2006). Moreover,

Nef has been reported to interact with, and modulate, the activity of numerous molecules involved in intracellular signal transduction, including (1) members of the Src family of tyrosine kinases, (2) serine/threonine kinases, (3) phosphatidylinositol 3-kinase (PI 3-kinase), (4) guanine nucleotide exchange factor Vav, and (5) calmodulin (Greenway et al., 2003; Hayashi et al., 2002; Renkema and Saksela, 2000). Taken together, Nef is an excellent candidate for directing the expression of a soluble factor that attracts monocytes and/or T cells into the brain. Of the various Nef variants that have been isolated, one the most studied is the Nef_{Bru} variant. It originates from HIV-1 Bru, formerly called LAV, which was derived from patient 'BRU' (Wain-Hobson et al., 1985). Less known is the variant Nef_{TH}, a myristoylation-deficient mutant that appeared during establishment of persistently HIV-1-infected TH4-7-5 astrocytoma cells resulting from infection of human astrocytoma 85HG66 cells with HIV-1 LAI-IIIB (Brack-Werner et al., 1992).

Using human astrocytic U251MG cells stably transfected with Nef_{Bru} or Nef_{TH} (Kohleisen et al., 1999), we tested the hypothesis that endogenous Nef triggers the release of a soluble factor capable of attracting monocytes. Additionally, we investigated the mechanism leading to this effect.

Results

Stable *nef*-expressing astrocytes secrete a chemotactic factor for monocytes

Cellular supernatants from human astrocytic U251MG-Nef cells were analyzed using a chemotaxis assay for a potential factor capable of inducing monocyte chemotaxis. Human monocytic THP-1 cells were used as read-out cells as they display many characteristics of primary human monocytes (Auwerx, 1991). Recombinant CCL2/MCP-1 at a final concentration of 25 ng/ml, which was previously determined as optimal (data not shown), was used as a positive control stimulus. As shown in Fig. 1, supernatants of three different U251MG cellular clones expressing nef_{Bru} (clones 2/8.2, 4/4.1, 4/4.2) induced chemotaxis of THP-1 cells that was statistically significant as compared with supernatants of U251MG-parental or -pNeo cells. Supernatants from U251MG-Nef_{TH} cells had less effect on THP-1 chemotaxis as compared with supernatants of U251MG-Nef_{Bru} cells.

Detection of differentially expressed proteins in supernatants of *nef*-expressing astrocytes

Using a protein array capable of detecting 79 different cytokines, chemokines and growth factors simultaneously, cellular supernatants from U251MG-Nef_{Bru} cells were screened to identify potential factors mediating monocyte chemotaxis. Supernatant from U251MG-parental cells was analyzed simultaneously and served as control. A quantitative comparison of each individual signal from both arrays revealed that nine proteins were present in the culture supernatant at different concentrations (Fig. 2). Of the nine proteins, three were chemokines: CCL2/MCP-1 and CXCL8/IL-8 were upregulated, and CXCL12/SDF-1 was downregulated. Moreover, interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and epidermal growth factor (EGF) were found in high concentrations in the supernatants of U251MG-Nef_{Bru} cells, whereas the concentrations of IL-6, IL-10 and oncostatin M were lower.

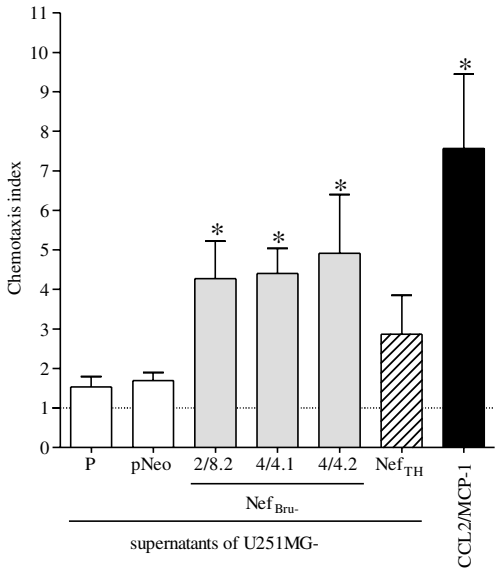


Fig. 1. Astrocytes expressing *nef* secrete a factor that is chemotactic for monocytes. A chemotaxis assay was performed using THP-1 cells. Cell culture supernatants from 16-hour incubations of astrocytic U251MG-parental (P) or stably transfected U251MG-Nef_{Bru} (clone 2/8.2, 4/4.1 and 4/4.2) or U251MG-Nef_{TH}, or with the vector carrying the neomycin resistance gene (-pNeo) were placed in the lower part of the chemotaxis unit. Recombinant CCL2/MCP-1 (25 ng/ml) was used as a positive control stimulus. Monocytic THP-1 cells were added into the upper part and allowed to migrate for 2 hours into the lower part. Subsequently, cell numbers in the lower part were counted and chemotaxis indexes were calculated as described in the Material and Methods. Data represent mean ± s.e.m. from five independent experiments; *, $P<0.05$.

Expression of CCL2/MCP-1 is upregulated in astrocytes stably expressing nef_{Bru}

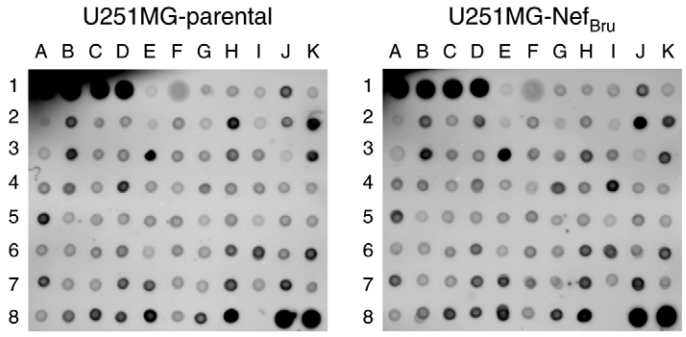
Of the two chemokines upregulated in the supernatants of U251MG-Nef_{Bru} cells detected with the protein array, only CCL2/MCP-1 is known to induce chemotaxis of monocytes (Leonard and Yoshimura, 1990) and T cells (Loetscher et al., 1994). The other, CXCL8/IL-8, is a chemoattractant for neutrophils (Lee et al., 1982). Therefore, only CCL2/MCP-1 was further investigated in terms of its regulation as a result of Nef expression.

To confirm upregulated CCL2/MCP-1 protein expression in U251MG-Nef cells, supernatants were analyzed with a CCL2/MCP-1-specific enzyme-linked immunosorbent assay (ELISA). CCL2/MCP-1 concentrations were determined in the supernatants of U251MG-Nef_{Bru} (clones 4/4.1, 4/4.2), -Nef_{TH}, -parental and -pNeo cells from several experiments where (1) incubation periods varied between 8 and 24 hours and (2) the initial cell density was different. Overall, CCL2/MCP-1 protein concentrations were significantly elevated in the supernatants of the U251MG cellular clones expressing nef_{Bru} versus U251MG-parental cells. However, there was no elevation detected in supernatants of U251MG-Nef_{TH} cells and, as expected, in supernatants of U251MG-pNeo cells, which were used as a negative control (Fig. 3A). A kinetic analysis revealed that CCL2/MCP-1 protein secretion occurred continuously and in a linear manner (Fig. 3B).

A

	A	B	C	D	E	F	G	H	I	J	K
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-alpha
2	I-309	IL-1 alpha	IL-1 beta	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12p40p70	IL-13	IL-15	IFN-gamma	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1beta
4	MIP-1 delta	RANTES	SCF	SDF-1	TARC	TGF-beta 1	TNF-alpha	TNF-beta	EGF	IGF-I	Angiogenin
5	Oncostatin M	Thrombopoietin	VEGF	PDGF-88	Leptin	BDNF	BLC	Ck beta 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3alpha	NAP-2	NT-3
8	NT-4	Osteoprotegerin	PARC	PIGF	TGF-beta 2	TGF-beta 3	TIMP-1	TIMP-2	Neg	Pos	Pos

B



C

	Human protein	ratio Nef/par.
2D	Interleukin-2 (IL-2)	1.89
2H	Interleukin-6 (IL-6)	0.41
2J	Interleukin-8 (IL-8/CXCL8)	4.59
2K	Interleukin-10 (IL-10)	0.57
3E	Monocyte chemotactic protein-1 (MCP-1/CCL2)	1.48
4D	Stromal-cell-derived factor 1 (SDF-1/CXCL12)	0.59
4G	Tumor necrosis factor-α (TNF-α)	2.22
4I	Epidermal growth factor (EGF)	4.70
5A	Oncostatin M	0.77

Fig. 2. Nef modulates cytokine and growth factor production in astrocytes. U251MG-Nef$_{Bru-4/4.2}$ and U251MG-parental cells were incubated in VLE-RPMI1640, 0.5% FCS for 16 hours. Supernatants were analyzed with RayBio® Human Cytokine antibody array V. (A) Scheme of the spotted primary antibodies. (B) Images after fluorescence scanning. (C) List of differentially regulated proteins. The ratios indicated are calculated by using the fluorescence intensities of the corresponding protein spots after background (Neg) correction and normalization of the intensities according to the mean intensities of the positive controls (Pos).

To evaluate whether the effect of Nef on CCL2/MCP-1 gene expression in astrocytes is a result of a direct endogenous action or a result of induction of a soluble factor that in turn induces CCL2/MCP-1 gene expression in a secondary manner, protein secretion was blocked by monensin, an inhibitor of the Golgi apparatus (Dinter and Berger, 1998). Subsequently, CCL2/MCP-1 protein production was investigated by intracellular CCL2/MCP-1 staining. As shown in Fig. 3C, CCL2/MCP-1 could only be detected in U251MG-Nef$_{Bru}$ cells and not in the U251MG-parental cells or in the U251MG-pNeo cells.

Additionally, to check whether Nef also increased CCL2/MCP-1 mRNA levels, total RNA of U251MG-Nef cells was analyzed using a ribonuclease protection assay designed to detect eight different chemokines, including CCL2/MCP-1 and CXCL8/IL-8 simultaneously. Total RNA from U251MG-pNeo cells served as control. High amounts of CCL2/MCP-1 mRNA were detected in U251MG cellular clones expressing *nef$_{Bru}$* (2/8.2, 4/4.1, 4/4.2) but only traces in U251MG-pNeo cells, and none in U251MG-Nef$_{TH}$ cells (Fig. 3D). The analysis also revealed that CXCL8/IL-8 mRNA was increased in U251MG-Nef$_{TH}$ cells only. No other chemokine mRNA, such as CCL1/I-309, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CXCL10/IP-10 or XCL1/lymphotactin, was detected in all cell types analyzed. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and the ribosomal L32 protein mRNA used for normalization displayed equal quantities in all samples investigated.

CCL2/MCP-1 is the exclusive chemotactic factor for monocytes secreted by *nef$_{Bru}$*-expressing astrocytes

CCL2/MCP-1 was the only chemokine detected in U251MG-Nef$_{Bru}$ cells that has the potential to induce chemotaxis of monocytes. However, additional factors might be present in the supernatants of U251MG-Nef$_{Bru}$ cells that are neither detectable using the protein array nor the ribonuclease protection assay. Therefore, the exclusive nature of CCL2/MCP-1 as the factor secreted by U251MG-Nef$_{Bru}$ capable of inducing chemotaxis of monocytes was verified.

First, supernatants of U251MG-Nef$_{Bru}$, -parental and -pNeo cells were incubated either with antibodies certified to neutralize exclusively the chemotactic activity of CCL2/MCP-1 or with isotype-matched control antibodies. A chemotaxis assay with monocytic THP-1 cells was performed and, where the neutralizing anti-CCL2/MCP-1 antibodies were applied, cell migration was reduced at the level of control cells. The isotype-matched control antibodies did not affect THP-1 chemotaxis in all supernatants of U251MG cells tested. Both a polyclonal set of anti-CCL2/MCP-1 and isotype-matched control antibodies (data not shown), as well as a monoclonal set, were applied (Fig. 4). To demonstrate the functionality of the antibody sets, recombinant CCL2/MCP-1 protein was used simultaneously as a positive control stimulus for THP-1 chemotaxis.

CCL2/MCP-1 exerts its chemotactic activity on monocytes/macrophages exclusively through the CC chemokine receptor CCR2 (Kurihara et al., 1997). To complete the findings, supernatant from U251MG-Nef$_{Bru-4/4.2}$ cells was tested for inducement of chemotaxis in U-937 cells, a human monocytic cell line lacking the CC chemokine receptor CCR2 (Zella et al., 1998). Lack of CCR2 expression in U-937 cells was confirmed by immunocytochemistry and reverse-transcriptase (RT)-PCR (Fig. 5A,B). Supernatants from U251MG-Nef$_{Bru-4/4.2}$ as well as U251MG-parental and -pNeo cells did not induce chemotaxis of monocytic U-937 cells, which is in contrast to recombinant CXCL12/SDF-1α used as a positive control stimulus (Fig. 5C).

Calmodulin is involved in Nef-induced CCL2/MCP-1 expression in astrocytes

In U251MG cells stably transfected with Nef$_{TH}$, both

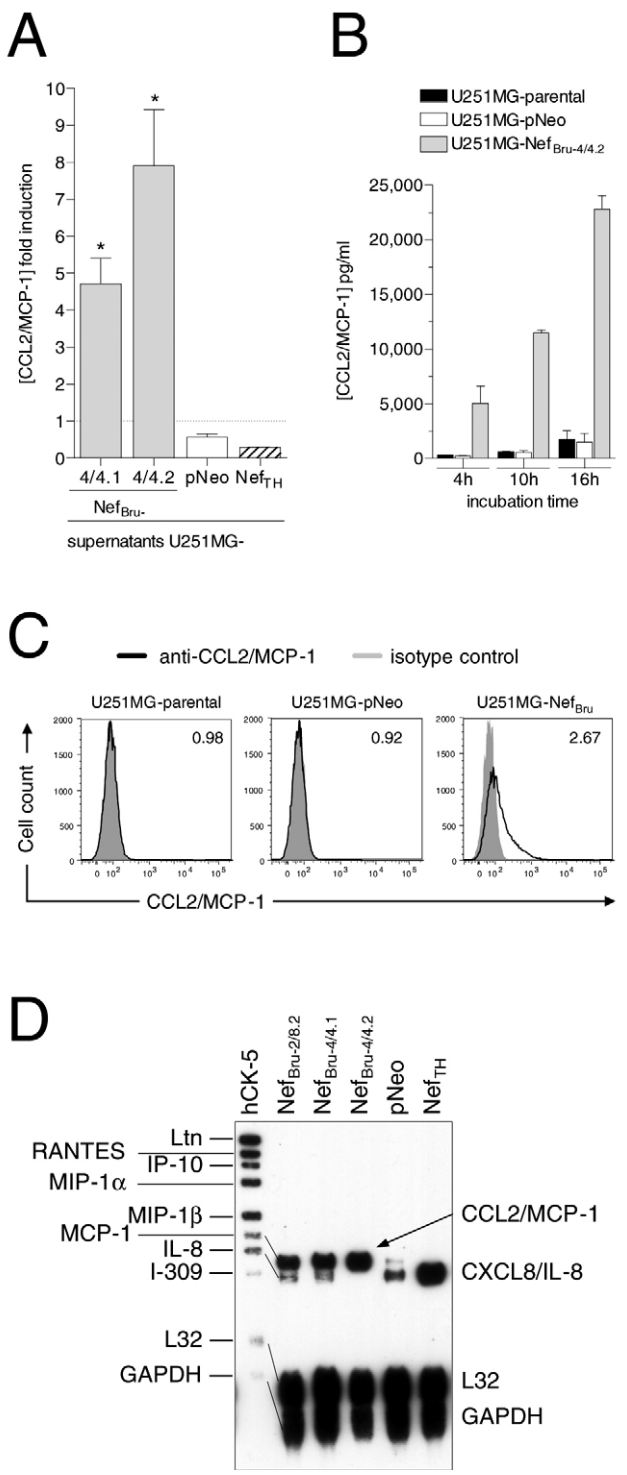


Fig. 3. CCL2/MCP-1 expression is increased in *nef*$_{Bru}$-expressing U251MG cells. (A) Astrocytic U251MG-Nef$_{Bru}$ (clones 4/4.1 and 4/4.2), -Nef$_{TH}$, -pNeo and -parental cells were seeded at a density of 2×10^5 or 4×10^5 cells/ml, incubated for 8, 16, 24 or 36 hours, and supernatants were subsequently analyzed with CCL2/MCP-1 ELISA. Fold inductions were calculated relative to the CCL2/MCP-1 protein concentrations determined in the supernatants of U251MG-parental cells. Data represent mean ± s.e.m. from at least six independent experiments; *, $P<0.05$. (B) Kinetic analysis of CCL2/MCP-1 protein secretion. Cells were incubated for periods as indicated and CCL2/MCP-1 protein concentrations determined by ELISA in the cellular supernatants. One representative experiment is shown. Data represent mean ± s.e.m. of duplicates. (C) Cells were incubated for 4 hours in the presence of monensin and subsequently stained intracellularly with PE-labeled mAb to CCL2/MCP-1 (black outline) or PE-labeled isotype-matched control antibody (gray). The ratio of the mean fluorescence intensities from CCL2/MCP-1-stained to isotype-stained cells is indicated in the upper right corner of each plot. (D) Total RNA was isolated from astrocytic U251MG-Nef$_{Bru}$ (clones 2/8.2, 4/4.1 and 4/4.2), -Nef$_{TH}$ and -pNeo cells, and analyzed by Multiprobe™ RNase protection assay using biotinylated hCK-5 as probe.

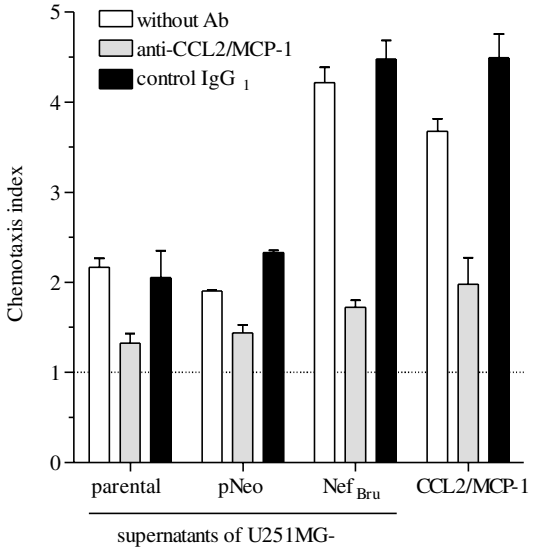


Fig. 4. CCL2/MCP-1 is the exclusive factor in the culture supernatants of U251MG-Nef$_{Bru}$ cells capable of inducing chemotaxis of monocytic THP-1 cells. Cell culture supernatants from *nef*$_{Bru}$-expressing astrocytes (clones 4/4.1 or 4/4.2) or rCCL2/MCP-1 (25 ng/ml) were placed into the lower part of the chemotaxis unit and THP-1 cells in the upper part. At 45 minutes prior to the assay, supernatants and rCCL2/MCP-1-containing wells were incubated with anti-CCL2/MCP-1 antibodies or isotype-matched control antibodies as indicated. One representative experiment of four independent experiments is shown. Data represent mean ± s.e.m. of triplicates.

CCL2/MCP-1 protein and mRNA expression was at the level of control cells (Fig. 3A,D). The primary amino acid sequence of Nef$_{TH}$ is characterized by a substitution of glycine with serine at position 2 (G2S), resulting in lack of the N-terminal myristoyl moiety (Kohleisen et al., 1992); myristoylation of Nef plays an important role in the interaction with calmodulin in vivo (Matsubara et al., 2005). These findings prompted us to investigate whether Nef$_{Bru}$-induced CCL2/MCP-1 expression could be inhibited by blocking calmodulin signaling. To check this possibility, U251MG-Nef$_{Bru}$ cells were treated with N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), a common calmodulin antagonist (Tanaka et al., 1982). RNA was analyzed using both ribonuclease protection assay and CCL2/MCP-1-specific RT-PCR. The data revealed that W7

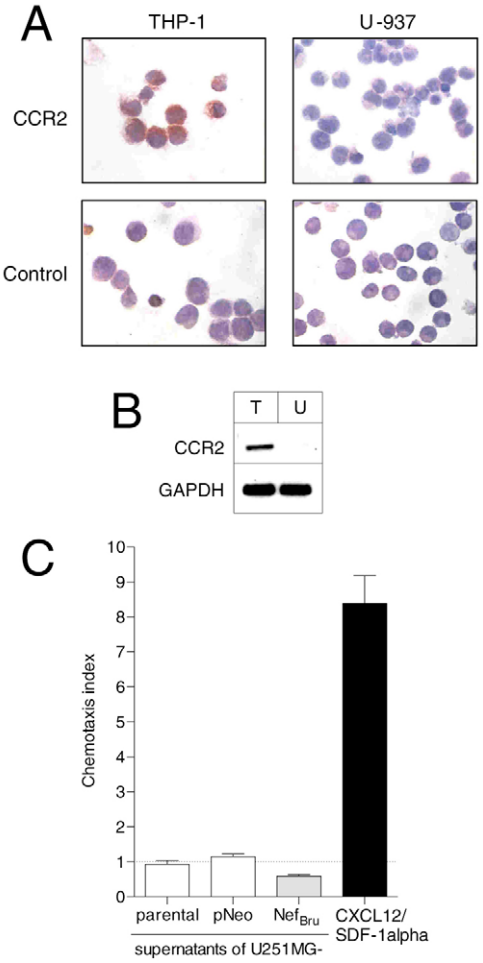


Fig. 5. Supernatants of U251MG-Nef$_{Bru}$ cells do not induce chemotaxis of CCR2-deficient monocytic cells.
(A) Immunocytochemistry analysis of monocytic THP-1 and U-937 cells using anti-CCR2 Ab. To exclude non-specific staining by the secondary antibody, anti-CCR2 Ab was substituted with distilled water (Control). Images were obtained using a microscope (Axiolab, Carl-Zeiss Jena) equipped with a ×40 objective and a ×10 ocular lenses. Original magnification, ×100. (B) CCR2-specific RT-PCR with total RNA originating from THP-1 cells (T) and U-937 cells (U). GAPDH served as internal control. (C) Supernatants of U251MG-Nef$_{Bru-4/4.2}$, -parental, -pNeo cells and rCXCL12/SDF-1α (25ng/ml) were placed into the lower parts of the chemotaxis unit, and U-937 cells in the upper parts. One representative experiment is shown. Data represent mean ± s.e.m. of triplicates.

(50 μM) dramatically reduced the level of CCL2/MCP-1 mRNA in U251MG-Nef$_{Bru}$ cells but not the levels of GAPDH or L32 mRNA, which were used as controls. Moreover, W7 had no effect on basal CCL2/MCP-1 mRNA level in U251MG-parental cells, which were used as control cells (Fig. 6A,B). The effect of W7 on CCL2/MCP-1 protein production was also tested with a CCL2/MCP-1-specific ELISA. W7 inhibited CCL2/MCP-1 protein production in U251MG-Nef$_{Bru}$ cells in a concentration-dependent manner (Fig. 6C). As little as 10 μM W7 reduced CCL2/MCP-1 protein content by 50% as compared with samples treated with the solvent only. In U251MG-parental and -pNeo cells,

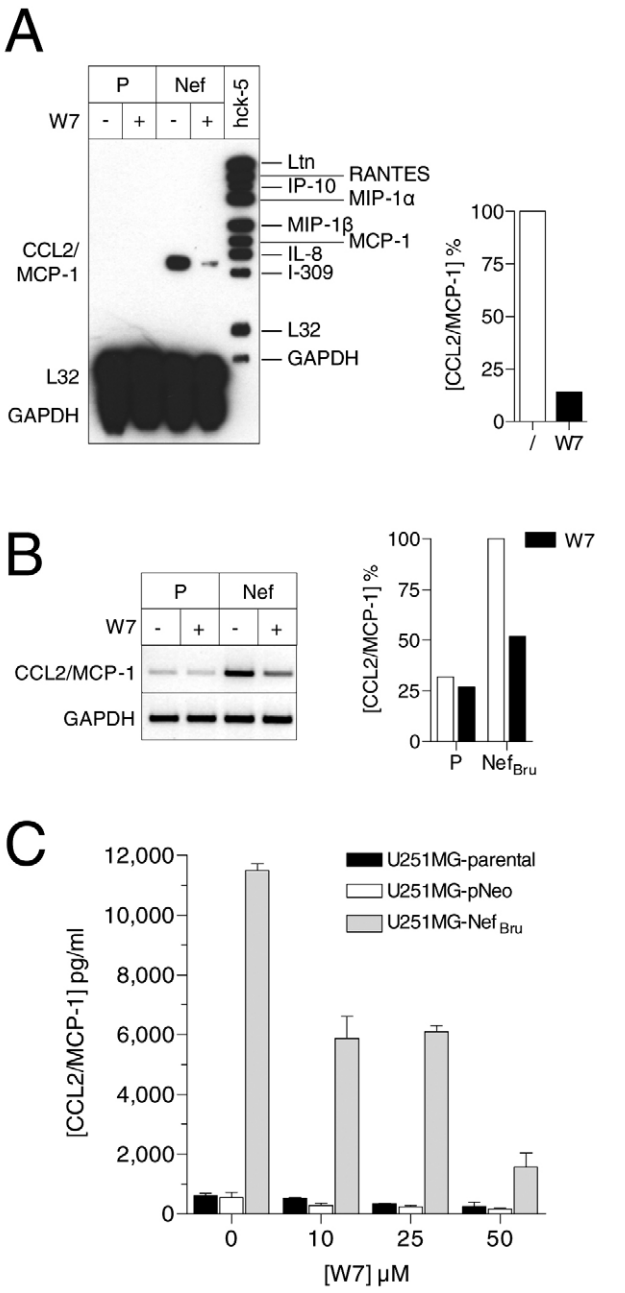


Fig. 6. Nef-induced CCL2/MCP-1 expression in astrocytes is dependent on calmodulin. U251MG-parental (P) and U251MG-Nef$_{Bru}$ cells (Nef) were treated with 50 μM W7 (+) or solvent (–) for 16 hours. Total RNA was isolated and analyzed with Multiprobe™ ribonuclease protection assay (A) and RT-PCR (B). The ratios of the CCL2/MCP-1 to GAPDH intensities were determined and the values obtained from the solvent-treated U251MG-Nef cells were set to 100%. One representative experiment of three independent experiments is shown. (C) Astrocytic U251MG-Nef$_{Bru}$, -pNeo and -parental cells were incubated for 8 hours with W7 at concentrations indicated, and CCL2/MCP-1 protein in the supernatants was determined by ELISA. One representative experiment is shown. Data represent mean ± s.e.m. of duplicates.

W7 had only a marginal influence on basal CCL2/MCP-1 protein production.

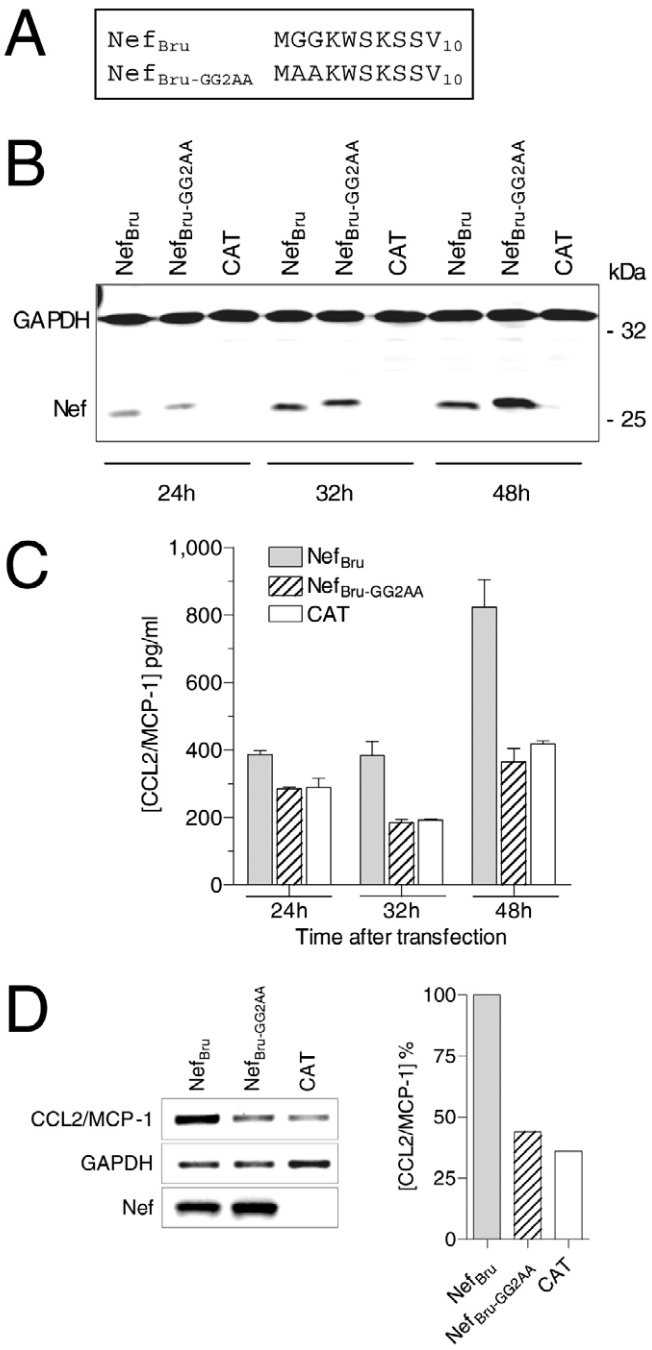


Fig. 7. Myristoylation-deficient Nef$_{Bru}$ does not increase CCL2/MCP-1 protein and mRNA production. U251MG-parental cells were transiently transfected with Nef$_{Bru}$, Nef$_{Bru-GG2AA}$ or CAT. The cell culture supernatants were replaced with medium 8, 24 and 32 hours after transfection. (A) Alignment of the N-terminal amino acid sequence of Nef$_{Bru}$ and Nef$_{Bru-GG2AA}$. (B). Simultaneous determination of Nef and GAPDH concentrations by western blotting. Cells were lysed after transfection as indicated. (C) CCL2/MCP-1 protein concentrations in the cell culture supernatants were determined using ELISA after transfection as indicated. Data represent mean ± s.e.m. of three separate transfection experiments. (D) Total RNA was isolated 48 hours after transfection and analyzed with RT-PCR. The ratios of the CCL2/MCP-1 to GAPDH intensities were determined and the values obtained from the Nef$_{Bru}$-transfected cells were set to 100%.

Myristoylation-deficient Nef$_{Bru}$ does not upregulate CCL2/MCP-1 expression in astrocytes

To test whether a lack of the N-terminal myristoyl moiety also affects CCL2/MCP-1 protein production of Nef$_{Bru}$-transfected cells, the Nef$_{Bru}$ nucleotide sequence was mutated in a manner resulting in the replacement of glycine with alanine at position 2 and 3 of the primary amino acid sequence (Nef$_{Bru-GG2AA}$) (Fig. 7A), preventing co-translational and potential post-translational myristoylation, respectively. The latter is probably a result of limited N-terminal proteolysis as previously shown by other proteins (Zha et al., 2000).

Nef$_{Bru}$, Nef$_{Bru-GG2AA}$ or chloramphenicol acetyl transferase (CAT) were transiently transfected into astrocytic U251MG-parental cells. Co-transfection of green fluorescent protein (GFP) indicated an overall transfection efficiency of about 40% (data not shown). Simultaneous quantification of Nef and GAPDH concentrations by western blotting demonstrated an equal expression yield of both Nef variants transfected (Fig. 7B). Non-myristoylated Nef$_{Bru}$ migrated slightly slower in the gel then myristoylated Nef$_{Bru}$, which was in accordance with recent findings with Nef$_{SF2}$ (Breuer et al., 2006). Increased CCL2/MCP-1 protein concentrations were found in the culture supernatants of Nef$_{Bru}$-transfected cells but not in Nef$_{Bru-GG2AA}$-transfected cells as compared with CAT-transfected cells (Fig. 7C). Additionally, RNA was analyzed using CCL2/MCP-1-, Nef- and GAPDH-specific RT-PCR. The data revealed that the mRNA of the both Nef variants was expressed in equal amounts, and CCL2/MCP-1 mRNA level was increased in Nef$_{Bru}$-transfected but not in Nef$_{Bru-GG2AA}$-transfected cells as compared with CAT-transfected cells (Fig. 7D).

Discussion

A dysregulated migration of monocytes/macrophages into tissues and organs is a frequently observed phenomenon in AIDS pathogenesis. Monocytes are attracted to sites of inflammation by several factors including chemokines such as CCL2/MCP-1 (Zlotnik and Yoshie, 2000). Elevated levels of CCL2/MCP-1 have been detected in the cerebrospinal fluid of HIV-1-infected individuals positively diagnosed with HAD/HIVE (Cinque et al., 1998; Conant et al., 1998). A polymorphism in the CCL2/MCP-1 promoter, leading to increased CCL2/MCP-1 expression and infiltration of mononuclear phagocytes into tissues, has been shown to correlate positively with the risk of HAD (Gonzalez et al., 2002). Recently, it was shown that CCL2/MCP-1 has a key role in infiltration of HIV-infected leukocytes into the central nervous system (Eugenin et al., 2006).

Astrocyte-derived CCL2/MCP-1 directs transmigration of leukocytes across the blood-brain barrier (Weiss et al., 1998), and astrocytes produce CCL2/MCP-1 after infection with HIV-1 in vitro and in vivo (Persidsky et al., 1999; Zink et al., 2001). Here, we have shown that there is a direct association between the elevated expression of CCL2/MCP-1 in astrocytes and the presence of HIV-1 Nef in these cells. Additionally, CCL2/MCP-1 has been identified as the exclusive factor induced by Nef-attracting monocytes. Notably, CCL2/MCP-1 also represents a chemotactic factor for T lymphocytes (Loetscher et al., 1994), which also infiltrate the brain of HIV-1-infected individuals (Petito et al., 2003). It can be suggested that Nef directly induced CCL2/MCP-1 expression in the

human astrocytic U251MG cell line since (1) CCL2/MCP-1 was continuously secreted, and (2) prevention of protein secretion did not affect its intracellular accumulation, indicating the non-involvement of a soluble factor with potential to induce CCL2/MCP-1 expression in a secondary manner. Regulation of Nef-induced CCL2/MCP-1 expression occurred at the mRNA level, and further studies should elucidate an increase of CCL2/MCP-1 transcription and/or mRNA stability. By contrast, elevated CCL2/MCP-1 mRNA levels were not detected by microarray analysis in astrocytes expressing either Nef or HIV-1 in other studies (Kim et al., 2004; Kramer-Hammerle et al., 2005a), probably as a result of a false negative result, a common problem when applying this technology (Hatfield et al., 2003).

Alternative hypotheses for elevated levels of CCL2/MCP-1 in the brain of HIV-1-infected individuals have been put forward. For example, recombinant HIV-1 Tat was found to induce CCL2/MCP-1 expression in astrocytes in vitro and in vivo (Conant et al., 1998; Pu et al., 2003; Weiss et al., 1999). However, detection of extracellular Tat as well as Nef in the brain has not been confirmed. Recently, intracellular Tat has been shown to stimulate the CCL2/MCP-1 promoter in human astrocytic cells (Abraham et al., 2003; Abraham et al., 2005). By contrast, in monocytes, Tat has been shown to be dispensable for HIV-mediated induction of CCL2/MCP-1 expression (Mengozzi et al., 1999). Therefore, it would be interesting to investigate whether knockout of *nef* or *tat* in HIV-1-infected astrocytes is sufficient to abrogate elevated CCL2/MCP-1 protein production.

Results from protein array analysis indicate that Nef modulates the expression of several cytokines in astrocytes, which in turn might affect neuronal integrity in a variety of ways. Besides the anti-inflammatory cytokine IL-10 (de Waal Malefyt et al., 1991), the IL-6 protein was also decreased in nef_{Bru}-expressing astrocytes. This downregulation of IL-6 by Nef may be of significance for HAD because it has previously been shown that an absence of IL-6 results in increased vulnerability of dopaminergic neurons to a neurotoxicant (Bolin et al., 2002), and the dopaminergic system in HIV-1-infected persons suffering from HAD is affected (Nath et al., 2000).

Elevated IL-2 could contribute to enhancement of CCL2/MCP-1-induced infiltration of T cells into the brain by triggering CCR2 expression on T cells (Loetscher et al., 1996). The role of elevated TNF-α in HAD has already been extensively discussed (Saha and Pahan, 2003). However, Nef-modified expression of TNF-α, IL-2, IL-6, IL-10, EGF and oncostatin M in astrocytes still awaits confirmation in a similar manner to CCL2/MCP-1.

Regarding CXCL8/IL-8, a higher protein concentration in nef_{Bru}-expressing astocytes and increased levels of mRNA were detected by RT-PCR (data not shown) but not by ribonuclease protection assay. As shown by ribonuclease protection assay, in nef_{TH}-expressing astocytes, CXCL8/IL-8 mRNA levels were strongly increased in contrast to CCL2/MCP-1. This correlated well with previous findings where astrocytoma 85HG-66 cells were infected with the corresponding parental virus strain of Nef_{TH} (Cota et al., 2000). Dysregulation of CXCL8/IL-8 probably plays a major role in the cognitive dysfunction associated with HAD since it has been shown that this CXC chemokine inhibits long-term potentiation in the CA1 region of the hippocampus, which is the seat of learning and memory (Xiong et al., 2003).

Previously, it was revealed that Nef is necessary and sufficient for inducement of the two CC chemokines, CCL3/MIP-1α and CCL4/MIP-1β, in HIV-1-infected macrophages (Swingler et al., 1999). However, elevated levels of these chemokines were not detected either at the mRNA or at the protein level in the cell type we used. Similarly, CXCL10/IP-10 was not detected, in contrast to a previous study using astrocytes infected with SINrep5-Nef virus. Here, a role of sequence variability within the *nef* gene was suggested to be responsible for its ability to induce this CXC chemokine. Although, in accordance with our findings, the authors also reported elevated CCL2/MCP-1 mRNA levels (van Marle et al., 2004).

During this study, it was demonstrated that the Nef_{TH} variant had no effect on CCL2/MCP-1 expression in astrocytic U251MG cells. Similarly, infection of astrocytoma 85HG-66 cells with the corresponding parental virus strain from which this Nef variant was isolated also had no effect on CCL2/MCP-1 expression (Cota et al., 2000). With respect to signaling, the most striking feature of Nef_{TH} as compared with Nef_{Bru} was the lack of the N-terminal myristoyl moiety. The importance of the N-terminal myristoyl moiety in Nef-induced CCL2/MCP-1 expression was confirmed by applying a myristoylation-deficient Nef_{Bru} variant. Intriguingly, myristoylation of Nef plays an important role in the interaction with calmodulin in vivo (Matsubara et al., 2005). By applying a calmodulin antagonist, evidence that signaling of Nef through calmodulin played a crucial role in Nef-induced CCL2/MCP-1 gene expression was obtained. Taken together, we conclude that Nef upregulates CCL2/MCP-1 expression in astrocytes by binding to calmodulin, which subsequently modulates calmodulin-dependent signaling pathways. Calcium/calmodulin (Ca^{2+}/CaM)-dependent protein kinases have been shown to regulate mitogen-activated protein kinases (MAPKs) (Enslen et al., 1996). Dysregulated MAPKs have been detected in the central nervous system of SIV-infected macaques (Barber et al., 2004), and it has been shown that Nef modulates MAPK activity in astrocytic U251MG cells (Robichaud and Poulin, 2000). Finally, activated p38 MAPK is necessary for CCL2/MCP-1 expression (Rovin et al., 1999).

To our knowledge, this report is the first to suggest that there is indeed a cell-biological consequence resulting from the ability of Nef to bind to calmodulin through its N-terminal myristoyl moiety. Moreover, it has been demonstrated that many other viral, bacterial and cellular proteins carry this moiety (Boutin, 1997; Maurer-Stroh and Eisenhaber, 2004). Thus, in future, it will probably be demonstrated that the myristoyl moiety from other proteins not only functions as a membrane anchor but also plays an important role in the interaction with calmodulin, leading to diverse cell biological phenomena.

The N-terminal domain of Nef is well conserved among the different variants and, moreover, it is very similar to the brain-specific acidic protein CAP-23/NAP-22 (Hayashi et al., 2002). CAP-23/NAP-22 binds to calmodulin in a myristoylation-dependent manner (Hayashi et al., 2000; Takasaki et al., 1999), and stimulates neuronal competence for axon regeneration (Bomze et al., 2001). In this context, it is interesting to note that a calmodulin antagonist blocked extracellular Nef-induced

IL-10 expression in monocytic U-937 cells (Brigino et al., 1997).

Nef is considered as a progression factor in AIDS because individuals harboring HIV-1 with deleted Nef are usually slow or non-progressors (Dyer et al., 1997), and macaques infected with a *nef*-deleted HIV-1 simian counterpart, SIV, remain disease-free for prolonged periods (Hofmann-Lehmann et al., 2003; Kestler et al., 1991). Moreover, as shown in a transgenic mouse model, it is sufficient to express HIV-1 *nef* to trigger an AIDS-like disease (Hanna et al., 1998; Simard et al., 2002). The N-terminal region of Nef including the myristoylation site has been shown to be crucial for the development of an AIDS-like disease in mice (Hanna et al., 2004). Here, we show that myristoylation of Nef is also crucial for CCL2/MCP-1 expression.

Provided that intercellular transfer of Nef is indeed a major occurrence in HIV-1-infected individuals (Qiao et al., 2006), upregulation of CCL2/MCP-1 by Nef should be found in many other cell types in the future. CCL2/MCP-1 is known to play an important role in the pathogenesis of severe diseases affecting the brain (Mahad and Ransohoff, 2003), heart (Kolattukudy et al., 1998; Lehmann et al., 1998), kidney (Viedt and Orth, 2002), skeletal muscle (Liprandi et al., 1999; Sell et al., 2006) and lung (Rose et al., 2003).

It has been shown that distinct subpopulations of monocytes, such as $CD14^+CD16^+$ and $CD14^+CD69^+$ are increased in AIDS patients, especially in those suffering from HAD (Pulliam et al., 1997). These cells accumulate in the brain of individuals suffering from HIVE (Fischer-Smith et al., 2001), and it has been suggested that HIV caused their recruitment from the bone marrow through a distinct mechanism (Gartner, 2000; Williams and Hickey, 2002). Recently, it has been shown that a potential inflammatory subset of monocytes, Ly6C(hi), is recruited from the bone marrow by the CCL2/MCP-1–CCR2 axis (Serbina and Pamer, 2006). Plasma levels and sera concentrations of CCL2/MCP-1 are elevated in HIV-1-infected individuals and correlate with viral load (Juffermans et al., 1999; Weiss et al., 1997). Taken together, elevated concentrations of CCL2/MCP-1 in the circulation of HIV-1-infected individuals, or even local production in the bone marrow, might have a systemic impact and could contribute to disease progression including HAD.

Consequently, it is plausible that Nef mediates its pathological effect on tissues and organs at least in part by upregulation of CCL2/MCP-1 expression. Further studies addressing CCL2/MCP-1 expression in Nef-transgenic animals and applying Nef-deleted virus should reveal whether Nef-induced CCL2/MCP-1 indeed plays a key role in AIDS pathology. The presented data should further encourage the development of a HAD/HIVE therapy targeted against CCL2/MCP-1. However, the chemokine CCL2/MCP-1 is something of a double-edged sword: it causes infiltration of monocytes and T cells, yet it protects neurons against apoptosis (Eugenin et al., 2003) and suppresses HIV-1 replication (Frade et al., 1997).

Materials and Methods

Materials

N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl (W7) was purchased from Merck Biosciences GmbH, dissolved in 50% ethanol at a stock concentration of 25 mM and further diluted in distilled water. Monensin sodium was purchased from Sigma-Aldrich, dissolved in methanol at a stock concentration of 30 mM. Recombinant human recombinant CCL2/MCP-1, mouse anti-human CCL2/MCP-1 monoclonal antibody (mAb) clone 24822.111, mouse IgG1 isotype control mAb clone 11711.11 and goat anti-human CCL2/MCP-1 polyclonal IgG were obtained from R&D Systems. Goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories. CCL2/MCP-1-specific enzyme-linked immunosorbent assay (ELISA) was purchased from GE Healthcare Europe GmbH. Human recombinant CXCL12/SDF-1α was from Sigma-Aldrich.

Cell culture and reagents

The human astrocytoma cell line U251MG was obtained from M. Brenner (National Institutes of Health, Bethesda, MD). The cell lines U251MG-Nef_{Bru} clones 2/8.2, 4/4.1 and 4/4.2, as well as U251MG-Nef_{TH} stably expressing *nef* from HIV-1_{Bru} (GenBank accession number K02013) or HIV-$1_{TH4\text{-}7\text{-}5}$ (GenBank accession number L31963) respectively, were established as reported (Kohleisen et al., 1999). Monocytic THP-1 and U-937 cells were from the German Collection of Cell Culture (DSMZ, Braunschweig, Germany). Cells were incubated at 37°C under 5% CO_2, 90% humidity, in VLE-RPMI 1640 medium certified to contain <0.01 endotoxin units/ml, supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (all from Biochrom AG).

Human protein cytokine array

Cell culture supernatants were screened with the RayBio® Human Cytokine antibody array V (RayBiotech). The assay was performed according to the manufacture's instructions with slight modifications: DyLight™ 647-conjugated streptavidin (Pierce Biotechnology) was used instead of horseradish peroxidase (HRP)-conjugated streptavidin. Using the Typhoon™ 9410 fluorescence scanner (GE Healthcare), positive signals were detected by applying an excitation wavelength of 633 nm and an emission filter of 670BP30 nm. Data were analyzed using ImageQuant™ TL software (GE Healthcare).

Ribonuclease protection assay

In vitro transcription of the BD RiboQuant™ Multi-probe template set hCK-5 (BD Biosciences) and ribonuclease protection assay was performed as described (Lehmann et al., 2001). Detection of RNA fragments was performed with the North2South® Chemiluminescent Detection Kit (Pierce Biotechnology). Signals were detected using a CCD camera and analyzed using Quantity One® Software (BioRad). Alternatively, the membrane was exposed to Hyperfilm™ ECL (GE Healthcare).

Reverse transcriptase-PCR (RT-PCR)

Amplification of CCL2/MCP-1 and GAPDH cDNAs were performed as described (Lehmann et al., 1998; Lehmann et al., 2002). Accordingly, human CCR2A (MCP-1RA) was amplified using the sense primer 5′-GGC TCA CTC TGC TGC AAA TG-3′ and the antisense primer 5′-TCT TTC CTG GTC TCA CTC CTG G-3′, and HIV-1 Nef was amplified using the sense primer 5′-GGA GCA ATC ACA AGT AGC AA-3′ and the antisense primer 5′-ATC AGG GAA GTA GCC TTG TG-3′. Gel pictures were acquired by the GelDoc 2000™ Gel Documentation System (BioRad), and the volume of each PCR product was determined using the Quantity One software (BioRad).

Chemotaxis assay

Monocytic THP-1 or U-937 cells were assayed in 96-well MultiScreen™-MIC plates equipped with 8.0 μm pore-sized hydrophilic polycarbonate filters (Millipore). Cell culture supernatants or recombinant chemokines diluted in VLE-RPMI 1640, 0.5% FCS or VLE-RPMI 1640, 0.5% FCS as control were placed in the lower chambers to a final volume of 150 μl. Before the assay, cells were grown in complete VLE-RPMI 1640 medium until saturation and split 1:2. After 16 hours, the living cells were counted using Trypan Blue (Sigma-Aldrich) dead cell exclusion staining, and resuspended to 1×10^6cells/ml in 37°C-tempered VLE-RPMI 1640, 0.5% FCS. Thereafter, a 75 μl cell suspension was placed in the upper chambers, and cells were allowed to migrate for 2 hours at 37°C, 90% humidity, 5% CO_2 into the lower part. Subsequently, cells that had migrated into the lower chambers were fixed with 1% paraformaldehyde (PFA) and cell numbers determined with a BD FACSCalibur flow cytometer equipped with a BD Multiwell™ AutoSampler. Data were analyzed using BD CellQuest™ software (BD Biosciences). The chemotaxis index is the ratio of the number of cells that migrated in response to a stimulus (cell culture supernatants or recombinant chemokines) to those that migrated towards VLE-RPMI 1640, 0.5% FCS.

Intracellular CCL2/MCP-1 stain

The cells were first incubated with monensin sodium salt (Sigma-Aldrich) at a final concentration of 3 μM for 45 minutes. Thereafter, the cells were washed once with medium tempered at 37°C and incubated in medium containing 3 μM monensin for a further 4 hours. BD Cytofix/Cytoperm™ solution (BD Biosciences) was used to stain cells intracellulary with R-Phycoerythrin (PE)-conjugated mouse anti-human CCL2/MCP-1 mAb clone 5D3-F7 or PE-conjugated mouse IgG1 isotype control clone MOPC-21 (BD Biosciences). Dead cells were stained with ethidium monoazide bromide (EMA). Cells were analyzed with a BD FACSCanto™ flow cytometer, and data were analyzed using FlowJo© software (Tree Star).

Immunocytochemistry

Monocytic cells were washed with PBS and attached to poly-lysin-coated slides (Sigma-Aldrich). Vecstatin Elite ABC Kit (Vector Laboratories) was used for immunocytochemistry performed as described previously (Lehmann et al., 1998). Anti-human CCR2 (BioConcept) was used as primary antibody.

Western blotting

The cell lysates were prepared by directly adding 1× SDS sample loading buffer to the cells followed by sonication. The blotted membranes were immunostained using mouse anti-Nef 3E6 mAb provided by K. Krohn through the National Institute for Biological Standards and Control Centralised Facility for AIDS Reagents, mouse anti-GAPDH mAb (Chemicon International) and goat anti-rabbit polyclonal Ab conjugated to HRP (New England Biolabs). ECL Plus was used as substrate, and positive signals were detected by fluorescence scanning (excitation wavelength 457 nm, emission filter 520BP30) using the Typhoon 9410 Fluorescence Scanner (GE Healthcare), and analyzed using ImageQuant TL software (GE Healthcare).

Transient transfection

Effectene® Transfection Reagent (Qiagen) was used to transfect plasmids into the cells according to the manufacturer's instructions. To monitor transfection efficiency, pTracer-SV40 (Invitrogen) expressing green fluorescent protein (GFP) was co-transfected. At 8 hours after adding the Effectene-DNA complex to the cells, cellular supernatants were replaced by complete medium, and cells further incubated at 37°C.

Plasmid construction

The *nef* gene was cloned from LAV-1 Bru Nef expression vector pTG1166 (Transgène) into the *Hin*dIII/*Bam*HI site of pcDNA5/FRT/TO© (Invitrogen) using the sense primer HIV-Nef-*Hin*dIII; 5′-ATC GAA GCT TAC CAT GGG TGG CAA GTG GTC-3′ for wild-type Nef and HIV-Nef_GG2AA-*Hin*dIII; 5′-ATC GAA GCT TAC CAT GGC TGC CAA GTG GTC-3′ to generate myristoylation-deficient Nef. HIV-Nef-*Bam*HI; 5′-TAC TAG TGG ATC CTC AGC AG-3′ was used as antisense primer. The plasmid pcDNA5/FRT/TO/CAT© (Invitrogen) containing the 32 kDa gene for chloramphenicol acetyl transferase (CAT) was used as a control for transient transfection.

Statistical analysis

GraphPad Prism 4 (GraphPad Software) was used for statistical analysis. The Wilcoxon Signed Rank Test was used to compare groups; a P value of less than 0.05 was considered significant. All tests were performed exactly and two tailed.

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