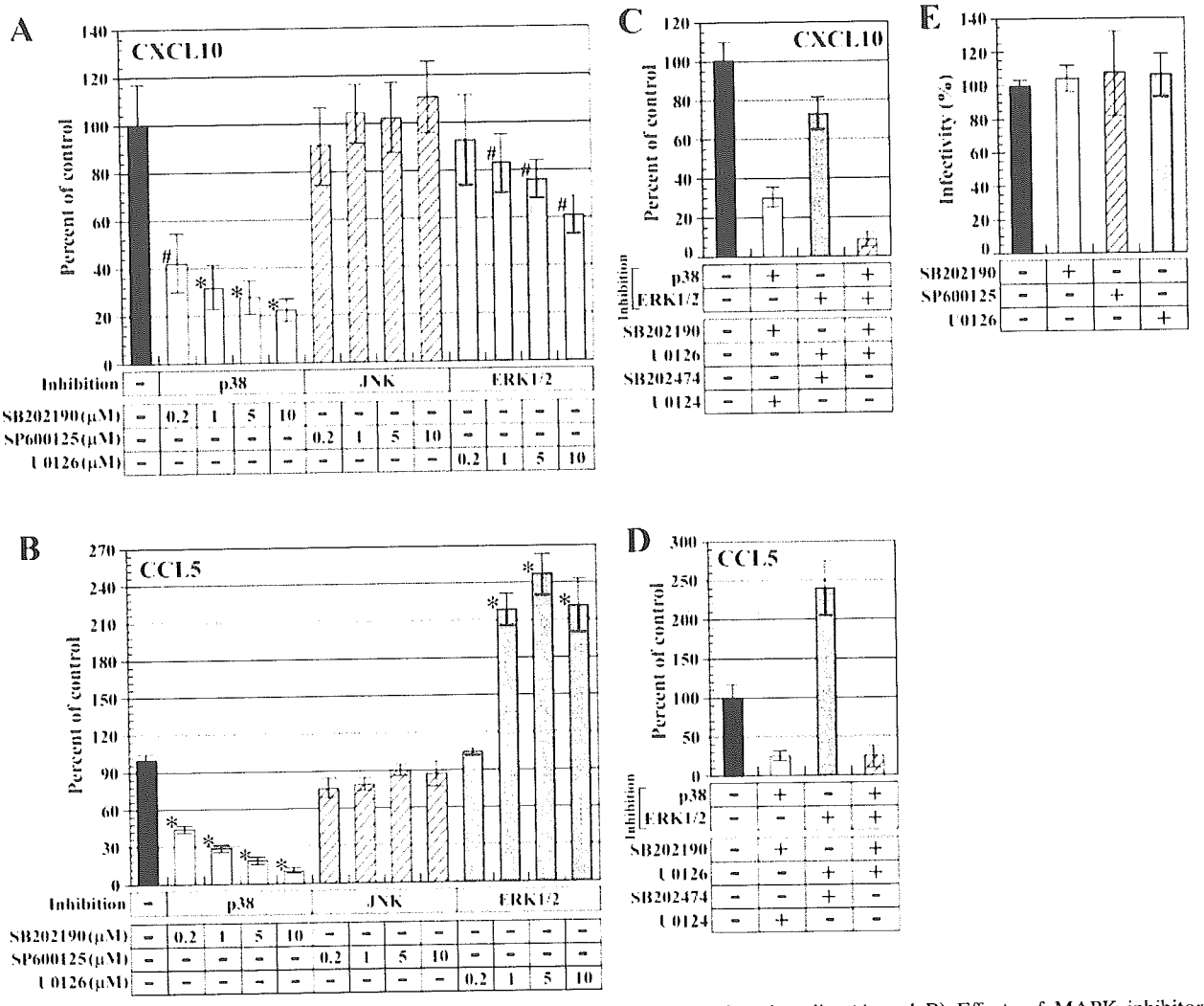

FIG. 4. MAPK signaling pathways regulate RV-induced chemokine expression in microglia. (A and B) Effects of MAPK inhibitors on chemokine expression in RV-infected cells. Ra2 cells were untreated (−) or treated with the indicated doses of SB202190, SP600125, and U0126 just prior to infection with CVS-11 virus. At 15 h postinfection, the protein contents of CXCL10 (A) and CCL5 (B) were determined. The results are shown as percentages of chemokine expression with reference to the values for the drug-untreated control. (C and D) Effect of simultaneous inhibitions of p38 and ERK1/2 pathways on RV-induced chemokine expression. The cells were untreated (−) or treated with SB202190 and U0126 (2.5 μM each) individually or in combination prior to infection with CVS-11 virus. SB202474 and U0124 were also used as controls to verify the specificities of SB202190 and U0126. After a 15-h incubation period, the protein levels of CXCL10 (C) and CCL5 (D) were determined. The percentages of chemokine expression were calculated with reference to the values for the drug-untreated control. (E) Effects of MAPK inhibitors on virus infectivity. Cells were pretreated with or without the indicated compounds (10 μM each) and infected with CVS-11 virus. At 24 h postinfection, virus infectivity was determined as described in the text. The results are shown as percentages of infectivity with reference to the values for the drug-untreated control. For each panel, the data are averages from three independent experiments, and the error bars represent standard deviations. Statistically significant differences are indicated by asterisks ($P < 0.01$) and pound signs ($P < 0.05$).

lowing the appropriate stimulus, IκB is phosphorylated by IκB kinase, ubiquitinated, and degraded by proteasomes. Degradation of IκB exposes the nuclear localization signal of NF-κB, and then NF-κB migrates to the nucleus and activates transcription (23). Since it remains unclear whether RV infection triggers the activation of the NF-κB pathway in mammalian cells, we examined the onset of NF-κB activation in RV-infected microglia (Fig. 5). Ra2 cells were uninfected or infected with CVS-11, and the phosphorylation and degradation of IκBα proteins were examined by Western blotting. In RV-infected cells, the signals of p-IκBα were detected between 10 and 15 h after infection (Fig. 5A, lanes 7 and 8), and only low, but detectable, levels of total IκBα were observed in RV-infected microglia at these time points (Fig. 5A, lanes 7 and 8). The alterations in the amounts of p-IκBα and total IκBα were not due to the difference in protein extracts loaded, as the protein levels of α-tubulin in each sample were comparable. As shown in Fig. 5B, the RV-induced phosphorylation and degradation of IκBα proteins were also observed for microglia, which had been infected with attenuated HEP-Flury virus (Fig. 5B, lane 4), and were markedly disrupted by the UV inactivation of RV virions (Fig. 5B, lanes 3 and 5). To confirm the NF-κB activation, microglia were mock infected or infected with CVS-11 virus, and the nuclear translocation of the NF-κB

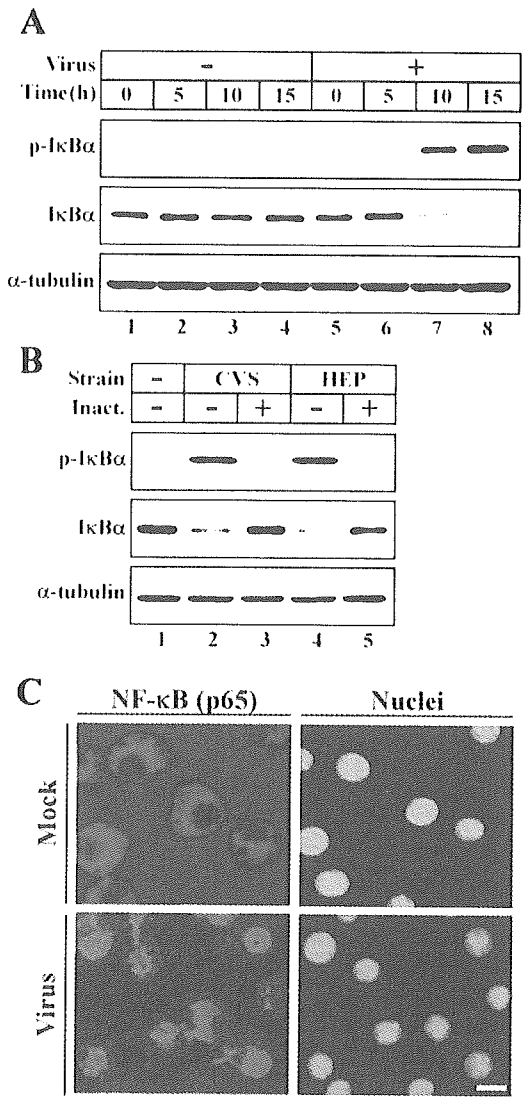

FIG. 5. RV infection activates the IκB–NF-κB signaling cascade in microglia. (A) Time course of IκBα phosphorylation and degradation in microglia following RV infection. Cells were mock infected (−) or infected with CVS-11 virus (+). After additional incubation for the indicated times, cells were harvested and subjected to Western blot analyses. The data are from one of three individual experiments with similar results. (B) Viral gene expression is required for the RV-induced phosphorylation of IκBα. Cells were incubated with or without CVS-11 and HEP-Flury virions, which had been untreated (−) or inactivated with UV irradiation (+) (Inact.). At 15 h postincubation, Western blot analyses were carried out as described for panel A. The data are from one of two individual experiments with similar results. (C) Nuclear translocation of NF-κB p65 in microglia infected with RV. Ra2 cells were mock infected (top) or infected with CVS-11 virus (bottom). At 15 h postinfection, the cells were fixed, and p65 proteins (left) and cell nuclei (right) were stained as described in the text. All images are confocal sections taken through the center of the cells. The experiments were repeated three times, and representative areas of each culture are shown. White bar, 10 μm.

p65 subunit was examined by confocal laser scanning microscopy (Fig. 5C). In mock-infected cells, the p65 molecules appeared to be dispersed in the cytoplasmic region (Fig. 5C, top). In contrast, when cells were infected with CVS-11, fluorescent signals of p65 were found primarily in the nuclei (Fig. 5C, bottom). These data demonstrate that RV infection of microglia results in the activation of the NF-κB signaling pathway.

RV infection stimulates chemokine expression via the IκB-NF-κB signaling pathway. We assessed whether the activation of the NF-κB signaling cascade is responsible for CXCL10 and CCL5 production in RV-infected microglia. Ra2 cells were preincubated with the increasing doses of CAPE, a chemical compound that has been shown to inhibit the nuclear translocation of NF-κB (37), or BAY 11-7082, an inhibitor of IκB phosphorylation (45), and then RV-induced chemokine expression in microglia was examined as described above. In the presence of either inhibitor, the RV-induced expression of CXCL10 (Fig. 6A) and CCL5 (Fig. 6B) was significantly abrogated in a dose-dependent fashion without affecting virus infectivity (Fig. 6C). Thus, these results indicate that RV infection stimulates CXCL10 and CCL5 production in microglia through activation of the IκB–NF-κB signaling cascade. We further investigated whether the activation of MAPK pathways is associated with NF-κB signaling in RV-infected microglia. Ra2 cells, which had been pretreated with MAPK inhibitors, were infected with CVS-11 virus, and the amounts of p-IκBα and total IκBα were examined by using Western blot analysis. Of the chemical compounds tested, the p38 inhibitor SB202190 partially but not completely diminished IκBα phosphorylation and degradation in RV-infected microglia (Fig. 6D, lane 3). As shown in Fig. 6E, the RV-induced phosphorylation of IκBα in SB202190-treated cells was reduced by about 72% compared to that in the untreated control. In the experiments shown in Fig. 6F, we examined the effect of p38 inhibitor on subcellular localization patterns of NF-κB p65 in RV-infected microglia. When Ra2 cells were pretreated with SB202190 and infected with CVS-11 virus, the RV-induced nuclear translocation of p65 proteins was inhibited (Fig. 6F, right) but not completely blocked (Fig. 6F, upper right panel). Taken together, these results suggest that the p38-mediated pathway is required for the efficient activation of NF-κB signaling in microglia in response to RV infection.

DISCUSSION

Microglia are resident immune effector cells within the CNS and are hence likely to encounter infectious agents at very early stages of infection, as well as at later stages, when peripheral leukocytes, such as lymphocytes and monocytes, are recruited into the brain parenchyma (26). Recruitment of leukocytes into the CNS is usually preceded by chemokine production from microglia and other CNS-resident cells, which is the first line of defense against neurotropic viruses (55).

It is well known that RV replication within the CNS occurs primarily in neurons and not in other CNS-resident cells, such as microglia and astrocytes. However, in vitro and in vivo studies provide evidence for the onset of viral gene expression in glial cells (17, 47, 49, 60), implying that although RV virions were taken up by glial cells, in which virus gene expression occurs, the production of virus progenies is impaired at the later stage of the viral replicative cycle. It is also reported that RV infection affects the characteristics of glial cells. Prosniak and colleagues have demonstrated that the mRNA expression levels of neuroleukin and the two isoforms of fibroblast growth

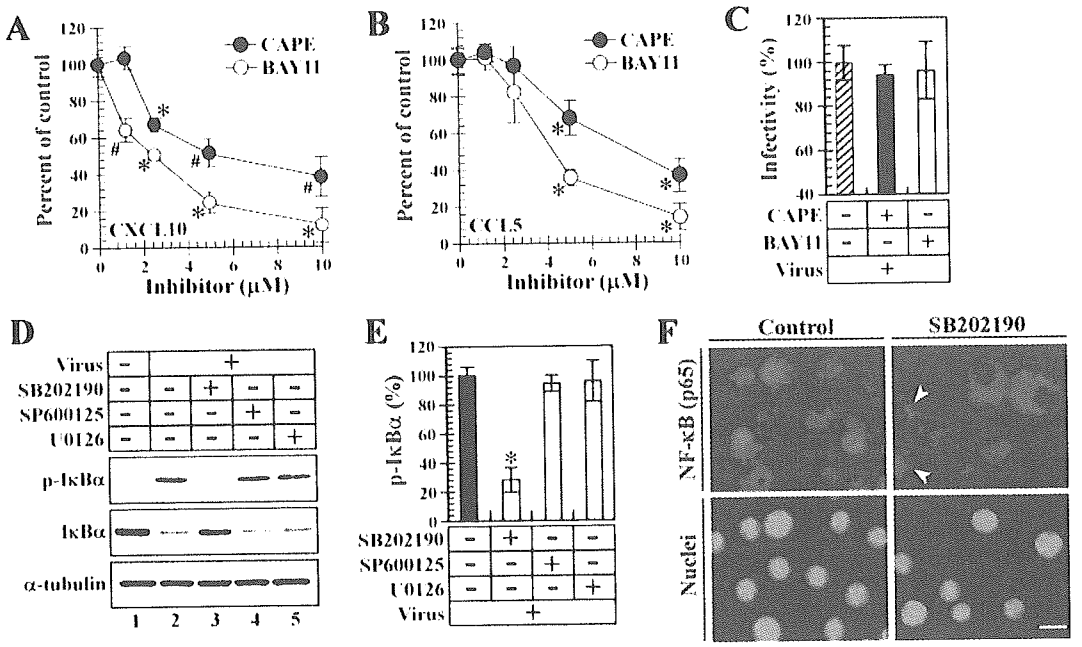

FIG. 6. RV infection stimulates chemokine expression via the NF-κB signaling pathway. (A and B) Effects of IκB and NF-κB inhibitors on the RV-induced expression of chemokines. Cells were untreated or treated with the indicated doses of CAPE and BAY 11-7082 and were infected with CVS-11 virus. At 15 h postinfection, the expression levels of CXCL10 (A) and CCL5 (B) were determined as described in the text. The results are shown as percentages of chemokine expression with reference to the values for the drug-untreated control. (C) Effects of IκB and NF-κB inhibitors on virus infectivity. Ra2 cells were pretreated with or without the indicated inhibitors (10 μM each) and infected with CVS-11 virus. At 24 h postinfection, the cells were fixed, and the percentages of virus plating were determined with reference to the values for the drug-untreated control. (D) IκB–NF-κB signaling is augmented by p38 activation in RV-infected microglia. Ra2 cells were pretreated with or without MAPK inhibitors (10 μM each) and infected with CVS-11 virus. After a 15-h incubation period, the cells were subjected to Western blot analyses. The data are from one of three separate experiments with similar results. (E) Quantitative analyses of IκBα phosphorylation in microglia following RV infection. The digital images of each blot shown in panel D were prepared, and the density of each band was quantified by image analysis. The percentages of band densities of p-IκBα were calculated with reference to the values for the drug-untreated control. For panels A, B, C, and E, mean values and standard deviations from the results of three individual experiments are shown, and significant differences are indicated by asterisks ($P < 0.01$) and pound signs ($P < 0.05$). (F) Effect of the p38 inhibitor on subcellular localization patterns of NF-κB p65 in RV-infected microglia. Cells were preincubated in the absence (left) or presence (right) of SB202190 and then infected with CVS-11 virus. At 15 h postinfection, the cells were stained for NF-κB p65 and cell nuclei. All images were taken through the centers of the cells. Similar results were obtained from two other experiments. Arrowheads indicate the p65 localization mentioned in the text. White bar, 10 μm.

factor-homologous factor 4 are significantly induced in RV-infected astrocytes in vitro and in vivo (47).

In the present study, as an initial step toward understanding the cellular response of microglia to RV infection, we have systemically examined the growth characteristics of RV and the activation of cellular signaling pathways leading to chemokine expression in these cell types. On the basis of our data, we provide a model for the cellular signaling events underlying microglial activation in response to RV infection (Fig. 7). We suggest here that RV virions are taken up by microglia into endosomal-lysosomal compartments and that virion uncoating occurs in the acidic environment of these vesicles. Although the virus-encoded proteins can be expressed in cytoplasms, the synthesis of the viral genome and the production of virus progenies were significantly impaired. As to the cellular response of microglia to RV infection, we also observed that the expression of two chemokines, CXCL10 and CCL5, is notably induced in RV-infected microglia. Furthermore, the data obtained here demonstrate that RV infection stimulates multiple signaling pathways mediated by NF-κB, p38, JNK, and ERK1/2 in microglia and that viral gene expression is required for the activation of these signal-transducing molecules. NF-κB-dependent signal transduction is a key process leading to the strong induction of CXCL10 and CCL5 expression in RV-infected microglia, and this signaling is indirectly augmented via the activation of the p38-mediated pathway. Our data also indicate that ERK1/2, but not JNK, partly contributes to the induction of CXCL10 expression and that it acts as a down-regulator of the excessive production of CCL5 in response to RV infection.

A recent study focusing on the overall expression profiles of host genes in the RV-infected brain has revealed that the predominant effect of RV infection is the down-regulation of gene expression (48). Consistent with these results in vivo, in the earlier study using culture of neuronal cells, we were unable to demonstrate marked changes in expression patterns of multiple host genes following RV infection (35). However, some reports indicate the RV-induced gene expression of a subset of cellular proteins, such as soluble factors and signal-transducing molecules, in the CNS (14, 48, 52). Galelli and colleagues demonstrated that the gene expression levels of chemokines, including CXCL10 and CCL5, are induced in mononuclear cell populations of the CNS infected with RV (14). Considering the potent chemotactic effects of CXCL10 and CCL5 on leukocytes, such as T cells and monocytes (39,

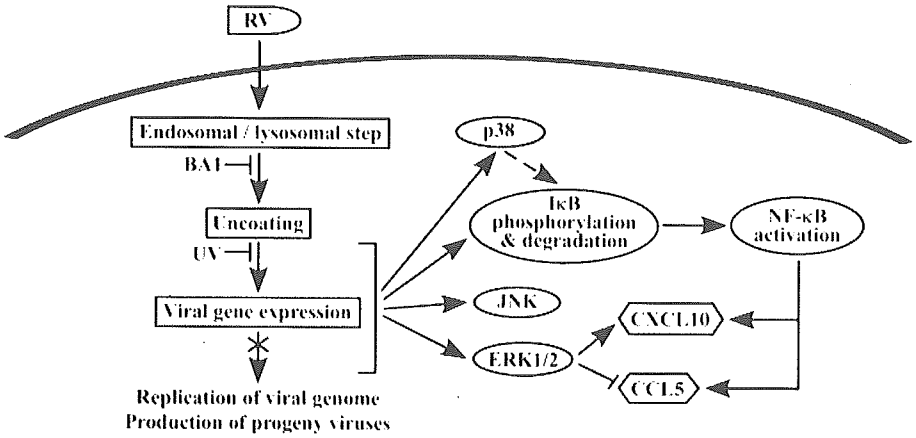

FIG. 7. Schematic model for the chemokine responses of microglia to RV infection. The model is based on the data in the present study and previous publications (see the text for the appropriate references). RV infection of microglia is initiated by the uptake of virions into endosomal-lysosomal compartments. Although microglia allows RV uncoating and expression of virus-encoded proteins, the replication of the viral genome and the production of virus progenies are impaired. RV infection of microglia leads to the activation of cellular signaling pathways mediated by MAPKs and IκB–NF-κB, and this process is triggered at the stage after viral gene expression. NF-κB signaling, which is additively augmented by p38 activation, is crucial for the expression of CXCL10 and CCL5 in the RV-infected microglia. The ERK1/2 pathway also participates in the induction of CXCL10 expression, while it acts as a down-regulator of the excessive production of CCL5 upon RV infection.

54), it is likely that the production of these chemokines is associated with the infiltration of mononuclear cells into the RV-infected CNS, as was reported previously (5, 14). Still, little information is available concerning the type of CNS-resident cells, which are able to produce these chemokines in response to RV infection. Our findings in the current study demonstrate the possibility that CNS-resident cells can produce CXCL10 and CCL5 via the recognition of RV infection. As for the activation of signal-transducing molecules evoked by RV infection, Dietzschold and colleagues previously suggested that the DNA-binding activities of AP-1, which is a downstream target of JNK pathway, and NF-κB are enhanced in the brains of RV-infected animals (9). The data obtained here provide direct evidence for the RV-induced activation of intracellular signaling pathways in CNS-resident cells.

Recent extensive studies have indicated that microglia intrinsically produces CXCL10 and CCL5 upon infection with a variety of neurotropic viruses, including cytomegalovirus (6), human immunodeficiency virus (58), herpes simplex virus (30), Newcastle disease virus (12, 63), Theiler's murine encephalomyelitis virus (42, 43), and Japanese encephalitis virus (7). However, the precise role of cell signaling molecules, especially that of MAPK subfamilies, in the virus-induced expression of these chemokines in microglia remains poorly understood. As for MAPK signaling being responsible for CXCL10 expression, a recent report suggests that the induction of CXCL10 production in microglia upon CMV infection is partly mediated by the p38 pathway (6). The results obtained in the present study indicate that ERK1/2, along with p38, is required for CXCL10 expression in RV-infected microglia. We also demonstrate that p38 and ERK1/2 participate in a different process leading to CXCL10 expression in microglia following RV infection, as the simultaneous inhibition of p38 and ERK1/2 additively decreased the CXCL10 levels. A previous study using culture of epithelial cells from peripheral tissue suggested that adenovirus entry into the cells induces CXCL10 expression via both the p38 and ERK1/2 pathways (62). Our findings are unique in that the activation of these MAPK pathways leading to CXCL10 expression is triggered at the step after virus entry, because UV-inactivated RV virions failed to induce MAPK phosphorylation.

As to CCL5 expression via MAPK signaling, it has been previously shown that the expression of this chemokine is induced via the p38 and ERK1/2 pathways in alveolar epithelial cells infected with respiratory syncytial virus (44). It has also been reported that p38, as well as JNK, is required for CCL5 production in bronchial epithelial cells following infection with influenza virus (28). On the other hand, a recent study focusing on herpes simplex virus-induced chemokine expression indicates that CCL5 responses in macrophages and fibroblasts against virus infection are induced by the p38-independent mechanism (31). From these studies, the contribution of MAPK signaling to CCL5 expression is considered to be dependent on the types of cells and viruses. With respect to the CCL5 response to neurotropic virus infection, Chen et al. (7) have demonstrated that the Japanese encephalitis virus-induced CCL5 production in mixed microglia-astrocyte cultures is partially induced via ERK1/2 activation. However, it remains unclear whether other MAPK pathways are responsible for virus-induced CCL5 production by microglia. The data shown in the present study constitute a body of evidence for virus-induced CCL5 expression through activation of the p38 signaling pathway in microglia. Furthermore, as a striking feature of chemokine expression in RV-infected microglia, we found that the activation of the ERK1/2 pathway negatively regulates virus-induced CCL5 expression. In view of our evidence that p38, as well as NF-κB, activates CCL5 expression in virus-infected microglia, it appears paradoxical that ERK1/2 inhibits the expression of this chemokine. However, the analysis of the kinetics of MAPK phosphorylation in RV-infected microglia demonstrated that p38 phosphorylation precedes the maximal activation of ERK1/2, rendering it likely that the ERK1/2 path-

way acts as a negative regulator of CCL5 expression. Previously, it has been suggested that the CCL5 response of microglia to human immunodeficiency virus infection is attenuated by the activation of p38 but not of ERK1/2 (58). Considering the strong chemotactic effect of CCL5 on a broad range of inflammatory cells (1, 54) and the lack of evidence for the MAPK-mediated down-regulation of CCL5 production in other cell types upon virus infection, it can be postulated that the MAPK signal pathways intrinsically adjust the level of CCL5 expression in microglia via recognition of virus infection, thereby controlling excessive leukocyte trafficking into the brain parenchyma.

An important body of knowledge has been accumulated about the chemokine expression mediated by the NF-κB pathway (32), while the NF-κB-dependent expression of CXCL10 and CCL5 in virus-infected microglia has been addressed in only a few studies (6, 7). In the present study, we show that NF-κB signaling pathway acts as a cardinal mediator of CXCL10 and CCL5 expression in virus-infected microglia, and our observation is consistent with earlier results from other investigators. As a remarkable feature of NF-κB-meditated chemokine expression, we found that NF-κB signaling can be augmented by p38 activation in RV-infected microglia. We also observed that the onset of this signal transduction was not completely blocked even in the absence of p38 activation, suggesting that the RV-induced expression of CXCL10 and CCL5 is achieved by two distinct mechanisms, the direct activation of NF-κB evoked by the recognition of virus infection and the p38-dependent transactivation of NF-κB signaling, both of which are required to fully activate chemokine expression in microglia. It is probable that the p38-independent activation of NF-κB in RV-infected microglia is mediated by the other signal-transducing molecules, such as phosphatidylinositol 3-kinase and double-stranded-RNA-activated protein kinase (33). Recent studies have demonstrated that the p38-dependent pathway activates NF-κB in response to stimuli, such as proinflammatory cytokines and environmental stress (8, 20, 56). However, much remains to be understood with regard to the convergence of these pathways during virus infection. Since IκB is not a substrate for p38 (56), it is likely that p38 may activate one or more of the signaling molecules upstream of IκB in the NF-κB cascade. Alternatively, as the p38 pathway participates in the induction of proinflammatory cytokines, such as interleukin 1β (33), which can activate the NF-κB pathway (4), it is possible that the RV-induced activation of p38 may facilitate proinflammatory cytokine expression, which subsequently induces NF-κB activation in an autocrine action. The strong induction of CXCL10 and CCL5 expression in RV-infected microglia may also be explained by a positive transcriptional synergy between NF-κB and the other transcription factors. It is well known that both CXCL10 and CCL5 are IFN-inducible chemokines (32), and in preliminary experiments, we observed that RV infection of microglia led to the induction of IFN-α and -β (data not shown). Accumulating evidence has indicated a transcriptional synergy between NF-κB and IFN-responsive transcription factors, such as signal transducer and activator of transcription (40, 41, 46). Thus, it is likely that IFNs secreted from RV-infected microglia stimulates IFN signaling in an autocrine manner, which subsequently facilitates chemokine gene expression in cooperation with NF-κB.

Overall, our study presented here provides an insight into the precise roles of intracellular signaling pathways mediated by MAPK and NF-κB in the regulation of chemokine expression in microglia through recognition of neurotropic virus infection.

ACKNOWLEDGMENTS

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Double-stranded RNA stimulates chemokine expression in microglia through vacuolar pH-dependent activation of intracellular signaling pathways

Kazuo Nakamichi,* Megumi Saiki,*,† Makoto Sawada,‡ Yutaka Yamamuro,† Kinjiro Morimoto* and Ichiro Kurane*

**Department of Virology 1, National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan*

†Department of Animal Science, College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa, Japan

‡Department of Brain Life Science, Research Institute for Environmental Medicine, Nagoya University, Nagoya, Aichi, Japan

Abstract

During neurotropic virus infection, microglia act as a source of chemokines, thereby regulating the recruitment of peripheral leukocytes and the multicellular immune response within the CNS. Herein, we present a comprehensive study on the chemokine production by microglia in response to double-stranded RNA (dsRNA), a conserved molecular pattern of virus infection. Transcriptional analyses of chemokine genes revealed that dsRNA strongly induces the expression of CXC chemokine ligand 10 (CXCL10) and CC chemokine ligand 5 (CCL5) in microglia. We also observed that the dsRNA stimulation triggered the activation of signaling pathways mediated by nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK). The microglial CXCL10 response to dsRNA was induced via NF-κB, p38, and JNK pathways, whereas the dsRNA-induced CCL5 production was dependent on JNK, but not on the other signal-transducing molecules tested. In addition, the acidic environment of intracellular vesicles was required for the activation of cellular signaling in response to dsRNA. Taken together, these results suggest that the recognition of dsRNA structure selectively induces the CXCL10 and CCL5 responses in microglia through vacuolar pH-dependent activation of NF-κB and MAPK signaling pathways.

Keywords: chemokine, double-stranded RNA, microglia, mitogen-activated protein kinase, nuclear factor-κB, Toll-like receptor 3.

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Because of the specialized blood–brain barrier and the relative lack of intraparenchymal leukocytes, the CNS as a healthy organ is immunologically quiescent (Barker and Billingham 1977). Microglia, the ontogenetic and functional equivalents of macrophages in somatic tissues, exert a central role in immune surveillance and host defense against infectious agents in the CNS (Dixon *et al.* 1991; Rock *et al.* 2004). Under normal conditions, microglia exist in a quiescent state lacking many of the effector functions and receptor expression patterns observed in macrophages. In response to brain infection with pathogenic microorganisms, especially that with neurotropic viruses, microglia readily transform into an activated state, acquiring numerous if not all the macrophage properties required to launch effective immune responses (Aloisi 2001). Once upon activation, microglia secrete various chemokines, which subsequently promote recruitment of peripheral leukocytes into the brain parenchyma and orchestrate a multicellular immune response against virus infection (Eugenin and Berman 2003).

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Address correspondence and reprint requests to Kazuo Nakamichi, PhD, Department of Virology 1, National Institute of Infectious Diseases, Shinjuku, Tokyo 162–8640, Japan.
E-mail: nakamich@nih.go.jp

Abbreviations used: BA1, bafilomycin A1; CAPE, caffeic acid phenethyl ester; CCL5, CC chemokine ligand 5; CXCL10, CXC chemokine ligand 10; dsRNA, double-stranded RNA; ERK1/2, extracellular signal-regulated kinases 1 and 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IκBα, inhibitory NF-κB α; IRF, IFN-regulatory factor; JNK, c-Jun N-terminal kinase; JAK, Janus kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; poly(I:C), polyinosinic-polycytidylic acid; TLR3, Toll-like receptor 3.

Chemokines are low molecular weight and structurally related molecules that are divided into four subfamilies, designated as C, CC, CXC, and CX_3C chemokine ligands based on the positions of their cysteine residues (Zlotnik and Yoshie 2000). Expression of most chemokines is regulated primarily at the level of transcription through activation of a specific set of transcription factors, such as nuclear factor κB (NF-κB) and interferon (IFN)-regulatory factor (IRF) (Melchjorsen *et al.* 2003). It has also been shown that the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38, participates in the activation of transcription factors that lead to chemokine expression (Mogensen and Paludan 2001).

Double-stranded RNA (dsRNA) represents a conserved molecular pattern of virus infection, which can be sensed by innate immune cells. In infected cells, this structure arises from viral genome comprising dsRNA, genome replication of single-stranded RNA viruses, or complementary mRNA encoded on opposite strands of DNA viruses that anneal to form dsRNA structures (Saunders and Barber 2003). Single-stranded RNAs with extensive secondary structures such as hairpin loop formations are also effective source of dsRNA (Saunders and Barber 2003). A growing body of evidence has demonstrated that the dsRNA structures are recognized by Toll-like receptor 3 (TLR3) or by the dsRNA-activated protein kinase, initiating downstream signaling that leads to consequential expression of chemokines and other types of immunomodulatory molecules (Mogensen and Paludan 2001). However, it remains totally unknown whether the recognition of dsRNA structure stimulates cellular signaling and consequential chemokine expression in microglia.

In this report, we have systemically analyzed the expression profiles of chemokine genes and the activation of signal-transducing molecules that contributes to microglial chemokine response to dsRNA. We demonstrate here that synthetic dsRNA, polyinosinic-polycytidylic acid [poly(I:C)], strongly induces the production of two chemokines, CXC chemokine ligand 10 (CXCL10) and CC chemokine ligand 5 (CCL5), in microglia through activation of NF-κB and MAPK signaling pathways.

Materials and methods

Reagents and antibodies

Granulocyte macrophage colony-stimulating factor was obtained from Genzyme (Cambridge, MA, USA). Poly(I:C), lipopolysaccharide (LPS) from *Escherichia coli* (serotype O111:B4), bovine serum albumin, and bafilomycin A1 (BA1), were purchased from Sigma (St. Louis, MO, USA). BAY 11-7082, caffeic acid phenethyl ester (CAPE), Janus kinase (JAK) inhibitor I, U0126, SB202190, and monensin were obtained from EMD Biosciences, Inc. (San Diego, CA, USA). SP600125 was purchased from Biomol (Plymouth Meeting, PA, USA). Rabbit antibodies against inhibitory NF-κB α (IκBα) and phosphorylated forms of ERK1/2 and JNK were purchased from Santa Cruz Biotechnology (Hercules, CA, USA). Antibodies specific for ERK1/2, p38, JNK, and α-tubulin, as well as horseradish peroxidase-linked secondary antibodies, were purchased from Sigma. Antibodies against phosphorylated p38 and IκBα were obtained from New England Biolabs (Beverly, MA, USA).

Cells

Microglial cell line, Ra2, was established by spontaneous immortalization of primary microglia from the normal brain tissue of neonatal C57BL/6 mouse (Sawada *et al.* 1998; Suzumura *et al.* 1998). Ra2 cells closely resemble primary microglia with respect to morphology, phagocyte function, expression of microglia-specific molecules, and high migrating activity to the brain (Sawada *et al.* 1998; Suzumura *et al.* 1998; Inoue *et al.* 1999; Kanazawa *et al.* 2000). Ra2 cells were cultivated in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA, USA), insulin (5 μg/mL), 0.2% glucose, granulocyte macrophage colony-stimulating factor (2 ng/mL), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cell cultures were maintained at 37°C in a humidified incubator containing 5% CO_2 in air.

Reverse transcription–polymerase chain reaction analysis

Semi-quantitative RT–PCR analysis to determine the transcription profiles of chemokine genes has been described in the previous report (Nakamichi *et al.* 2004). Ra2 cells were plated at a density of 2.4×10^6 in the 60-mm diameter dishes, and were incubated in Eagle's minimum essential medium containing 0.5% fetal calf serum, 0.2% glucose, and the above antibiotics (hereafter called test medium) with or without poly(I:C) (50 μg/mL). After incubation of the cells for the appropriate times, total RNA was extracted by using Isogen (Nippon Gene, Tokyo, Japan), and the first-strand complementary DNAs (cDNAs) were generated from RNA preparations by using oligo-(dT) primer and reverse transcriptase (Takara Bio Inc., Shiga, Japan). PCR primers were synthesized on the basis of the published oligonucleotide sequences for cDNA amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and chemokines (Neumann *et al.* 1999; Lacroix-Lamande *et al.* 2002), and their specificities and optimum PCR conditions have been described previously. The absence of contaminating genome DNA was verified by PCR using RNA preparations not treated with reverse transcriptase. PCRs were performed with serially fivefold-diluted cDNA preparation as described previously (Nakamichi *et al.* 2004), and the amplified products were separated by using 1.8% agarose gel electrophoresis and visualized by ethidium bromide staining. Real-time PCR was performed on each cDNA preparation using the above-mentioned primers, LightCycler DX400 (Roche, Penzberg, Germany), and LightCycler DNA Master SYBR Green I (Roche) according to the manufacturer's protocol. The relative amounts of chemokine cDNA were normalized with reference to those of GAPDH cDNA.

Measurement of chemokine production

Ra2 cells were plated at a density of 2×10^6 per well in the six-well culture dishes and stimulated with poly(I:C) as described above. After incubation for the appropriate times, the culture fluids were separated, and the protein levels of CXCL10 and CCL5 contents were determined

by using DuoSet ELISA Development kits for mouse IP-10/CRG-2/CXCL10 and RANTES/CCL5 (R & D Systems Inc., Minneapolis, MN, USA), respectively, according to the manufacturer's protocols.

Western blot analysis

The activation of NF-κB and MAPK pathways was measured by immunoblotting using antibodies against phosphorylated forms of IκBα, ERK1/2, p38, and JNK as described elsewhere (Nakamichi *et al.* 2004). Ra2 cells, which had been plated at a density of 7×10^6 in the 90-mm-diameter culture dishes, were incubated in test media with or without poly(I:C) (50 μg/mL). At the appropriate time points, the cells were washed with phosphate-buffered saline and lysed directly with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitors (Complete Mini; Roche), and phosphatase inhibitor cocktails (Sigma). Extracts were clarified by centrifugation at 12 000 *g* for 20 min at 4°C. Each sample, containing 10 μg of proteins, was separated under reducing conditions in 0.4% sodium dodecyl sulfate–12% polyacrylamide gels, and was transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The blots were blocked with 2% bovine serum albumin in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl) containing 0.1% Tween 20 and incubated with the primary antibodies. The proteins were reacted with the horseradish peroxidase-linked secondary antibodies and visualized with an enhanced-chemiluminescence Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ, USA) and photographed using an enhanced-chemiluminescence mini camera (Amersham Biosciences). For quantification of IκBα and MAPK phosphorylation, the digital images of each blot were prepared, and the band densities were measured by using Scion Image (Scion Corp., Frederick, MD, USA) according to the manufacturer's instruction.

Inhibition of cellular signaling pathways

Inhibition of IκB/NF-κB and MAPK signaling pathways was performed essentially as described previously (Tibbles *et al.* 2002; Jaramillo *et al.* 2003; Wu *et al.* 2003; Nakamichi *et al.* 2004). Briefly, the cells were incubated for 1 h at 37°C in test media containing BAY 11-7082, CAPE, JAK inhibitor I, U0126, SB202190, SP600125, BA1, or monensin just prior to the experiment, and were stimulated with poly(I:C) (50 μg/mL) or LPS (50 ng/mL) in the presence of each inhibitor. After incubation of the cells for the appropriate times, the cells and supernatants were subjected to the above-described analyses. Under the assay conditions, these inhibitors did not induce any cytotoxic effects as judged by a dye exclusion test using trypan blue (Nakamichi *et al.* 2001).

Statistics

The significance of differences between groups was statistically determined by Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Chemokine expression in microglia in response to double-stranded RNA

In order to elucidate whether the recognition of dsRNA induces chemokine expression in microglia, we first assessed the transcription patterns of multiple chemokine genes in microglial Ra2 cells following stimulation with poly(I:C), which is a synthetic dsRNA extensively used as a functional analogue of viral dsRNA (Mogensen and Paludan 2001). Ra2 cells were incubated in the absence or presence of poly(I:C), and the relative mRNA levels of three major chemokine subfamilies, CXC, CC, and CX_3C chemokines, were examined by semiquantitative RT–PCR (Fig. 1). When Ra2 cells were treated with poly(I:C), the strong signals of

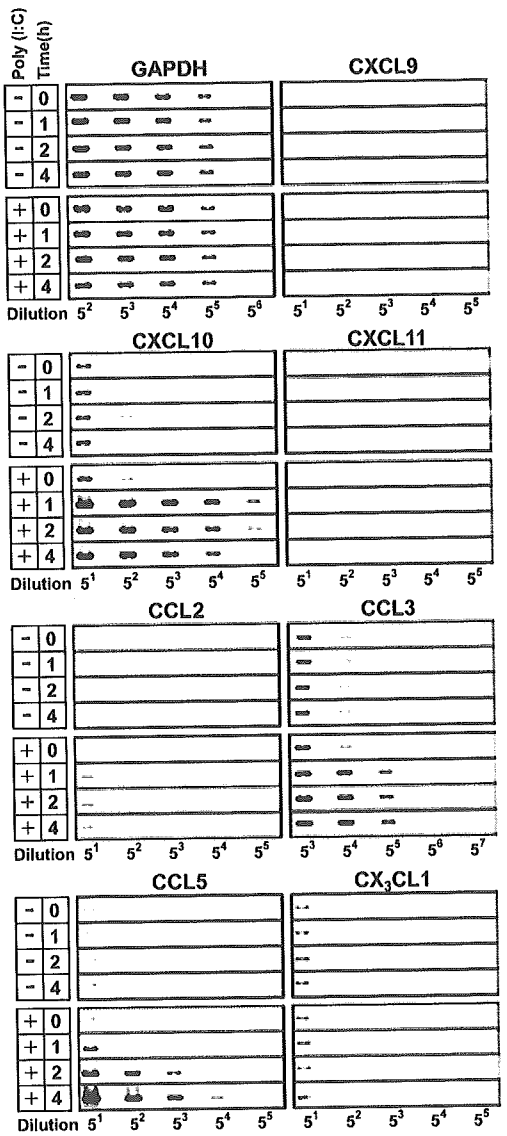

Fig. 1 Effect of double-stranded RNA on chemokine gene expression in microglia. Microglial cells were incubated with (+) or without (–) polyinosinic-polycytidylic acid [poly(I:C), 50 μg/mL]. At the indicated time points, total RNAs were extracted from each culture and subjected to RT–PCR analyses for the chemokine gene expression by using serially five-fold diluted cDNA preparations. The data are from one of three independent experiments with similar results.

amplified products of CXCL10 and CCL5 were observed at highly diluted concentrations of PCR templates between 1 and 4 h after stimulation compared to those of mock-stimulated cells. We also observed that the poly(I:C) treatment increases the transcription levels of CCL2 and CCL3 within fivefold through 25-fold ranges, but the induction of these chemokines appeared to be weaker than that of CXCL10 and CCL5. Based on these results, we sought to systemically investigate the mechanism underlying the strong induction of CXCL10 and CCL5 in microglia in response to dsRNA. In the experiments shown in Figs 2(a) and (b), the kinetics of CXCL10 and CCL5 transcription were monitored quantitatively by using real-time PCR method as described above. When Ra2 cells were stimulated with poly(I:C), the expression levels of CXCL10 and CCL5 genes peaked around 2 h (Fig. 2a) and 6 h (Fig. 2b) after stimulation, respectively, and they decreased at later time points. To assess whether the dsRNA-induced transcription of CXCL10 and CCL5 genes correlates with the enhanced protein production, we measured the levels of CXCL10 and CCL5 in culture supernatants of microglia following dsRNA stimulation. Figures 2(c) and (d) show the kinetics of CXCL10 and CCL5 production in microglia in the presence or absence of poly(I:C). In the poly(I:C)-treated microglia, the onsets of CXCL10 and CCL5 expression were detected after 4 h of incubation, and the chemokine levels were significantly increased between 4 and 16 h after incubation compared to those in mock-treated cells ($p < 0.05$). When microglia were incubated with the increasing concentrations of poly(I:C) for 16 h, the production of either chemokine was promoted in a dose-dependent manner (Figs 2e and f). Taken together, these results indicate that the recognition of dsRNA pattern greatly induces the gene expression and protein production of two chemokines, CXCL10 and CCL5, in microglia.

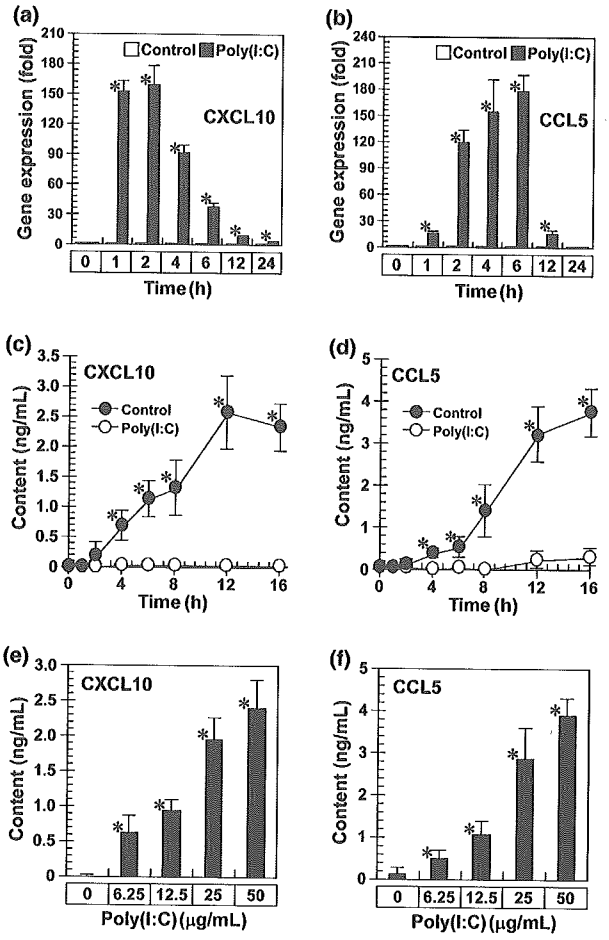

Fig. 2 CXC chemokine ligand 10 (CXCL10) and CC chemokine ligand 5 (CCL5) responses in microglia upon stimulation with double-stranded RNA (dsRNA). (a and b) Kinetics of chemokine gene expression in microglia following dsRNA stimulation. Ra2 cells were incubated with or without polyinosinic-polycytidylic acid [poly(I:C), 50 μg/mL], and the relative amounts of CXCL10 and CCL5 cDNAs were determined by using real-time PCR method as described in the text. The results are shown as *n*-fold increase in the amounts of chemokine cDNA with reference to the cDNA levels in poly(I:C)-untreated cells 0 h after incubation. (c and d) Time course of chemokine production in microglia following dsRNA treatment. Microglial cells were incubated with or without poly(I:C), and the protein contents of CXCL10 (c) and CCL5 (d) in culture supernatants were determined by ELISA. (e and f) Dose-dependent effect of dsRNA on chemokine production in microglia. Ra2 cells were incubated with the indicated concentrations of poly(I:C) for 16 h, and the protein contents of CXCL10 (e) and CCL5 (f) were determined as described above. Mean values and standard deviations from results of three separate experiments are shown. Statistically significant differences are indicated by asterisks ($p < 0.05$).

Roles of nuclear factor-κB and janus kinase signaling in microglial chemokine responses to double-stranded RNA

The enhanced production of CXCL10 and CCL5 in the dsRNA-treated microglia implies that dsRNA may stimulate undefined cellular signaling pathway underlying the chemokine expression. Considering the important roles of NF-κB signaling cascade in transcriptional regulation of chemokine gene expression (Baeuerle and Henkel 1994; Melchjorsen *et al.* 2003), we examined whether the dsRNA-induced chemokine responses in microglia are achieved by NF-κB pathway. NF-κB is normally found in the cytoplasm in a latent form associated with IκB, of which various isoforms exist (Karin and Ben Neriah 2000). Following the appropriate stimulus, IκB is phosphorylated by IκB kinase, ubiquitinated, and degraded proteasomes, leading to nuclear translocation of NF-κB and consequential activation of transcription (Karin and Ben Neriah 2000). In the experiments shown in Fig. 3(a), we monitored the IκBα phosphorylation and degradation as hallmarks of the activation of NF-kB signaling cascade in the dsRNA-stimulated microglia. When microglia were treated with poly(I:C), the onset of IκBα phosphorylation was readily seen after 0.5 h of

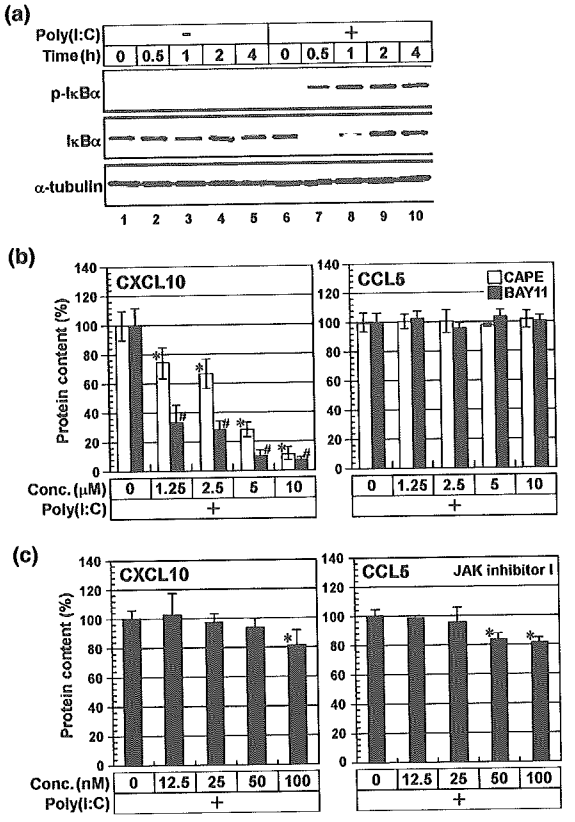

Fig. 3 Effect of nuclear factor κB (NF-kB) and Janus kinase (JAK) inhibitors on the double-stranded RNA (dsRNA)-induced chemokine responses in microglia. (a) Time course of inhibitory NF-κB α (IκBα) phosphorylation and degradation in microglia upon dsRNA stimulation. Microglial cells were incubated with (+) or without (–) polyinosinic-polycytidylic acid [poly(I:C), 50 μg/mL] and subjected to Western blot analyses of phosphorylated and total IκBα at the time points indicated. The amounts of α-tubulin were also assessed to monitor the equal loading of protein extracts. The data are from one of three individual experiments with similar results. (b and c) The dsRNA-induced chemokine production in microglia in the presence of NF-κB and JAK inhibitors. The cells were pretreated with the indicated doses of caffeic acid phenethyl ester (CAPE) and BAY 11-7082 (BAY11) (b) or JAK inhibitor I (c) just prior to the stimulation with poly(I:C) (50 μg/mL). After a 15-h incubation period, the protein levels of CXC chemokine ligand 10 (CXCL10) and CC chemokine ligand 5 (CCL5) were determined by ELISA. The percentages of chemokine contents were calculated with reference to the values for drug-untreated control. Mean values and standard deviations from results of three separate experiments are shown. Statistically significant differences are indicated by * and # ($p < 0.05$).

incubation (Fig. 3a, lane 7), and the strong signals of phosphorylated IκBα were detected at later time points (Fig. 3a, lanes 8–10). In the presence of poly(I:C), the total amounts of IκBα proteins were markedly decreased between 0.5 and 1 h after incubation (Fig. 3a, lanes 7 and 8), and the nuclear translocation of NF-κB was observed within the same time frame as IκBα degradation (data not shown). We assessed whether the activation of NF-κB pathway is responsible for CXCL10 and CCL5 production in the dsRNA-stimulated microglia (Fig. 3b). Ra2 cells were preincubated with the increasing doses of CAPE, a chemical compound that has been shown to inhibit nuclear translocation of NF-κB (Natarajan *et al.* 1996), or BAY 11-7082, an inhibitor of IκBα phosphorylation (Pierce *et al.* 1997), and then the poly(I:C)-induced production of CXCL10 and CCL5 proteins was determined as described above. As shown in Fig. 3(b), treatment of microglia with CAPE or BAY 11-7082 significantly diminished the poly(I:C)-induced CXCL10 production ($p < 0.05$), and maximal concentrations of these compounds (10 μM) abrogated the CXCL10 levels by about 90% compared to those of drug-untreated cells. On the other hand, the poly(I:C)-induced CCL5 production in microglia was unchanged, even in the presence of high concentrations of inhibitors tested. These results demonstrate that the NF-κB signaling pathway mediates the dsRNA-induced production of CXCL10, but not that of CCL5, in microglia. As both CXCL10 and CCL5 are known to be IFN-inducible chemokines (Melchjorsen *et al.* 2003), we next assessed the possibility that dsRNA activates JAK signaling via any undefined mechanism, thereby facilitating chemokine production in microglia. As shown in Fig. 3(c), when Ra2 cells were treated with increasing doses of potent inhibitor direct against JAK and tyrosine kinase 2 (Thompson *et al.* 2002), the dsRNA-induced production of CXCL10 and CCL5 was slightly decreased at high doses of this compound that did not induce cytotoxicity.

Role of mitogen-activated protein kinase pathways in microglial chemokine responses to double-stranded RNA

Seeing that the NF-κB and JAK inhibitors did not completely block the dsRNA-induced CCL5 production in microglia, we hypothesized that the other signaling pathways to CCL5 expression might be operating in microglia. Based on these observations, and on the important roles of MAPK-mediated signaling pathways in gene expression, the next set of experiments was carried out to examine whether the dsRNA recognition triggers the MAPK activation in microglia. Figure 4 shows the time course of MAPK phosphorylation in microglia in the presence or absence of dsRNA. When microglia were treated with poly(I:C), the phosphorylated forms of ERK1/2 and JNK1/2 were readily observed at time points up to 1 h (Fig. 4, lanes 7 and 8), whereas the amounts of phosphorylated p38 was increased gradually between 1 and 4 h after incubation (Fig. 4, lanes 8–10), indicating that the dsRNA activates MAPK signaling pathways in microglia. We further investigated the contribution of MAPK pathways to the dsRNA-induced production of CXCL10 and CCL5 in microglia. The cells were pretreated with the increasing concentrations of

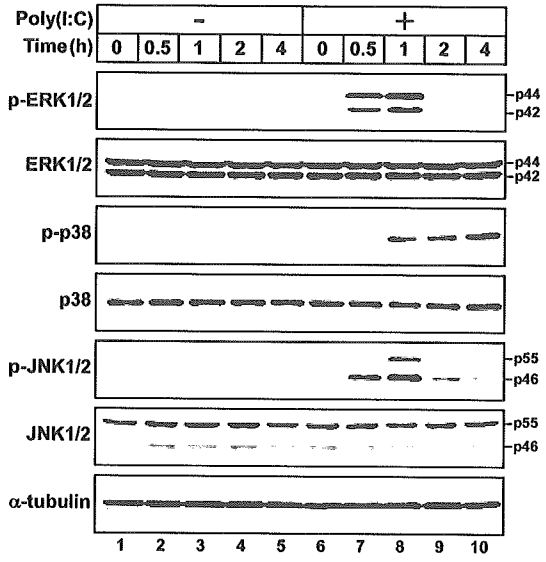

Fig. 4 Activation of mitogen-activated protein kinase (MAPK) signaling pathways in microglia following double-stranded RNA stimulation. Microglial cells were incubated in the presence (+) or absence (–) of polyinosinic-polycytidylic acid [poly(I:C), 50 μg/mL], and were subjected to Western blot analyses of phosphorylated and total MAPK molecules at the indicated time points. Data are from one of three individual experiments with similar results.

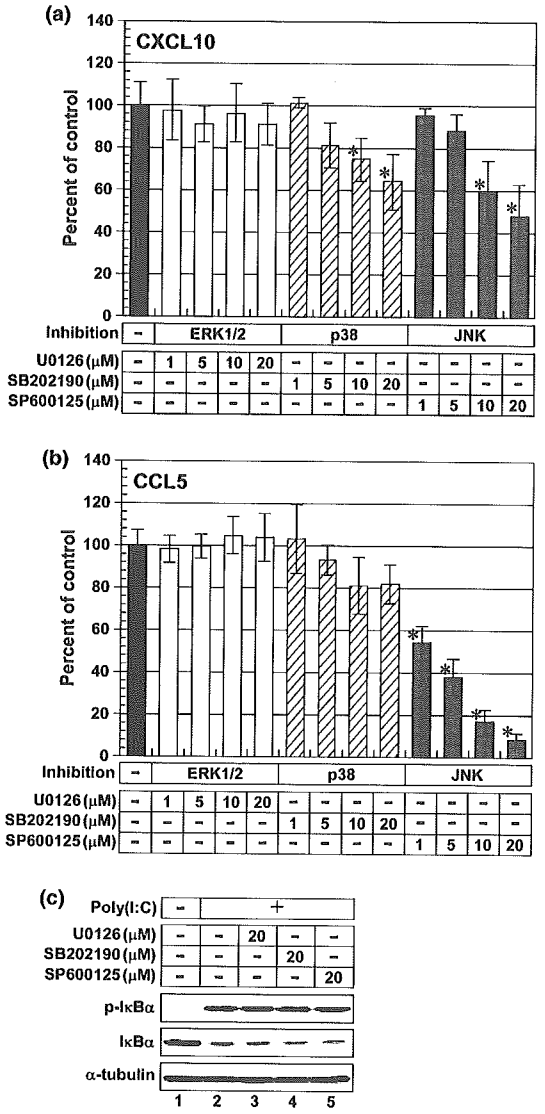

Fig. 5 Mitogen-activated protein kinase (MAPK) pathways participate in the double-stranded RNA (dsRNA)-induced chemokine production in microglia. (a and b) Effect of MAPK inhibitors on microglial chemokine response to dsRNA. Microglial cells were pretreated with the indicated doses of chemical compounds and then stimulated with polyinosinic-polycytidylic acid [poly(I:C), 50 μg/mL]. After a 15-h incubation period, the amounts of CXC chemokine ligand 10 (CXCL10) (a) and CC chemokine ligand 5 (CCL5) (b) were determined as described in the text. The percentages of chemokine contents were calculated with reference to the values for drug-untreated control. Mean values and standard deviations from results of three independent experiments are shown. Statistically significant differences ($p < 0.05$) are indicated by asterisks (comparison to the first bar from left). (c) Effect of MAPK inhibitors on inhibitory NF-κB α (IκBα) phosphorylation and degradation. Microglial cells, which had been pretreated with the indicated chemical compounds, were stimulated with poly(I:C) (50 μg/mL) for 1 h, and were subjected to Western blot analyses of phosphorylated-IκBα, total IκBα, and α-tubulin as described in the legend to Fig. 3(a). The data are from one of three separate experiments.

U0126, SB202190, and SP600125 to inhibit the activation of ERK1/2, p38, and JNK, respectively, and then the production of CXCL10 (Fig. 5a) and CCL5 (Fig. 5b) in microglia upon stimulation with poly(I:C) was determined as described above. As shown in Fig. 5(a), the inhibition of p38 and JNK led to dose-dependent reduction in the poly(I:C)-induced CXCL10 response in microglia, whereas ERK1/2 inhibitor had little or no effect on CXCL10 production. These results suggest that the dsRNA-induced CXCL10 response was partly mediated by p38 and JNK pathways in microglia. The protein levels of CCL5 were severely reduced in the presence of JNK inhibitor, and the maximal diminution of CCL5 production was observed when the cells were treated with the highest dose of this compound (20 μM). We also observed that the CCL5 expression was slightly decreased in microglia treated with p38 inhibitor compared to that in drug-untreated cells, but these differences were not statistically significant. These observations indicate that the dsRNA-induced production of CCL5 in microglia is primarily achieved through the activation of JNK signaling pathway. As shown in Fig. 5(c), the levels of IκBα phosphorylation and degradation evoked by poly(I:C) were not affected by the treatments with each MAPK inhibitor, rendering it unlikely that the dsRNA-induced MAPK activation mediates the chemokine production via indirect augmentation of NF-κB activity.

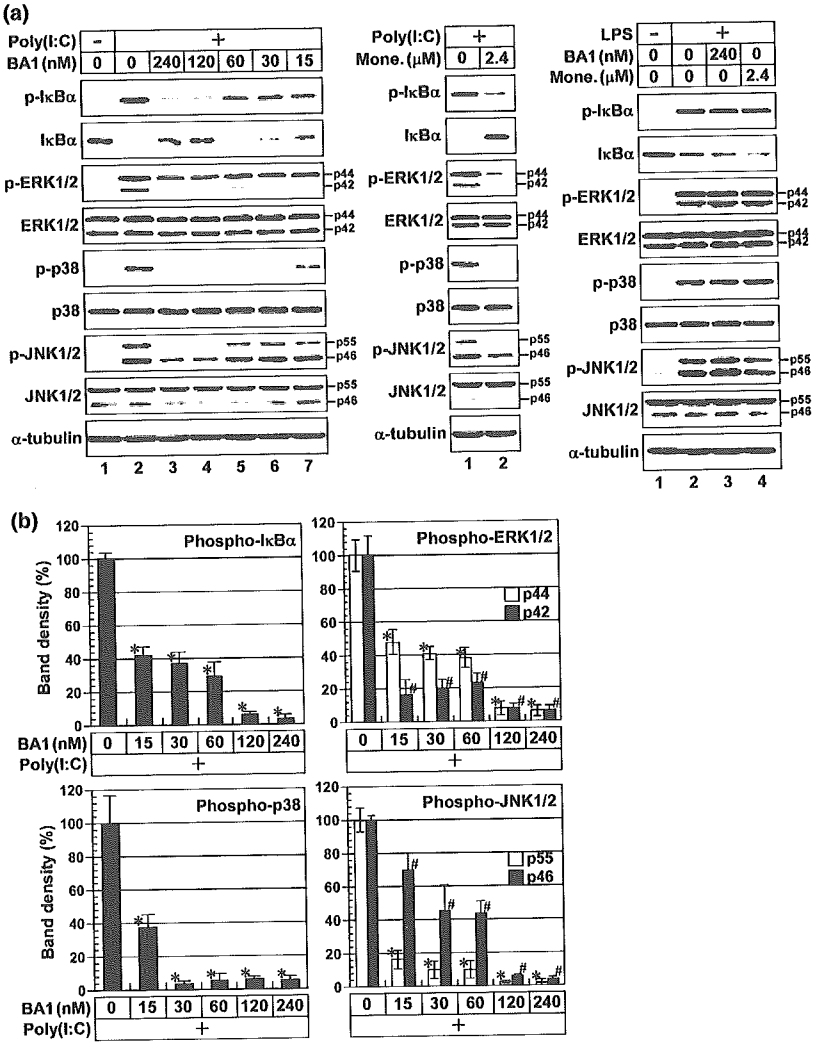

Fig. 6 Effect of endosomal pH-neutralizing agents on the activation of signaling pathways in the double-stranded RNA (dsRNA)-stimulated microglia. (a) Microglial cells were pretreated with the indicated doses of bafilomycin A1 (BA1) and monensin (Mone.) and then stimulated with polyinosinic-polycytidylic acid [poly(I:C), 50 μg/mL] or lipopolysaccharide (LPS: 50 ng/mL) for 1 h. The protein extracts from each culture were subjected to Western blot analyses as described in the text. The data are one from three independent experiments with similar results. (b) The digital images of each blot shown in (a) were prepared, and the density of each band was quantified by image analysis. The percentages of band densities of phosphorylated proteins were calculated with reference to the values for the drug-untreated control. Mean values and standard deviations from results of three separate experiments are shown. Statistically significant differences ($p < 0.05$) are indicated by asterisks and hashes (comparison to the values for drug-untreated cells).

Requirement of vacuolar acidification for double-stranded RNA-induced activation of signaling pathways in microglia

Recent studies using culture of dendritic cells suggested that TLR3 molecules, which recognize dsRNA pattern, are localized in intracellular vesicles (Matsumoto *et al.* 2003; Funami *et al.* 2004). In the experiments shown in Fig. 6, we examined whether the acidic environment of these vesicles is required for the dsRNA-induced activation of signaling pathways in microglia. To prevent endosomal acidification, microglia are pretreated with the indicated concentrations of BA1, a selective and potent inhibitor of vacuolar proton pump (H^+-ATPase) (Gagliardi *et al.* 1999) or monensin, an ion carrier that increases vesicular pH (Prabhananda and Kombrail 1992), and were then stimulated with poly(I:C). We also examined the effects of BA1 and monensin on the LPS-induced activation of signal-transducing molecules, which is known to have low sensitivity to vacuolar pH neutralization in macrophages (Weber and Levitz 2000; He and Kogut 2003). As shown in Fig. 6(a), the dsRNA-evoked phosphorylation of IκBα, ERK1/2, p38, and JNK1/2 in microglia was dramatically decreased in the presence of BA1 and monensin, whereas the treatment with these compounds had little or no effect on the LPS-induced phosphorylation of signal-transducing molecules. To assess the effect of endosomal neutralization on the activation of signal-transducing molecules more quantitatively, Western blotting was independently repeated three times, and the relative amounts of phosphorylated IκBα and MAPK proteins were measured by using image analyses as described above. As shown in Fig. 6(b), the dsRNA-induced phosphorylation of IκBα, ERK1/2, and JNK1/2 was significantly decreased in the presence of BA1 ranging from 15 to 60 nM, and a more dramatic diminution was seen when high doses of this compound were added (120 and 240 nM). The levels of p38 phosphorylation were readily abrogated in the presence of BA1 at doses between 30 and 240 nM. Therefore, these results demonstrate that the endosomal acidification is

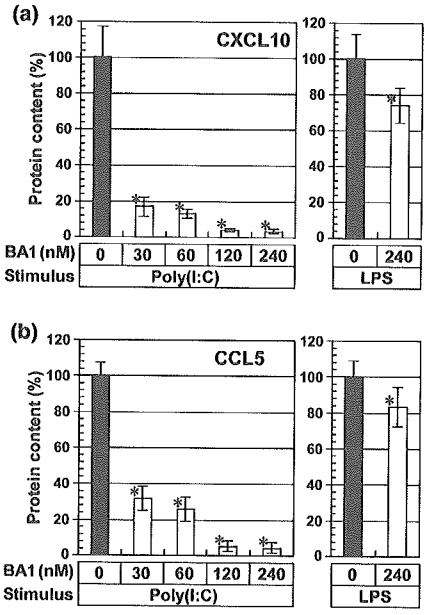

Fig. 7 Effect of endosomal pH-neutralizing agents on the double-stranded RNA-induced production of CXC chemokine ligand 10 (CXCL10) and CC chemokine ligand 5 (CCL5) in microglia. The cells were pretreated with the indicated concentrations of bafilomycin A1 (BA1) and then stimulated with polyinosinic-polycytidylic acid [poly(I:C), 50 μg/mL] or lipopolysaccharide (LPS: 50 ng/mL) for 15 h. The protein contents of CXCL10 (a) and CCL5 (b) were determined as described in the text. The percentages of chemokine contents were calculated with reference to the values for drug-untreated control. Mean values and standard deviations from results of three individual experiments are shown. Statistically significant differences ($p < 0.05$) are indicated by asterisks.

required for the activation of signaling pathways in microglia through recognition of dsRNA structure. If the dsRNA-induced production of CXCL10 and CCL5 was attributable to the activation of NF-κB and MAPK pathways, then the neutralization of vesicular pH should interfere with the production of these chemokines. As shown in Figs 7(a) and (b), the production of CXCL10 and CCL5 in the poly(I:C)-stimulated microglia were dose-dependently decreased in the presence of BA1, and the treatment with maximal concentration of this compound (240 nM) nearly completely diminished the dsRNA-induced chemokine production in microglia. In contrast, the LPS-induced production of either chemokine had low sensitivity to vacuolar pH neutralization. These data show that the dsRNA-induced expression of CXCL10 and CCL5 in microglia is mediated by the vacuolar pH-dependent activation of intracellular signaling pathways.

Discussion

The blood–brain barrier serves as a protective mechanism for the brain by preventing entry of most pathogens from free access to the CNS. However, neurotropic viruses enter the CNS in infected migratory leukocytes, by intraneuronal transfer from peripheral nerves, or by transcytosis across blood–brain barrier via direct infection of cerebral endothelial cells (Zhang and Tuomanen 1999). Microglia are resident immune effector cells within the CNS and are hence likely to encounter infectious agents at very early stages of infection, as well as at later stages, when peripheral leukocytes are recruited into the brain parenchyma (Kreutzberg 1996). It is well known that CXCL10 and CCL5 exert potent chemotactic effects on peripheral leukocytes such as T cells and monocytes (Schall *et al.* 1988; Neville *et al.* 1997). Recent extensive studies have indicated that these chemokines are produced by microglia upon infection with a variety of neurotropic viruses (Lokensgard *et al.* 2001; Palma and Kim 2001; Si *et al.* 2002; Cheeran *et al.* 2003; Chen *et al.* 2004; Olson and Miller 2004).

In this report, we present a comprehensive study on the induction of chemokines by measuring and correlating events from cellular signaling to chemokine responses in microglia upon stimulation with dsRNA, a conserved molecular pattern of virus infection. On the basis of our data, we suggest that the recognition of dsRNA structure strongly induces the expression of CXCL10 and CCL5. To the best of our knowledge, we believe this to be the first report showing the dsRNA-induced expression of these chemokines in microglia. Previous study suggested that the poly(I:C) stimulation of microglia has no effect on the gene expression levels of CCL5 and other chemokines (Olson and Miller 2004), and these observations are contrasted by our current data. However, these differing results can be explained by the kinetics of CCL5 gene expression. In the previous investigation, the level of CCL5 transcription was investigated at 24 h post-stimulation with poly(I:C), whereas its expression appeared to be triggered at earlier time points, as demonstrated in the present study.

The data obtained in the current study also demonstrate that the microglial CXCL10 response to dsRNA was primarily achieved by NF-κB pathway, and was partly mediated through p38 and JNK activation. As the dsRNA-induced IκBα phosphorylation was not diminished by p38 and JNK inhibitors, it is postulated that these MAPK pathways mediate CXCL10 expression without affecting the NF-κB activity. Previous studies suggested that several stimuli, such as lipopolysaccharide, CD40 ligand, human immunodeficiency virus Tat protein, IFN-γ, and cytomegalovirus infection, activate CXCL10 expression in microglia via cellular signaling mediated by NF-κB, signal transducer and activator of transcription-1, p38, ERK1/2, phosphatidylinositol 3-kinase (D'Aversa *et al.* 2002; Si *et al.* 2002; Cheeran *et al.* 2003; Delgado 2003; D'Aversa *et al.* 2004). In addition to these signal-transducing molecules, our current data constitute the first evidence that the JNK-mediated pathway contributes to microglial CXCL10 response.

Recent studies suggested that the dsRNA-induced CCL5 expression in peripheral tissue-derived cell types, such as epithelial cells, fibroblasts, and macrophages, are mediated through activation of NF-κB, IRF3, dsRNA-activated protein kinase, and phosphatidylinositol 3-kinase (Gern *et al.* 2003; Guillot *et al.* 2004; Ieki *et al.* 2004; McWhirter *et al.* 2004). Although the cellular signaling, which regulates microglial CCL5 response to dsRNA, has not been reported, the accumulating evidences suggest that the microglial activation evoked by several stimuli results in CCL5 expression via signaling pathways that involve NF-κB, ERK1/2, and nuclear factor interleukin-6 (Hu *et al.* 1999; Delgado *et al.* 2002; Jang *et al.* 2002; Kim *et al.* 2002; Chen *et al.* 2004; D'Aversa *et al.* 2004). In the present study, we demonstrated that the JNK activation is crucial for the dsRNA-induced CCL5 response in microglia. To our knowledge, the present study is the first report demonstrating a JNK-dependent CCL5 production during microglial activation. It is probable that the dsRNA-induced stimulation of JNK pathways leads to downstream activation of transcription factors, culminating in transcription of CCL5 gene (Melchjorsen *et al.* 2003).

Our results obtained in this study seem to be unique in that the CXCL10 and CCL5 responses in microglia are differentially regulated by intracellular signaling pathways. It is known that the promoter regions of CXCL10 and CCL5 genes contain recognition sites for NF-κB and activator protein-1, a downstream target of JNK pathways (Melchjorsen *et al.* 2003). However, the dsRNA-induced CCL5 production in microglia was not accompanied by NF-κB activation, despite the fact that the inhibition of this signaling markedly diminished CXCL10 response. We also observed that the dsRNA-induced production of CCL5 in microglia was notably impaired in the absence of JNK activation when compared to that of CXCL10. Much remains to be understood about this differential regulation of CXCL10 and CCL5 responses in microglia upon dsRNA recognition. However, considering the strong chemotactic effects of either chemokine on inflammatory cells, it seems reasonable that the gene expression of CXCL10 and CCL5 in microglia is intrinsically up-regulated by a specific set of transcription factors, thereby controlling the excessive leukocyte trafficking into the brain parenchyma.

The poly(I:C)-induced production of CXCL10 and CCL5 was slightly decreased by treatment with JAK inhibitor. These results imply that JAK signaling partly mediates chemokine production in dsRNA-stimulated microglia. In preliminary experiments, the poly(I:C) treatment did not lead to the activation of JAK signaling within the same time frame as the activation of NF-κB and MAPK pathways (data not shown). Thus, it seems possible that cytokines, such as type 1 IFNs, secreted from dsRNA-treated microglia autocrinely stimulates JAK signaling, which subsequently facilitates chemokine expression at later time points.

The striking finding in this study is that the acidic environment within intracellular vesicles is crucial for the dsRNA-induced activation of cellular signaling pathways and the consequential production of CXCL10 and CCL5 in microglia. Although a previous report indicated that endosomal/lysosomal compartments are required for the maturation of cellular proteases (Sastradipura *et al.* 1998), the roles of these vesicles in microglial function, especially that in cellular activation upon pathogen recognition, remains to be elucidated. The results from two recent studies using peripheral tissue-derived cells imply that the NF-κB signaling is triggered by the poly(I:C)/TLR3 association in intracellular vesicles and that the endosomal acidification is required for the dsRNA-induced activation of IFN-β promoter and interleukin-12 production (Matsumoto *et al.* 2003; Funami *et al.* 2004). Our data obtained in the current study provide evidence that the dsRNA-evoked activation of MAPK, as well as that of NF-κB, depends on acidic milieu of intracellular vesicles.

In conclusion, the results obtained in the present study demonstrate that the recognition of dsRNA structure triggers the activation of NF-κB and MAPK signaling pathways, leading to the strong induction of CXCL10 and CCL5 production in microglia. Our study provides an insight into the mechanism involved in microglial responses to the conserved molecular pattern of virus infection.

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