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*J Immunol* 2000; 164:3222-3228; doi: 10.4049/jimmunol.164.6.3222

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p38 Mitogen-Activated Protein Kinase and c-Jun-NH2-Terminal Kinase Regulate RANTES Production by Influenza Virus-Infected Human Bronchial Epithelial Cells

Kousei Kujime,* Shu Hashimoto,2* Yasuhiro Gon,* Kazufumi Shimizu,† and Takashi Horie*

Airway epithelial cells which are the initial site of influenza virus (IV) infection are suggested to participate in airway inflammatory response by expressing various cytokines including RANTES; however, the intracellular signal that regulates RANTES expression has not been determined. In the present study, we examined the role of p38 mitogen-activated protein (MAP) kinase, extracellular signal-regulated kinase (Erk), and c-Jun-NH2-terminal kinase (JNK) in RANTES production by IV-infected human bronchial epithelial cells. The results showed that IV infection induced increases in p38 MAP kinase, Erk, and JNK phosphorylation and activity. SB 203580, PD 98059, and CEP-1347 attenuated IV-infection induced p38 MAP kinase activity, Erk activity, and JNK activity, respectively. SB 203580 and CEP-1347 attenuated RANTES production by 45.3% and 45.2%, respectively, but a combination of these inhibitors additively attenuated by 69.1%. In contrast, PD 98059 did not attenuate. Anti-IL-1α mAb, anti-IL-1β mAb, anti-TNF-α mAb, anti-IL-8 mAb, anti-IFN-β mAb, anti-RANTES mAb, and a combination of these mAbs did not affect IV infection-induced increases in p38 MAP kinase, Erk, and JNK phosphorylation, indicating that each cytokine neutralized by corresponding Ab was not involved in IV infection-induced phosphorylation of MAP kinases. N-acetylcysteine (NAC) did not affect IV infection-induced increases in MAP kinase phosphorylation, whereas NAC attenuated RANTES production by 18.2%, indicating that reactive oxygen species may act as a second messenger leading to RANTES production via p38 MAP kinase- and JNK-independent pathway. These results indicate that p38 MAP kinase and JNK, at least in part, regulate RANTES production by bronchial epithelial cells. The Journal of Immunology, 2000, 164: 3222–3228.

Received for publication October 6, 1999. Accepted for publication December 30, 1999.

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1 Abbreviations used in this paper: IV, influenza virus; MAP, mitogen-activated protein; Erk, extracellular signal-regulated kinase, JNK, c-Jun-NH2-terminal kinase; BEC, human bronchial epithelial cells; NAC, N-acetylcysteine; MEK-1, MAP/Erk kinase kinase-1; ROS, reactive oxygen species.
Materials and Methods

Virus stock

IV strain A/Udon/307/72 (H3N2) was grown in Madin-Darby canine kidney cells (American Type Culture Collection, Manassas, VA) in DMEM (Nissui, Tokyo, Japan) and semipurified by two cycles of differential centrifugation from the infected culture supernatants. Virus stock was stored at −80°C.

Cells and reagents

BEC line NCI-H292 was obtained from American Type Culture Collection. NCI-H292 and BEC line NCI-H292 in culture medium which is RPMI 1640 (Nissui) supplemented with 10% heat-inactivated FCS (MitsubishiKasei, Tokyo, Japan), streptomycin and penicillin (Meiji Pharmaceutical, Tokyo, Japan). SB 203580 and PD 98059 were obtained from Calbiochem-Novabiochem (La Jolla, CA) and New England Biolabs (Beverly, MA), respectively. CEP-1347 was kindly provided by Cephalon (West Chester, PA). SB 203580, PD 98059 and CEP-1347 were dissolved in DMSO. N-acetylcycteine (NAC) was obtained from Sigma (St. Louis, MO). Anti-human IL-1α mAb, anti-human IL-1β mAb, anti-human TNF-α mAb, and anti-human IL-8 mAb were obtained from Genzyme (Cambridge, MA). Polyclonal rabbit anti-human IFN-β Ab and anti-human RANTES mAb were obtained from Chemicon (Temecula, CA) and PeproTech (London, U.K.), respectively.

Cell cultures

The cells were plated onto tissue culture plate (no. 1007, Falcon, Oxonard, CA) for Western blot analysis and in vitro kinase assay and 24-well flat-bottom tissue culture plate (Corning, Corning, NY) for cytokine production, and cultured using culture medium at 37°C in humidified 5% CO2 atmosphere. When the cells were grown in subconfluent conditions, the culture medium was replaced with serum-free RPMI 1640, and the cells were cultured for 16 h. To examine IV infection-induced Erk, p38 MAP kinase, and JNK phosphorylation and activity in cytokine production, the cells were infected with IV at a multiplicity of infection (moi) of 2. The cells for analysis of p38 MAP kinase, Erk, and JNK phosphorylation were lysed at the desired times as indicated after IV infection. To examine the effect of SB 203580, PD 98059 and CEP-1347 on p38 MAP kinase activity, Erk activity, and JNK activity, respectively, and RANTES production by IV-infected BEC, the cells that had been incubated with SB 203580, PD 98059, or CEP-1347 or a combination of these inhibitors for 1 h were infected with IV. The cells were cultured for 6 h for the analysis of MAP kinase activity and for 24 h for the determination of cytokine production. At the end of 24-h cultivation, the culture supernatants were harvested and centrifuged, and the supernatants were collected, filtered with a Millipore filter, and stored at −80°C until assay. To examine the effect of anti-cytokine mAb to IV infection-induced p38 MAP kinase, Erk and JNK phosphorylation, analysis of threonine and tyrosine phosphorylation, the cells were infected with IV and then cultured using culture medium at 37°C in humidified 5% CO2 atmosphere.

Western blot analysis of p38 MAP kinase, Erk, and JNK

Analysis of threonine and tyrosine phosphorylation of p38 MAP kinase was performed using an anti-phosphorylated threonine and tyrosine of p38 MAP kinase Ab (anti-phospho-specific p38 MAP kinase Ab, New England Biolabs), which is specific for active p38 MAP kinase. Equal amounts of protein (50 μg/lane) were separated by 15% SDS-PAGE, transferred to membranes, and probed using phosphorylation-state independent p38 MAP kinase-specific Ab (affinity purified rabbit polyclonal IgG) to determine total p42/p44 MAP kinase levels, phosphorylation-state independent p42/p44 MAP kinase Ab (affinity purified rabbit polyclonal IgG) to determine total p42/p44 MAP kinase levels, or phosphorylation-state independent JNK-specific Ab (affinity purified rabbit polyclonal IgG) to determine total JNK levels, respectively.

p38 MAP kinase and Erk kinase assay

The activity of p38 MAP kinase was analyzed by commercially available kits (p38 MAP Kinase Assay Kit, New England Biolabs). The kit employs two different Abs, anti-p38 MAP kinase Ab which is specific for p38 MAP kinase and does not cross-react with ERK1/2, or JNK, and anti-phospho-specific activating transcription factor-2 (ATF-2) Ab to detect p38 MAP kinase-induced phosphorylation of ATF-2. p38 MAP kinase activity was analyzed by a specific immunoprecipitation with anti-phospho-specific p32/p44 MAP kinase Ab followed by an in vitro kinase assay of its substrate, ATF-2, according to the manufacturer’s instruction, as described previously (14). The activity of Erk was analyzed by commercially available kits (MAP Kinase Assay Kit, New England Biolabs). The kit employs two different Abs, anti-phospho-specific p42/p44 MAP kinase Ab which is specific for active p42/p44 MAP kinase and does not cross-react with p38 MAP kinase or JNK, and anti-phospho-specific Elk-1 Ab to detect p42/p44-induced phosphorylation of Elk-1. Erk activity was analyzed by a specific immunoprecipitation with anti-phospho-specific p32/p44 MAP kinase Ab followed by an in vitro kinase assay of its substrate, Elk-1, according to manufacturer’s instructions, as described previously (30). Briefly, the cell lysate containing 200 μg of protein was incubated with anti-p38 MAP kinase Ab to selectively immunoprecipitate p38 MAP kinase or anti-phospho-specific p42/p44 MAP kinase Ab to selectively immunoprecipitate active p42/p44 MAP kinase from cell lysates, and the immunoprecipitates were incubated with ATP-2 fusion protein or Elk-1 fusion protein in the presence of ATP, a process which allowed immunoprecipitated active p38 MAP kinase to phosphorylate its substrate, ATF-2, and p42/p44-induced phosphorylation of ATF-2, which was separated by a 15% SDS-PAGE, transferred to membranes, and blotted with anti-phospho-specific ATF-2 Ab or anti-phospho-specific Elk-1 Ab. The membrane was incubated with HRP-conjugated anti-rabbit IgG Ab and HRP-conjugated anti-biotin Ab and then the membrane was incubated with 10 ml of the ECL solution and exposed on Kodak XAR film for 1 min.

JNK kinase assay

The activity of JNK was analyzed by commercially available kits (SAPK/JNK Assay Kit, New England Biolabs). The kit employs an N-terminal c-Jun fusion protein bound to Sepharose beads to selectively pull down JNK from cell lysates, after which the kinase reaction is conducted in the presence of unlabeled ATP. c-Jun phosphorylation is selectively measured using phospho-specific c-Jun Ab that specifically measures JNK-induced phosphorylation of c-Jun. Analysis of activity of JNK was performed according to the manufacturer’s instruction, as described previously (14). Briefly, the cell lysate containing 200 μg of protein was mixed with 2 μg of c-Jun fusion protein beads and then incubated. After microcentrifugation, the pellet was washed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer with ATP. Equal amounts of protein (5 μg/lane) were separated by 15% SDS-PAGE, transferred to a membrane, and blotted with anti-phospho-c-Jun Ab. The membrane was incubated with HRP-conjugated anti-rabbit IgG Ab and HRP-conjugated anti-biotin Ab and then the membrane was incubated with 10 ml of the ECL solution and exposed on Kodak XAR film for 1 min.

Measurement of RANTES

The concentration of RANTES in the culture supernatants from BEC were measured by commercially available ELISA kits (Amersham International, Aylesbury, U.K.). ELISA was performed according to the manufacturer’s instructions. All samples were assayed in duplicate.

Statistical analysis

Statistical significance was analyzed by using ANOVA. A p value <0.05 was considered significant.
Results

IV infection induces p38 MAP kinase, Erk, and JNK phosphorylation

To determine whether IV infection could induce the threonine and tyrosine phosphorylation of p38 MAP kinase, Erk, and JNK, BEC were infected with IV, and p38 MAP kinase, Erk, and JNK in the cells were immunooblotted. Amounts of phosphorylated threonine and tyrosine of p38 MAP kinase in IV-infected cells increased at 1 h, were maximal at 6 h, and sustained between 8 and 12 h; thereafter, they returned to near basal levels at 24 h (Fig. 1a, upper panel). Amounts of phosphorylated threonine and tyrosine of Erk in IV-infected cells increased at 30 min, were maximal at 6 h, and sustained between 8 and 12 h; thereafter they returned to near basal levels at 24 h (Fig. 1b, upper panel). Amounts of phosphorylated threonine and tyrosine of JNK in IV-infected cells increased at 4 h and were maximal at 6 h; thereafter they sustained between 8 to 12 h and returned to near basal levels at 24 h (Fig. 1c, upper panel). The lower panels of Fig. 1a showed that equal amounts of p38 MAP kinase protein were immunoblotted with phosphorylation-independent p38 MAP kinase-specific Ab regardless of time of culture periods, indicating that IV infection-induced p38 MAP kinase phosphorylation occurred in the absence of changes in p38 MAP kinase protein levels. Similarly, IV infection induced in Erk and JNK phosphorylation occurred in the absence of changes in Erk protein levels and JNK protein levels (lower panels of Fig. 1, b and c).

IV infection induces p38 MAP kinase, Erk, and JNK activity, and SB 203580, PD 98059 and CEP-1347 attenuate IV infection-induced p38 MAP kinase, Erk, and JNK activity, respectively

Activation of p38 MAP kinase, Erk, and JNK is mediated by dual phosphorylation of the threonine and tyrosine residues of p38 MAP kinase, Erk, and JNK (9, 13, 31), respectively. Increases in the threonine and tyrosine phosphorylation of p38 MAP kinase, Erk, and JNK in IV-infected BEC shown in Fig. 1 reflect activation state of p38 MAP kinase, Erk, and JNK. In addition to analysis of the threonine and tyrosine phosphorylation of p38 MAP kinase, Erk, and JNK, we next examined whether IV infection could induce p38 MAP kinase, Erk, and JNK activity, and the effect of SB 203580 on p38 MAP kinase activity, the effect of PD 98059 on Erk activity and the effect of CEP-1347 on JNK activity in IV-infected BEC. IV infection induced p38 MAP kinase activity as demonstrated by the increased phosphorylation of its substrate, ATF-2 (Fig. 2a), Erk activity as demonstrated by the increased phosphorylation of its substrate, Elk-1 (Fig. 2b), and JNK activity as demonstrated by the increased phosphorylation of c-Jun (Fig. 2c). SB 203580, PD 98059, and CEP-1347 attenuated IV infection-induced increases in p38 MAP kinase activity (Fig. 2a), Erk activity (Fig. 2b), and JNK activity (Fig. 2c), respectively. SB 203580, PD 98059, and CEP-1347 did not attenuate IV infection-induced increases in Erk and JNK activity, p38 MAP kinase and JNK activity, and p38 MAP kinase and Erk activity, respectively (data not shown). Addition of DMSO vehicle alone did not attenuate IV infection-induced increases in p38 MAP kinase, Erk, and JNK activity (data not shown).
SB 203580 and CEP-1347, but not PD 98059, attenuates IV infection-induced RANTES production

RANTES production was reportedly induced by IV infection (7). IV infection induced p38 MAP kinase, Erk, and JNK phosphorylation and activity, and SB 203580, PD 98059, and CEP-1347 attenuated IV infection-induced increases in p38 MAP kinase, Erk, and JNK activity, respectively (Figs. 1 and 2). These observations suggested that IV infection-induced RANTES production might be mediated through p38 MAP kinase-, Erk-, and JNK-dependent pathway. To test this possibility, BEC that had been incubated with various concentrations of SB 203580, PD 98059 or CEP-1347 for 1 h were infected with IV, and the concentrations of RANTES in the culture supernatants were determined at 24 h after IV infection. The results are expressed as the mean ± SD in six different experiments. *1, p < 0.01 compared with RANTES concentrations in BEC cultured without inhibitor; *2, p < 0.01 compared with RANTES concentrations in BEC cultured without inhibitor and those in BEC cultured either with SB 203580 or CEP-1347 only.

SB 203580 and CEP-1347 additively attenuate IV infection-induced RANTES production

SB 203580 and CEP-1347 partially attenuated IV infection-induced RANTES production. From these results, we next examined the effect of a combination of SB 203580 (10 μM) and CEP-1347 (1 μM) on RANTES production. SB 203580 and CEP-1347 additively attenuated RANTES production by 69.1%, but a complete inhibition was not seen (Fig. 4). Addition of DMSO vehicle alone did not affect RANTES production by IV-infected BEC (data not shown).

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Influenza infection-induced p38 MAP kinase, Erk, and JNK phosphorylation is not mediated with cytokine

Because IV infection has been shown to induce the production of various cytokines by BEC (6–8), it might be possible that cytokine produced by IV-infected BEC is responsible for the induction of p38 MAP kinase, Erk, and JNK phosphorylation. To test this possibility, anti-IL-1α mAb, anti-IL-1β mAb, anti-TNF-α mAb, anti-IL-8 mAb, anti-IFN-β Ab, and anti-RANTES mAb were added to the culture, and then BEC were infected with IV. The threonine and tyrosine phosphorylation of p38 MAP kinase, Erk, and JNK were analyzed at 6 h after IV infection. As shown in Fig. 5, each Ab and a combination of all Abs did not affect IV infection-induced increases in p38 MAP, Erk, and JNK phosphorylation. In addition, we measured the concentrations of IL-1α, IL-1β, TNF-α, IL-8, IFN-β, and RANTES in the culture supernatants from IV-infected BEC at 6 h after IV infection by a specific ELISA. The concentrations of these cytokines were below the assay sensitivity limit. Each Ab used in this study had sufficient Ab concentrations for neutralizing corresponding cytokine activity of assay sensitivity limit.

The effect of NAC on IV infection-induced p38 MAP kinase, Erk, and JNK phosphorylation and RANTES production

Because it has been shown that IV infection induces the generation of reactive oxygen species (ROS) (32) and that ROS can induce MAP kinase phosphorylation (33–35), we examined the effect of NAC on the threonine and tyrosine phosphorylation of p38 MAP kinase, Erk, and JNK to clarify the involvement of ROS in IV infection-induced p38 MAP kinase, Erk, and JNK phosphorylation. The results showed that NAC did not affect of IV infection-induced increases in p38 MAP kinase, Erk, and JNK phosphorylation, indicating that ROS which can be scavenged by NAC were not involved in IV infection-induced phosphorylation of p38 MAP kinase, Erk, and JNK phosphorylation (Fig. 6). Finally, it has been shown that IV infection activates NF-κB leading to IL-8 expression and antioxidants inhibit IV infection-induced NF-κB activation (32), we examined the effect of NAC on IV infection-induced RANTES production. The results showed that NAC attenuated RANTES production by 18.2% (Fig. 7). The total number of the cells and cell viability at the end of the culture period of each experiment, determined by trypan blue exclusion dye, did not differ with culture conditions, suggesting that IV infection-induced RANTES production and the attenuation by inhibitors and NAC of RANTES production did not result from cell cytotoxicity.

Discussion

IV infection induced the threonine and tyrosine phosphorylation of p38 MAP kinase, Erk, and JNK. A time-course study of phosphorylation of these kinases showed similar kinetics. Many extracellular stimuli induce MAP kinase phosphorylation and activity. It is generally observed that the maximal MAP kinase phosphorylation and activity is seen within 60 min after the stimulation. In this study, the maximal phosphorylation of p38 MAP kinase, Erk, and JNK were observed at 6 h after IV infection. Therefore, we speculated that cytokines produced by IV-infected BEC and/or ROS generated by IV-infected BEC might be responsible for the induction of MAP kinase phosphorylation, because various cytokines produced by the infection of IV (6–8) and ROS have been shown to induce MAP kinase phosphorylation (33–35). The results were: 1) each neutralizing Ab to corresponding cytokine as described above did not affect IV infection-induced increases in p38 MAP kinase, Erk, and JNK phosphorylation; 2) IL-1α, IL-1β, TNF-α, IL-8, IFN-β, and RANTES in the culture supernatants from IV-infected BEC at 6 h postinfection were not detected by specific

![FIGURE 5](http://www.jimmunol.org/)
Consequently, rescue motoneurons undergoing apoptosis and inhibit JNK activity production. In addition, SB 203580 and CEP-1347 additively increase and induce simultaneously p38 MAP kinase, Erk, and JNK activity. A total of 10 μM SB 203580 and 50 μM PD 98059 were used in this study to examine the inhibitory effect of these inhibitor on RANTES production, because the previous studies with analysis of the role of p38 MAP kinase and Erk in eliciting various biological responses including cytokine expression showed that these concentrations of inhibitors almost completely inhibited (27, 28, 36, 37). A total of 1 μM CEP-1347 can rescue motoneurons undergoing apoptosis and inhibit JNK activity (29). Consequently, 10 μM SB 203580, 50 μM PD 98050, and 1 μM CEP-1347 employed in this study were sufficient concentrations to examine the signal transduction pathway. IV infection induced simultaneously p38 MAP kinase, Erk, and JNK activity. A total of 10 μM SB 203580, 50 μM PD 98059, and 1 μM CEP-1347 almost completely attenuated IV infection-induced increases in p38 MAP kinase, Erk and JNK activity, respectively. In the production of RANTES, 10 μM SB 203580 caused a 45.3% decrease and 1 μM CEP-1347 caused a 45.2% decrease in RANTES production, whereas 50 μM PD 98059 did not inhibit RANTES production. In addition, SB 203580 and CEP-1347 additively attenuated RANTES production by 69.1%. The inhibition by a combination of these inhibitors was statistically significant compared with that by SB 203580 or CEP-1347 only. These results indicated that p38 MAP kinase and JNK, but not Erk, at least in part participate in regulating RANTES production. The incomplete inhibition of RANTES production induced by a combination of SB 203580 and CEP-1347 observed in this study might suggest that parallel pathways which are p38 MAP kinase- and JNK-independent pathway may regulate RANTES production. One possible signal that may regulate IV infection-induced RANTES production by BEC is ROS.

It has been shown that IV infection activates NF-κB leading to IL-8 expression and antioxidants inhibit IV infection-induced NF-κB activation (32). In this study, NAC did not affect IV infection-induced increases in p38 MAP kinase and JNK phosphorylation, whereas NAC attenuated RANTES production by 18.2%. Collectively, these results indicated that ROS generated by IV infection might act as a second messenger leading to the induction of RANTES production via p38 MAP kinase- and JNK-independent pathway.

The mechanism of activation and the function of MAP kinases have been extensively studied. A variety of extracellular stimuli activate p38 MAP kinase and elicit a variety of cellular functions. p38 MAP kinase-mediated cytokine expression has been well documented (14, 15, 36, 37). Recent studies have indicated the involvement of Erk and the coordinate regulation by p38 MAP kinase and Erk in cytokine expression in various cells (18–21). However, our results showed that Erk was not involved in IV infection-induced RANTES production by BEC. Phosphorylation and catalytic activation of JNK in cells has been implicated in the intracellular signal promoting apoptosis (9, 38). In addition, the JNK pathway was recently shown to play a role in cytokine expression (22, 23). However, little is known about the role of JNK in IV-induced RANTES production in BEC. Our results with JNK-mediated RANTES production by IV-infected BEC indicate new evidence on the role of JNK in airway epithelial cells, which is the regulation of RANTES production. RANTES are produced by a variety of cells (39–41). In human umbilical vein endothelial cells, Erk has been shown to regulate RANTES production (42). Collectively, the signal transduction pathway regulating RANTES production may be cell type-specific.

IV infection induces the expression of various cytokines including RANTES in airway epithelial cells. Eosinophilia, neutrophilia, and high levels of IL-8 and eosinophilic cationic protein are seen in induced sputum from bronchial asthma patients infected with IV (43). RANTES that exhibits a chemotactic activity for eosinophils has been shown to play an important role in the production of airway inflammation of asthmatics through the recruitment of eosinophils into the site of airway inflammation (26). Our results with the role of p38 MAP kinase- and JNK-dependent pathway in RANTES production by IV-infected BEC are important in understanding the pathogenesis of IV-induced asthma exacerbation.

Finally, MAP kinase cascades are connected with the activation of various transcription factors that participate to various extents in the inducible expression of gene-encoding cytokine. The promoter of the gene-encoding RANTES contains sequences for the binding several nuclear transcription factors including NF-κB and AP-1 (44, 45). These transcription factors participate to various extents in the inducible expression of the gene encoding RANTES. p38 MAP kinase has been implicated in the activation of multiple transcription factors, including NF-κB (46). JNK has been implicated in the activation of multiple transcription factors, including AP-1 (9, 47). Recently, Hiura et al. (48) have shown that JNK and ELISA; and 3) NAC did not affect IV infection-induced increases in p38 MAP kinase, Erk, and JNK phosphorylation. These results indicated that cytokines as described above and ROS were not involved in IV infection-induced phosphorylation of p38 MAP kinase, Erk, and JNK. However, a role of cell-associated and intracellular cytokines in the induction of MAP kinase phosphorylation remains to be determined. It is also of interest to identify responsible IV-specific molecule for the induction of MAP kinase phosphorylation and determine a relationship between IV growth and the induction of MAP kinase phosphorylation. It is likely that IV replication is more directly related to the induction of MAP kinase phosphorylation, because a time-course of the induction of MAP kinase phosphorylation is more directly related to the induction of MAP kinase phosphorylation. It is likely that IV replication is more directly related to the induction of MAP kinase phosphorylation, because a time-course of the induction of MAP kinase phosphorylation is more directly related to the induction of MAP kinase phosphorylation.
NF-κB response elements are involved in RANTES gene activation in macrophage cell line. RAW 264.7 cells, stimulated by LPS. We have not investigated and identified downstream targets of p38 MAP kinase and JNK leading to RANTES gene expression (47, 49). Therefore, further study should be done to clarify the links between the activation of these kinases and the expression of RANTES gene.

From the data presented in this paper, we conclude that p38 MAP kinase and JNK, at least in part, regulate RANTES production by IV-infected BEC.

Acknowledgments

We thank Dr. Yuzuru Matsuda (Kyowa-Hakko Kogyo Company, Ltd.) and Drs. Jeffery Vaughn and Matthew Miller (Cephalon Inc.) for the generous gift of CEP-1347.

References