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BRIEF COMMUNICATION

Role of MSK1 in the Induction of NF-κB by the Chemokine CX3CL1 in Microglial Cells

Marcos Galán-Ganga[1,2] · Ángel J. García-Yagüe[1,2] · Isabel Lastres-Becker[1,2]

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Abstract

Microglial cells are essential mediators of neuroinflammatory processes involved in several pathologies. Moreover, the chemokine fractalkine (CX3CL1) is essential in the crosstalk between neurons and microglia. However, the exact roles of CX3CL1, CX3CL1 receptor (CX3CR1) and microglia signalling are not fully understood in neuroinflammation. In addition, the findings reported on this subject are controversial. In this work, we investigated whether CX3CL1 induced pro-inflammatory signalling activation through NF-κB pathway. We were able to show that CX3CL1 activates the pro-inflammatory pathway mediated by the transcription factor NF-κB as an early response in microglial cells. On the other side, CX3CR1-deficient microglia showed impaired NF-κB axis. Phospho-kinase assay proteome profiles indicated that CX3CL1 induced several kinases such as MAPK's (ERK and JNK), SRC-family tyrosine kinases (YES, FGR, LCK and LYN) and most interesting and also related to NF-κB, the mitogen- and stress-activated kinase-1 (MSK1). Knockdown of MSK1 with short interfering RNAs decreased partially MSK1 protein levels (about 50%), enough to decrease the mRNA levels of *Il-1β, Tnf-α* and *iNos* triggered by stimulation with CX3CL1. These results indicate the relevance of CX3CL1 in the activation of the pro-inflammatory NF-κB signalling pathway through MSK1 in microglial cells.

Keywords Neuroinflammation · Microglia · CX3CR1 · P65 · NF-kB · Nrf2

Introduction

Microglia act as the resident macrophage cells within the brain and are implicated in the regulation of inflammation, apoptosis, phagocytosis of cell debris, synaptic connectivity and synaptic pruning, which are essential aspects for sculpting neural circuits (Mecca et al. 2018). Microglia are key cellular mediators of neuroinflammatory processes related to the expression of key inflammatory mediators as well as reactive oxygen species (ROS). Activation of microglia induces the release of pro-inflammatory mediators that favour the permeabilisation of the blood–brain–barrier, with subsequent infiltration of peripheral leukocytes inside of the central nervous system (CNS), including T cells and macrophages (Chen et al. 2016). Inflammatory factors produced by microglia and astrocytes can damage local tissue and, can further increase inflammation and glial activation, leading to a vicious inflammatory cycle (chronic neuroinflammation) (Cherry et al. 2014). Chronic microglial activation is associated with neurodegenerative diseases such as Alzheimer's disease (Streit et al. 2004; Cuadrado et al. 2018; Lastres-Becker et al. 2014; Regen et al. 2017), Parkinson's disease (Castro-Sanchez et al. 2018; Lastres-Becker 2017; Lastres-Becker et al. 2012) and traumatic brain injury (Corps et al. 2015; Donat et al. 2017). Therefore, the crosstalk between neurons and microglia is of paramount value in the brain. Fractalkine (CX3CL1)/CX3CR1 signalling represents the most important communication channel between neurons and microglia (Mecca et al. 2018). The ligand CX3CL1 is expressed in neurons and its receptor CX3CR1 in microglia (Ransohoff 2016; Bisht et al. 2016), which facilitates

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✉ Isabel Lastres-Becker
ilbecker@iib.uam.es

[1] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Instituto de Investigación Sanitaria La Paz (IdiPaz), Instituto de Investigaciones Biomédicas "Alberto Sols" UAM-CSIC, C/ Arturo Duperier, 4, 28029 Madrid, Spain

[2] Department of Biochemistry, School of Medicine, Universidad Autónoma de Madrid, Madrid, Spain

a privileged association between these two cell types and also plays an essential role in microglial activation (Sheridan and Murphy 2013). In vitro and in vivo models have indicated a role of CX3CL1 in neuroprotective and anti-inflammatory functions (Pabon et al. 2011; Desforges et al. 2012). However, a comparable amount of evidence shows the opposite effect, sustaining CX3CL1 role in neuroinflammation and neurotoxicity (Hanzel et al. 2014; Zanier et al. 2016; Sheridan and Murphy 2013). Consequently, CX3CL1 appears to have anti-inflammatory/neuroprotective activity in some settings, whereas it contributes to neurotoxicity in others (Sheridan and Murphy 2013; Lauro et al. 2015). The role of the CX3CL1 / CX3CR1 axis becomes even more important due to its relationship with neuroinflammation associated with different pathologies. For example, it has been observed that CX3CL1 is upregulated in brain inflammation (Pan et al. 1997) and the expression of CX3CL1 and CX3CR1 undergoes dynamic regulation both in the course of traumatic brain and spinal cord injury (Poniatowski et al. 2017).

In a previous paper, we demonstrated that hippocampal HT22 cells expressing the human TAU(P301L) mutant protein produce CX3CL1, which in microglia activates AKT, inhibits glycogen synthase kinase-3β (GSK-3β) and upregulates the transcription factor NRF2/NFE2L2 and its target genes including heme oxygenase 1. NRF2 is the master regulator of redox homeostasis and seems to play a role in the conversion of microglia from the pro-inflammatory to the anti-inflammatory phenotype (Lastres-Becker et al. 2012, 2014, 2016; Rojo et al. 2010). However, NRF2 can be activated by the transcription factor NF-κB, the master regulator of inflammation (Rushworth et al. 2012) indicating crosstalk between NF-κB and NRF2, and a balance between pro-inflammation/anti-inflammation. These results prompted us to investigate whether NF-κB pathway is involved in CX3CL1/CX3CR1 microglial function.

Materials and Methods

Cell Culture

Primary astrocytes and microglia were prepared from neonatal (P0-P2) mouse cortex from *Cx3cr1*$^{+/+}$ and *Cx3cr1*$^{-/-}$ and grown and isolated as described in (Lastres-Becker et al. 2014). Briefly, neonatal (P0-P2) mouse cortex was mechanically dissociated and the cells were seeded onto 75 cm^2 flasks in DMEM:F12 supplemented with 10% FCS and penicillin/streptomycin. After 2 weeks in culture, flasks were trypsinized and separated using CD11b MicroBeads for magnetic cell sorting (MACS Miltenyi Biotec, Germany). Microglial and astroglial cultures were at least 99% pure, as judged by immunocytochemical criteria. Medium was changed to Dulbecco's Modified Eagle Medium:F12 (DMEM:F12) serum-free without antibiotics 16 h before treatment. Immortalized microglial cell line (IMG) isolated from the brains of adult mice, were purchased from Kerafast Inc., and were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 2 mM L-glutamine, in 5% CO_2 at 37 °C, 50% relative humidity. Medium was changed to serum-free DMEM without antibiotics 16 h before treatments. CX3CL1 was obtained from PreproTech (Catalog# 400-26) and solubilized in water at 46 µM and used at 100 nM.

Preparation of Nuclear and Cytosolic Extracts

IMG cells were seeded in p100 plates (1.5×10^6 cells/plate). IMG cells were treated with CX3CL1 (100 nM) and samples were collected at different time points. Cytosolic and nuclear fractions were prepared as described previously (Rojo et al. 2004). Briefly, cells were washed with cold PBS and harvested by centrifugation at 1100 rpm for 10 min. The cell pellet was resuspended in 3 pellet volumes of cold buffer A (20 mM HEPES, pH 7.0, 0.15 mM EDTA, 0.015 mM EGTA, 10 mM KCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 µg/ml leupeptin) and incubated on ice for 30 min. Then, the homogenate was centrifuged at 500 *g* for 5 min. The supernatants were taken as the cytosolic fraction. The nuclear pellet was resuspended in five volumes of cold buffer B (10 mM HEPES, pH 8.0, 0.1 mM EDTA, 0.1 mM NaCl, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 µg/ml leupeptin). After centrifugation in the same conditions indicated above, the nuclei were resuspended in loading buffer containing 0.5% SDS. The cytosolic and nuclear fractions were resolved in SDS-PAGE and immunoblotted with the indicated antibodies.

Immunoblotting

Whole-cell lysates were prepared in RIPA-Buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1 mM EGTA, 1% Igepal, 1% sodium deoxycholate, 0.1% SDS, 1 mM PSMF, 1 mM Na_3VO_4, 1 mM NaF, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin). Whole cell lysates, cytosolic and nuclear fractions containing 25 µg of whole proteins from IMG-treated cells were loaded for SDS-PAGE electrophoresis. Immunoblots were performed as described in (Cuadrado et al. 2014). The primary antibodies used are described in Supplementary Table S1.

Analysis of mRNA Levels by Quantitative Real-Time PCR

Total RNA extraction, reverse transcription, and quantitative polymerase chain reaction (PCR) was done as detailed in previous articles (Lastres-Becker et al. 2014). Primer sequences are shown in Supplementary Table S2. Data analysis was based on the ΔΔCT method with normalization of the raw data to housekeeping genes (Applied Biosystems). All PCRs were performed in triplicates.

Phospho-Kinase Assay Proteome Profiler

Primary microglia from *Cx3cr1*$^{+/+}$ and *Cx3cr1*$^{-/-}$ mice were collected as described above and 150,000 cells were plated on p60 to 80% of confluency for 16 h. IMG were seeded at 300,000 cells/p60. Then, the medium was replaced with serum-free DMEM without antibiotics for 24 h before CX3CL1 (100 nM) treatment for 1 h. Cells were then lysed and total proteins were extracted with lysis buffer supplemented by the kit. According to the manufacturer's protocol, 200 µg cell lysates were incubated with each human phospho-kinase array (R&D Systems, Minneapolis, MN). Cell lysates were diluted and incubated overnight with nitrocellulose membranes in which capture and control antibodies against 43 different kinases and transcription factors, have been spotted in duplicate. The arrays were washed to remove unbound proteins and were incubated with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied and a signal was produced at each capture spot corresponding to the amount of phosphorylated protein bound. Pixel densities on developed X-ray film were collected and analyzed by ImageJ.

siRNA Assays

siRNA used to knockdown mouse MSK1 (ON-TARGETplus Mouse Rps6ka5 (73086) siRNA—SMARTpool, Dharmacon, Catalog #L-040751-00-0005) and control scrambled siRNA sequence (Silencer® Select Negative Control siRNA #1, Ambion Cat#: 4390843). IMG cells were seeded at 500,000 cells/p60. Then, cells were transfected with 25 nM siRNAs following DharmaFECT General Transfection Protocol (Dharmacon). After 24 h, cells were transfected with 12.5 nM siRNAs and after 24 h used for experiments and harvested for analysis.

Statistical Analyses

Data are presented as mean ± SEM. To determine the statistical test to be used, we employed GraphPad Instat 3, which includes the analysis of the data to normal distribution via the Kolmogorov–Smirnov test. In addition, statistical assessments of differences between groups were analysed (GraphPad Prism 5, San Diego, CA) by unpaired Student's *t* tests when normal distribution and equal variances were fulfilled, or by the non-parametric Mann–Whitney test. One and two-way ANOVA with post hoc Newman–Keuls test or Bonferroni's test were used, as appropriate.

Results

CX3CL1 Induces NF-κB-p65 and Pro-inflammatory Cytokines Expression in Microglial Cells

To assess whether CX3CL1 was able to activate pro-inflammatory signalling pathways in microglial cells, we first analysed the subcellular distribution of the transcription factor NF-κB-p65, a master regulator of the inflammatory response. Immortalized microglial cells (IMG) were maintained under serum-free conditions for 16 h and then stimulated with CX3CL1 (100 nM) and data were collected at different time points. After 30 min of treatment, p65 started to translocate to the nucleus (Fig. 1a, b) and reached a maximum at 120 min, indicating that CX3CL1 is able to activate pro-inflammatory response as an early event. Similar results were obtained by immunofluorescence study, where we observed that after 2 h of treatment with CX3CL1 there is an increase of p65 expression both in the cytosol and in the nucleus (Fig. 1c). This was corroborated by the observation that mRNA and protein levels of the pro-inflammatory factors IL-1β, IL-6 and TNF-α increased after CX3CL1 treatment (Fig. 1d–f). Our results indicate that CX3CL1 is able to activate the pro-inflammatory pathways as a prompt molecular event in microglial cells.

To gain more insight into the role of the CX3CL1/CX3CR1 axis on NF-κB signalling, we analysed the expression pattern of NF-κB pathway in *Cx3cr1*-deficient primary microglia. Our results show that the absence of CX3CR1 leads to a 50% decrease in the mRNA expression of *Rela* (p65) as well as *Il-1β* and *Il-6* (Fig. 2a–c). These results are specific for CX3CR1-expressing microglia given that astrocytes obtained in the same purification setting did not show alteration in *Rela* (p65) expression (Fig. 2d).

CX3CL1 Triggers Several Kinase Pathways

Although the implication of several signalling pathways involved in the modulation of CX3CL1 actions has been described, such as PI3K/AKT (Lastres-Becker et al. 2014; Lyons et al. 2009), most of the data were obtained in neuronal cell types (Sheridan and Murphy 2013). Therefore, we investigated what signalling pathways could be activated by CX3CL1 using a kinase proteome profiler assay

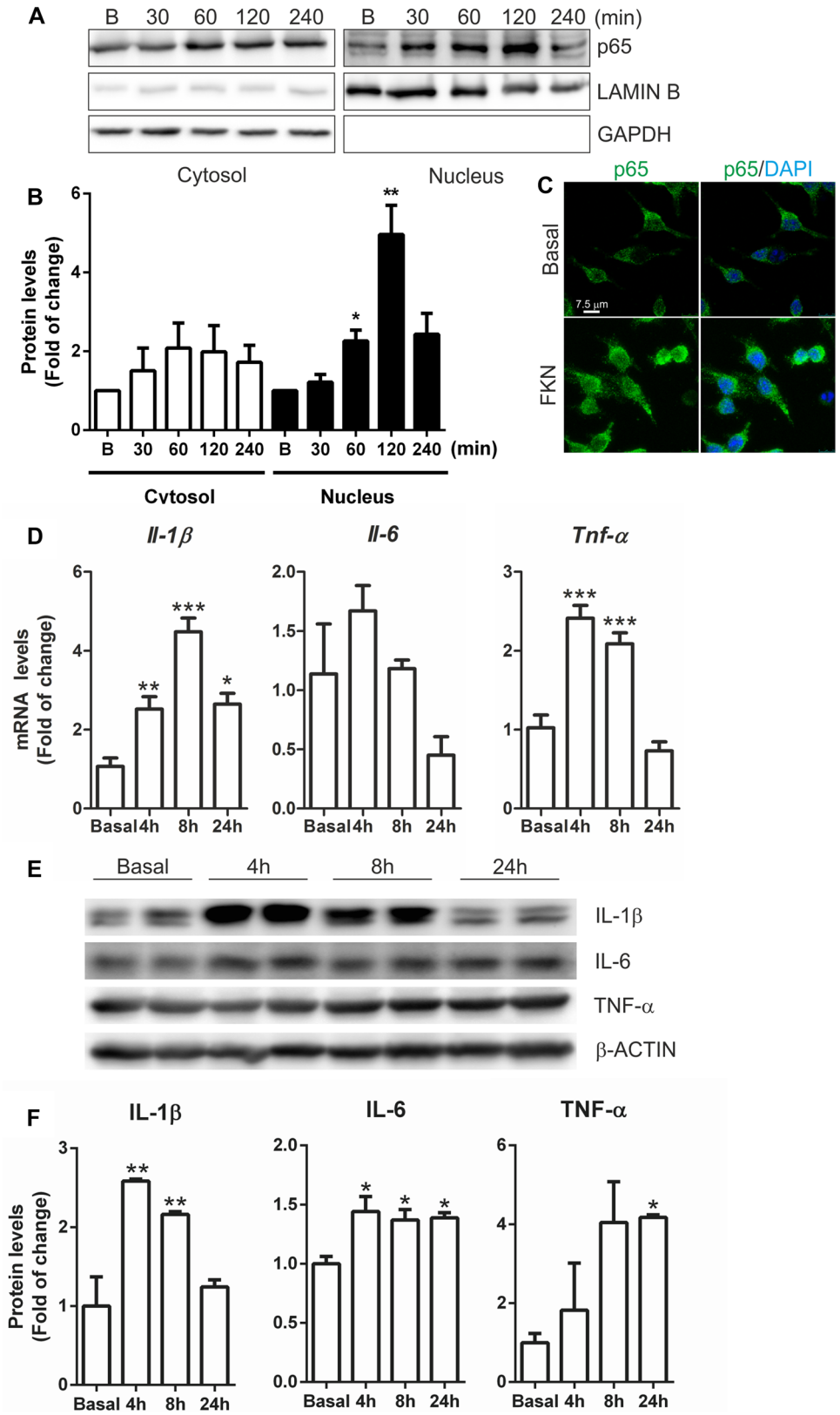


Fig. 1 *CX3CL1 induces NF-κB signalling in IMG microglia*. Cells were incubated in the presence of recombinant CX3CL1 (100 nM) for 30, 60, 120 and 240 min as indicated. **a** Analysis by subcellular fractionation in immunoblots: NF-κB levels (top); LAMIN B level was used as nuclear protein loading control (middle); GAPDH levels used as cytosol protein loading control (bottom). **b** Densitometric quantification of representative blots from A normalized for GAPDH levels for cytosol and LAMIN B levels for the nucleus. Bars indicate mean of $n = 2–3$ samples ± SEM. Asterisks denote significant differences $*p < 0.05$ and $**p < 0.01$, comparing the indicated groups with the basal condition according to a one-way ANOVA followed by Newman–Keuls post-test. **c** Immunohistochemistry for p65 in IMG microglial cells, which were serum-starved for 16 h and then treated with CX3CL1 (100 nM, 2 h.). **d** Cells were incubated in the presence of recombinant CX3CL1 (100 nM) for 4, 8 and 24 h and quantitative real-time PCR determination of messenger RNA for *Il-1β, Il-6* and *Tnf-α* was analysed and normalised by *Actb* (β-Actin) messenger RNA levels. **e** Cells were incubated in the presence of CX3CL1 for 4, 8 and 24 h. Immunoblot analysis in whole cell lysates of protein levels of IL-1β, IL-6, TNF-α and β-actin as loading control. **f** Densitometric quantification of representative blots from C normalized for β-ACTIN levels. Bars indicate mean of $n = 3–4$ samples ± SEM. Asterisks denote significant differences $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, comparing the indicated groups with the basal condition according to a one-way ANOVA followed by Newman–Keuls post-test

(Fig. 2e; Suppl. Fig. 1) in IMG cells treated with CX3CL1 (100 nM) for 1 h. Interestingly, the results indicate that CX3CL1 treatment induced the activation of two members of MAPK: extracellular signal-regulated kinase (ERK) and c-Jun *N*-terminal kinase (JNK) (Fig. 2e, f). The MAPKs are crucial players for microglial IL-1β production (Kim et al. 2004) and transmit a broad range of extracellular signals to mediate various intracellular responses in the brain (Kaminska et al. 2009). We reported previously that CX3CL1 was able to induce the PI3K/AKT/GSK-3β pathway (Lastres-Becker et al. 2014) in BV2 cells, which activates the transcriptional factor NRF2. These results are corroborated with these experiments in IMG cells (Fig. 2e, f). Our results showed that CX3CL1 also activated 5′-prime-AMP-activated protein kinase (AMPK), which has critical roles in regulating growth and reprogramming metabolism, as well as autophagy and cell polarity (Mihaylova and Shaw 2011). Furthermore, it has been reported that microglial polarization to the M2 phenotype and reduction of oxidative stress are mediated through activation of AMPK and NRF2 pathways (Wang et al. 2018), what confirms our results. Our data shows that CX3CL1 treatment activates SRC-family tyrosine kinases like YES, FGR, LCK and LYN in IMG. Perhaps, this activation is associated with the acquisition of such pro-inflammatory phenotype of the microglia as reported by Socodato et al. (Socodato et al. 2015). Interestingly, it has been reported that NF-κB activity can be regulated mainly by IκB family members, although the importance of additional mechanisms controlling the nuclear transcription potential of NF-κB is generally accepted. Mitogen- and stress-activated kinase-1 (MSK1) has been identified as a nuclear kinase for p65 since MSK1 associates with p65 in a stimulus-dependent way. This effect represents, together with phosphorylation of nucleosome components such as histone H3, an essential step leading to selective transcriptional activation of NF-κB-dependent gene expression (Vermeulen et al. 2003). Our data suggest that CX3CL1 is able to induce NF-κB signalling through MSK1 activation (Fig. 2E).

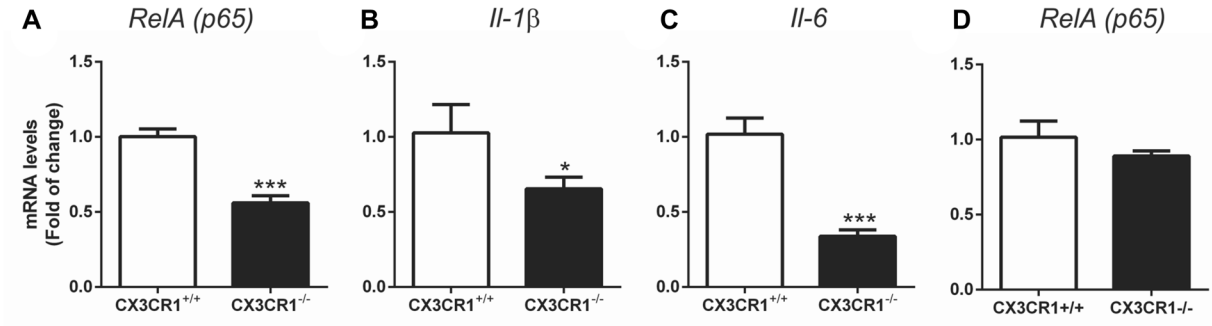
CX3CL1 Prompted MSK1-Dependent NF-κB-Signalling Activation

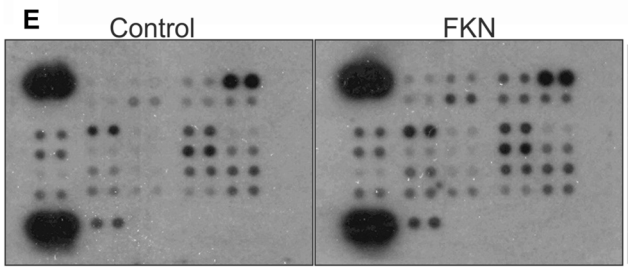
Previous studies suggest that MSK1, a downstream kinase of ERK1/2 and p38 (Fig. 4) (Kefaloyianni et al. 2006), positively regulates NF-κB-p65 signalling pathways (Peng et al. 2012). MSK1 enhances p65 transcriptional capacity by promoting phosphorylation of p65 at serine 276 and phosphorylates histone-3 at serine 10, which in turn promotes a permissive chromatin structure for transcriptional activity induced by NF-κB-p65. Our results show that CX3CL1 activated MSK1 (Fig. 2e). Hence, we investigated whether MSK1 downregulation could modulate the pro-inflammatory mechanisms triggered by CX3CL1 stimulation in microglia. For this purpose, we knocked down MSK1 with short interfering RNAs (siRNAs). IMG cells were transfected with siRNA against MSK1 (Fig. 3a) or scramble siRNA as control. Partial knockdown of the protein (about 50%, Fig. 3a) was able to reduce mRNA levels of *Il-1β, Tnf-α* and *iNos* produced by stimulation with CX3CL1 (Fig. 3b–d, respectively), indicating the relevance of MSK1 activation in the pro-inflammatory signalling pathway induced by CX3CL1/CX3CR1.

Discussion

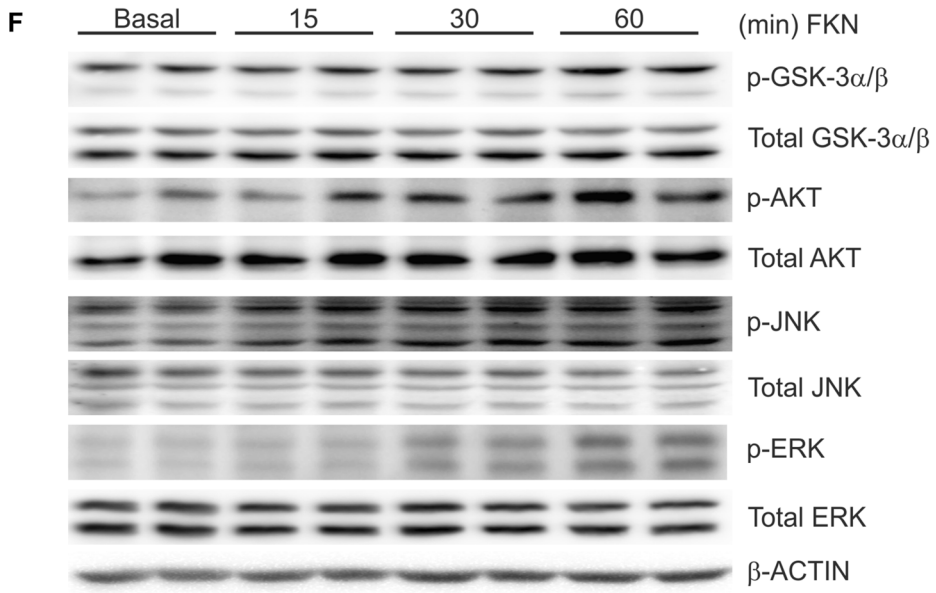
The chemokine axis CX3CL1/CX3CR1, with it is uniquely functional and structural characteristics, has been found to participate in inflammation (Jones et al. 2010) although its function has originated a huge controversy (Lauro et al. 2015; Sheridan and Murphy 2013). To gain insight into CX3CL1/CX3CR1 implications in inflammation, we studied its role in NF-κB signalling pathway in microglial cells. We showed for the first time that treatment of immortalized microglial cells (IMG) with CX3CL1-induced NF-κB-p65 translocation to the nucleus and increased pro-inflammatory cytokines expression (Fig. 1). The activation of NF-κB is an event that occurs relatively at short periods of time (between 30 min and 2 h), compared to the activation of the anti-inflammatory pathway of the transcription factor NRF2, which is delayed as previously shown (Lastres-Becker et al. 2014). These data indicate that CX3CL1 is able to activate NF-κB as an early pro-inflammatory event followed sequentially by the activation of NRF2 as an anti-inflammatory retarded fashion. These results are in agreement with previous reports, which indicate that after inflammatory challenge microglia execute a pro-inflammatory program including NF-κB activation followed by NRF2 anti-inflammatory retarded induction (Rojo et al. 2014; Cuadrado et al. 2014). This could explain why CX3CL1/CX3CR1 axis activation could have a dual role in microglial function, depending on the state of the cell and on how long after the challenge the cell state is analysed.

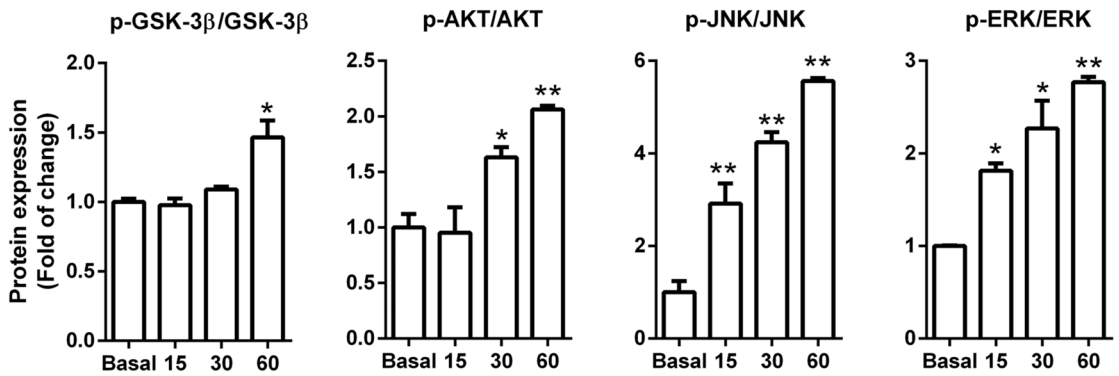
On the other hand, we showed for the first time that the absence of CX3CR1 impaired mRNA levels of NF-κB-p65 and genes regulated by this transcription factor in primary microglia (Fig. 2). Although it had been described that NF-κB can reverse-regulate CX3CL1 and CX3CR1 during CLP-induced sepsis (Raspe et al. 2013) and that CX3CL1 stimulated by NF-κB-dependent inflammatory signals induced aortic smooth muscle cell proliferation (Chandrasekar et al. 2003), our data confer a firm evidence of the relationship between CX3CL1/CX3CR1 and the signalling pathway of NF-κB as well as its involvement in the pro-inflammatory process in microglial cells.





Protein	Fold of change	Protein	Fold of change
ERK1/2/3	3.622 ± 0.391 *	CREB	1.278 ± 0.027 *
JNK1/2/3	2.019 ± 0.041 *	LYN	1.376 ± 0.073 *
GSK-3α/β	1.105 ± 0.001 **	LCK	1.267 ± 0.077 *
MSK1/2	2.054 ± 0.192 *	YES	1.655 ± 0.006 *
AMPKα1	2.134 ± 0.03 *	FGR	1.790 ± 0.280 *
AKT1/2/3	1.322 ± 0.022 **	FAK	1.903 ± 0.279 *





◂**Fig. 2** *CX3CR1 receptor implications in the transcription factors NF-κB signalling pathway and protein phosphorylation profiling induced by CX3CL1 in IMG cells*. Primary cultures of microglia from control wild-type mice (*Cx3cr1*$^{+/+}$) and Cx3cr1-knockout mice (*Cx3cr1*$^{-/-}$) were used. Quantitative real-time PCR determination of messenger RNA levels of NF-κB-regulated genes coding *RelA* (**a**), *Il-1β* (**b**) and *Il-6* (**c**) respectively, normalized by *Actb* (β-Actin) messenger RNA levels. **d** Astrocytes from *Cx3cr1*$^{-/-}$ mice do not show any alterations in the transcription factors NF-κB signalling. Primary cultures of astrocytes from *Cx3cr1*$^{+/+}$ and *Cx3cr1*$^{-/-}$ mice were used. Quantitative real-time PCR determination of messenger RNA levels of NF-κB-regulated gene coding RelA. Asterisks denote significant differences $*p<0.05$ and $***p<0.005$, assessed by t-student analysis. **e** IMG cells were treated with CX3CL1 (100 nM) for 1 h. Array spots were visualized in accordance with the manufacturer's instructions. The intensity of each spot was measured as described in "Materials and Methods". The table shows the relative fold change of proteins with significant difference upon CX3CL1 treatment, setting 1 for control (no treatment of CX3CL1). The data are shown as an average of two individual sets of samples. **f** IMG cells were incubated in the presence of recombinant CX3CL1 (100 nM) for 15, 30 and 60 min, as indicated. Analysis of total cell lysates in immunoblots: p-GSK-3α/β vs total GSK-3α/β levels; p-AKT vs total AKT levels; p-JNK vs total JNK levels and p-ERK vs total ERK levels. Densitometric quantification of representative blots is shown. Asterisks denote significant differences $*p<0.05$ and $**p<0.01$, comparing the indicated groups with the basal condition according to a one-way ANOVA followed by Newman–Keuls post-test

To determine the signalling pathways activated by the CX3CL1/CX3CR1 axis, we performed a phospho-kinase assay proteome profiler, which indicated that CX3CL1 triggers several kinase pathways such as ERK1/2, p38 and JNK that converge to MSK1-dependent NF-κB signalling activation (Figs. 2, 4). Interestingly, our data reveal the implication of MSK1 in NF-κB activation by CX3CL1 in microglia. Moreover, these results were corroborated in siMSK1 microglial cells, which displayed impaired NF-κB signalling after CX3CL1 treatment (Fig. 3). In addition, inflammatory gene expression is also co-determined by MAPK signalling (Vanden Berghe et al. 1998), identifying the downstream MSK1 as an essential NF-κB p65 S276 kinase (Vermeulen et al. 2003), but our results are the first evidence that the activation of microglia by CX3CL1 induces NF-κB signalling pathway through MSK1. Therefore, it is interesting to note the possibility that pharmacological treatments that modulate neuroinflammation may be altering the signalling pathway of MSK1/NF-κB as well. For example, dimethyl fumarate (DMF), which is an inducer of NRF2 signalling, with anti-inflammatory effects (Cuadrado et al. 2018; Lastres-Becker et al. 2016) is able to reduce the antigen presenting capacity of dendritic cells via suppression of NF-κB and MSK1 signalling (Peng et al. 2012). Further experiments are

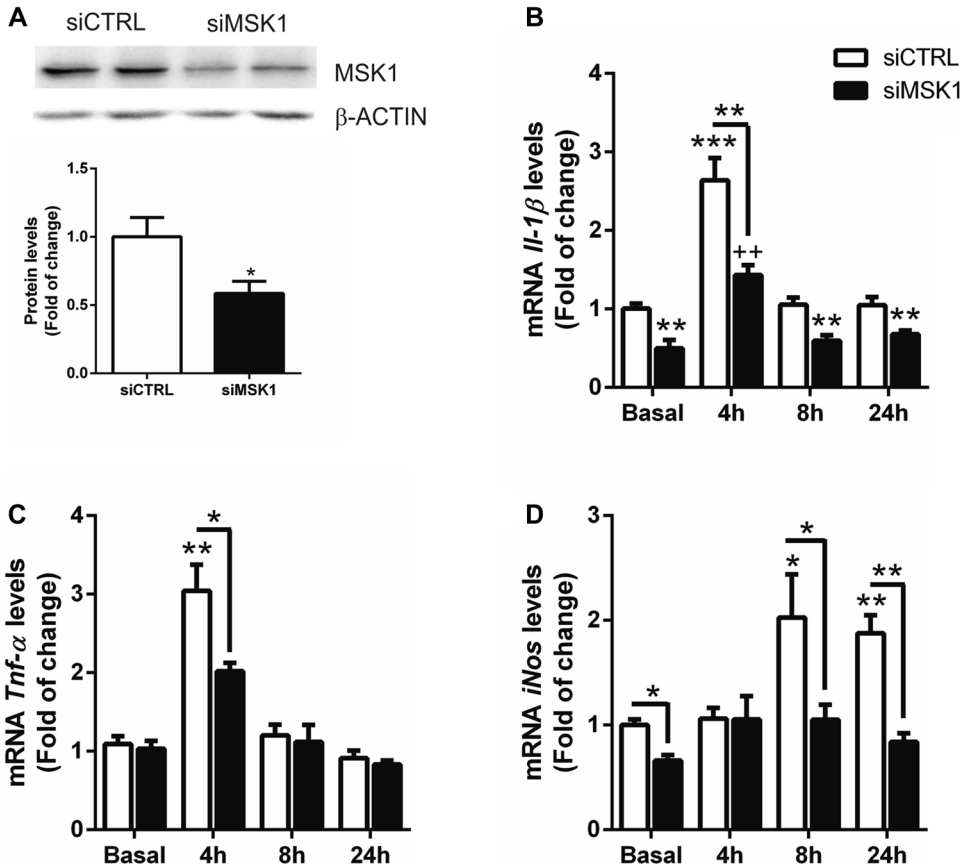


Fig. 3 *CX3CL1 activates NF-κB signalling in an MSK1-dependent manner*. **a** IMG cells were transfected with siRNAs for MSK1 or with a control scrambled siRNA. Cells were lysed at 48 h after siRNA transfection. Whole-protein lysates were immunoblotted with specific antibodies as indicated in the panels. **b**–**d** *Il-1β, Tnf-α* and *iNOS* mRNA levels, respectively, were analysed after siRNA knockdown and CX3CL1 (100 nM) treatment for 4, 8 and 24 h. Values are mean ± SEM (n = 4). Statistical analyses were performed with one-way ANOVA followed by Newman–Keuls multiple-comparison test. $*p<0.05$, $**p<0.01$, and $***p<0.001$ vs IMG cells transfected with scrambled siRNA

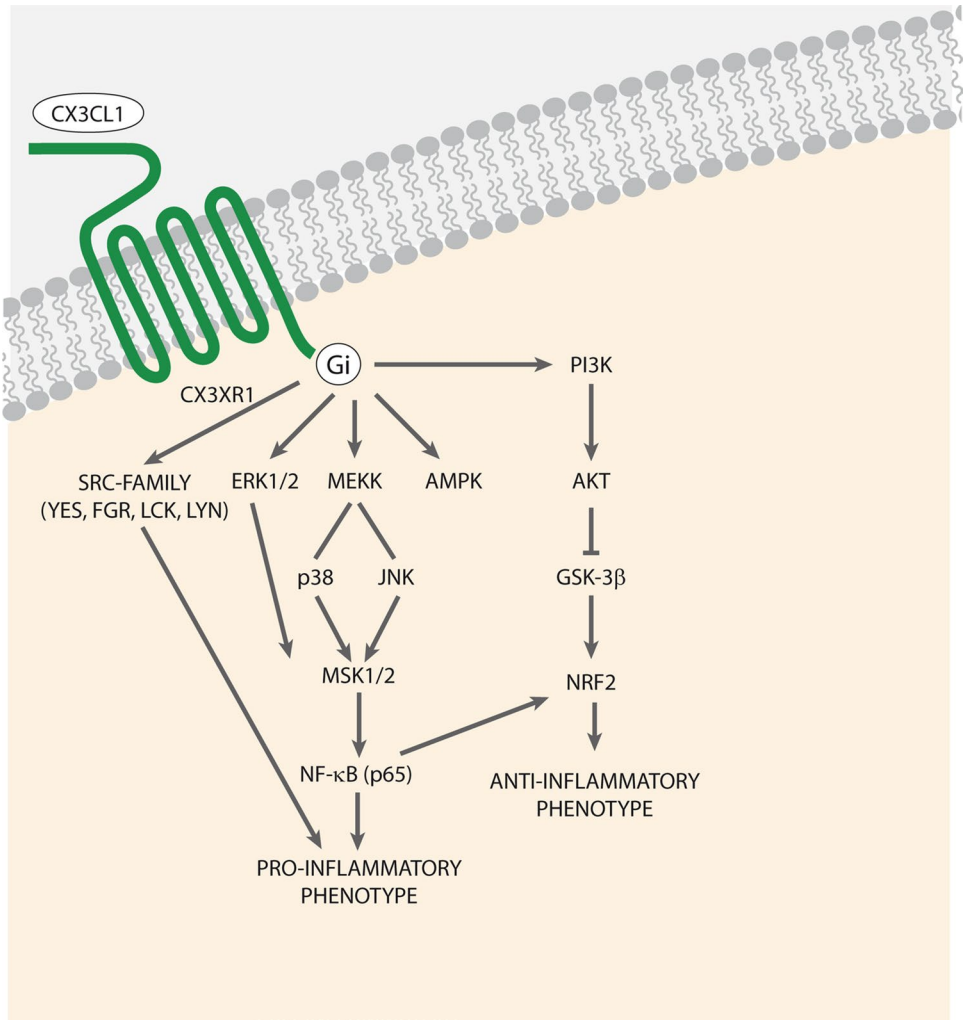


Fig. 4 *Molecular mechanisms implicated in CX3CL1/CX3CR1 signalling*. Scheme of the signalling pathways involved in the activation of the transcription factors NF-κB and NRF2, respectively, driven by CX3CL1/CX3CR1 activation

necessary to determine which kinases are essential in the activation pathway of CX3CL1/ MSK1/NF-κB.

It is also necessary to consider the possibility that the CX3CL1/CX3CR1 axis could be a biomarker and pharmacological target in neuroinflammation related to various pathologies. CX3CL1 levels in serum and cerebrospinal fluid (CSF) have been suggested to serve as a biomarker for reflecting symptomatic severity in osteoarthritis (Huo et al. 2015) or monitoring treatment outcomes (Wojdasiewicz et al. 2014). More related to the central nervous system, it has been also observed that CSF levels of soluble CX3CL1 are significantly higher in traumatic brain injury (TBI) patients than in healthy controls, suggesting that CX3CL1 function might be regulated by chemokine cleavage after TBI. Furthermore, it is plausible that CX3CL1/CX3CR1 signalling will also play a role in TBI pathogenesis. Interestingly, CX3CR1+ blood-derived monocytes, appear to contribute to axonal damage in the dorsal column crush model of spinal cord injury. Therefore, the effects of CX3CR1 modulation of TBI pathology need to be taken carefully before this signalling pathway can be considered for therapeutic procedures. (Gyoneva and Ransohoff 2015). In the same line of evidence, it has been shown that inhibition of CX3CL1/CX3CR1 signalling pathway promotes the recovery of neurological functioning after the occurrence of an early ischemic stroke (Liu et al. 2017). But as we have explained before, there are many discrepancies surrounding the benefit or harmfulness of the CX3CL1/CX3CR1 axis. Thus, in contrast to what we have just discussed, it has also been observed that the soluble isoform of CX3CL1 is sufficient for neuroprotection after exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a Parkinson's disease (PD) model. Specifically, it has been shown that the soluble CX3CL1 isoform reduces impairment of motor coordination, decreases dopaminergic neuron loss, and ameliorates microglial activation and proinflammatory cytokine release resulting from MPTP exposure. (Morganti et al.

2012). In addition, in our laboratory, we have shown that CX3CR1-deficiency exacerbates α-synuclein-A53T induced neuroinflammation and neurodegeneration in a mouse model of PD (Castro-Sanchez et al. 2018). All the data seem to indicate that, depending on the type of pathology, the CX3CL1/CX3CR1 axis may have different implications.

Overall, the present work suggests the involvement of MSK1/NF-κB activity in CX3CL1/CX3CR1 signalling and points to MSK1/NF-κB as possible pharmacological targets for therapeutic strategies to modulate neuroinflammation.

Author Contributions ILB contributed to conception and design of the study. MGG, AJGY and ILB acquisition and analysis of data. ILB contributed to drafting the manuscript and figures.

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Compliance with Ethical Standards

Conflict of interest None of the authors has a conflict of interest to declare. The authors alone are responsible for the content and writing of the paper.

Ethical Approval All experiments were performed by certified researchers according to regional, national, and European regulations concerning animal welfare and animal experimentation, and were authorized by the Ethics Committee for Research of the Universidad Autónoma de Madrid and the Comunidad Autónoma de Madrid, Spain, with Ref PROEX 279/14, following institutional, Spanish and European guidelines (Boletín Oficial del Estado (BOE) of 18 March 1988 and 86/609/EEC, 2003/65/EC European Council Directives).

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