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Signaling Pathways Involved In Dengue-2 Virus Infection Induced RANTES Overexpression

[1]Ying-Ray Lee, [1,2]Huan-Yao Lei, [2]Shun-Hua Chen, [3]Jen-Reng Wang, [2]Yee-Shin Lin, [3]Trai-Ming Yeh, [4]Ching-Chuan Liu, and [2]Hsiao-Sheng Liu
[1]Institute of Basic Medical Sciences
[2]Departments of Microbiology and Immunology
[3]Medical Laboratory Science and Biotechnologyand Pediatrics
[4]College of Medicine, National Cheng Kung University

Abstract: Dengue viruses participate in liver inflammation by inducting the expression of various chemokines including Regulated on Activation Normal T-cell Expressed and Secreted (RANTES). However, the underlying signaling remains unknown. Here, we reveal that Ras, Raf-1 and three mitogen-activated protein kinases (MAPKs) p38, extracellular signal-regulated kinase (Erk), and c-jun-NH_2-terminal kinase (JNK) can be activated or phosphorylated in dengue-2 virus infected hepatocyte and epithelial cells by western blotting and confirmed by dominant negative mutants of ras, raf-1, p38, Erk, and JNK. The Tet-off inducible plasmids harboring dengue-2 virus prM, core, E or NS1 gene were utilized to reveal their role in RANTES activation. However, no effect was detected among the genes tested indicating that they are either dispensable or not sufficient for RANTES activation. Taken-together, Ras, Raf-1, JNK, Erk and p38 related signaling pathways are essential for the activation of RANTES by dengue-2 virus. The knowledge gathered will shed light on developing a novel therapeutic approach to block inflammatory infiltrates through decreasing RANTES expression.

Keywords: RANTES, MAPK signaling pathway, dengue-2 virus

INTRODUCTION

The mosquito-borne dengue virus (which has four serotypes) is a member of the family *Flaviviridae*. It occurs predominantly in the tropical and subtropical regions of the world and may cause 100 million cases of dengue fever (DF), 500,000 cases of Dengue Hemorrhagic fever (DHF), and 25,000 deaths. A total of 2.5 billion people are at the risk of dengue virus infection each year[1]. The major symptoms are either asymptomatic or self-limited known as DF. The more severe forms of dengue virus infection, DHF and DSS, are characterized by plasma leakage and may be life threatening[2]. DHF is a severe febrile disease characterized by abnormalities in homeostasis and increased vascular permeability. Further progression of DHF may result in DSS, which is a form of hypovolemic shock associated clinically with hemonconcentration, which may lead to death. The clinical features include plasma leakage, bleeding tendency, and liver involvement[3, 4]. Liver involvement is common in dengue virus infected patients with mild elevation of serum transaminases. Dengue virus related dysfunction of hematological, vascular, and hepatic system causes the manifestations of DHF/DSS.

Dengue virus can infect patient liver and cause hepatitis. Elevated serum transaminase levels were found in dengue patients, and the level of AST elevation correlated with that of hemorrhage[5, 6]. In dengue virus related hepatitis, the level of AST is higher than that of ALT, while the hepatitis induced by other types of viruses have more ALT than AST indicating virus type specific pathogenesis. Dengue virus also can induce apoptosis in human hepatoblastoma cell line[7, 8]. Previously, Lin *et al*.[9], demonstrated that RANTES was induced via oxidative stress dependent and independent pathways, which further activates NF-IL6 in dengue-2 virus infected hepatoma cell lines. Moreover, RANTES was preferentially induced in liver cells by dengue virus but not enterovirus and coxsackievirus. Patients with dengue virus infection have increased RANTES serum levels compared to those with other viral infections[9].

Corresponding Author: Hsiao-Sheng Li, Department of Microbiology and Immunolog, College of Medicine, National Cheng Kung University 1 University Road, P.Box 70101,Tainan, Taiwan

RANTES is a member of the C-C chemokine, which was originally cloned from $CD8^+$ T lymphocytes[10]. The natural ligands of RANTES, CCR5 chemokine receptor, is the fusion/entry cofactor for M-tropic HIV-1, and RANTES can antagonize the infection of $CD4^+$ cells to M-tropic strains of HIV-1[11-13]. The importance of RANTES in virus-induced pathogenesis was demonstrated by its role in the pathogenesis of HIV infection. RANTES can recruit lymphocytes and NK cells to the sites of inflammation [14-17]. Whether liver damage caused by dengue virus is a direct effect of virus replication or an indirect effect of RANTES-mediated inflammation deserves further investigation. The balance between virus elimination and tissue damage might affect the severity of the disease. Because liver is the major site for the synthesis of most coagulation factors, reduced levels of coagulation factors are either the results of increased consumption or impaired synthesis. The latter is the likely consequence of liver injury.

Many extracellular stimuli elicit specific biological responses through activation of mitogen-activated protein kinase (MAPK) cascades[18]. Mammalian MAP kinase superfamily has been molecularly characterized: extracellular signal-regulated kinase (Erk), p38 MAP kinase, and c-Jun-NH_2-terminal kinase (JNK) etc. MAP kinase p38 and JNK are activated by environmental stresses including hyperosmotic shock, abnormal temperature, UV irradiation and inflammatory cytokines. They play important roles in apoptosis and cytokine expression[19-24]. Erk is activated by mitogenic stimuli and is essential for cell proliferation and differentiation[25, 26]. Erk and JNK also involves in the signaling cascades of various inflammatory mediators including cytokines and chemical mediators[27-32].

RANTES could be activated differently by various stimuli, indicating a wide range of control at the transcriptional level[33-36]. In a previous study, HIV infection can activate RANTES through phosphorylation of Erk[37]. And Influenza virus infection can up-regulate RANTES expression through p38 and JNK[38]. Dengue virus can induce DNA binding activity of NF-IL-6 and up-regulates RANTES expression[9]. However, whether p38 and JNK are involved in dengue virus related RANTES activation remaining unclear.

All together, the signaling pathways of RANTES activation in dengue-2 virus infected hepatocyte and endothelial cells were reported in this study.

MATERIALS AND METHODS

Cell line and virus: Chang liver cells (a non-malignant human liver epithelial cell line)[39], and Hep3B (human hepatoma cell lines) cells were cultured in Dulbecco's modified Eagle's medium, (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Trace BioSciences, Australia), penicillin (200 units/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 incubator. The human microvascular endothelial cell-1 (HMEC-1) cells were cultured in endothelial basal medium MCDB 131 (GIBCO, USA), supplemented with 10% FBS (TRACE, Australia), 1% L-glutamine, 1% penicillin, hydrocortisone (1 μg mL^{-1}) (SIGMA, USA), and 10 ng mL^{-1} epidermal cell growth factor (EGF) (GIBCO, USA). For virus infection experiments, cells were adsorbed with dengue-2 virus at the titer of 100 m.o.i. at 37°C for 2 hr. The cells were then washed three times with phosphate buffered saline (PBS) and incubated at 37°C in DMEM (GIBCO-BRL, USA), supplemented with 2% FBS (TRACE, Australia), penicillin (200 unit/ml) and streptomycin (100 μg mL^{-1}).
Dengue-2 virus (PL046 isolated from Taiwan) was maintained in C6/36 cells. The titer was evaluated in BHK-21 cells by plaque assay. Dengue-2 virus was heated at 56°C for 30 min as the inactivated virus[40].

Western blot analysis of p38 MAP kinase, Erk, and JNKL: Analysis of threonine and tyrosine phosphorylation of p38 MAP kinase was performed using an anti-phosphorylated threonine and tyrosine of p38 MAP kinase antibody (anti-phospho-specific p38 MAP kinase Ab, New England Biolabs, USA), which was specific for active p38 MAP kinase and did not cross-react with Erk and JNK. Analysis of threonine and tyrosine phosphorylation of Erk was performed using an anti-phosphorylated threonine and tyrosine of p42/p44 MAP kinase antibody (anti-phospho-specific p42/p44 MAP kinase Ab, New England Biolabs, USA), which was specific for active p42/p44 MAP kinase and did not cross-react with p38 MAP kinase and JNK. Analysis of threonine and tyrosine phosphorylation of JNK was performed using an anti-phosphorylated threonine and tyrosine of JNK antibody (anti-phospho-specific JNK Ab, New England Biolabs, USA), which was specific for active JNK and did not cross-react with p38 MAP kinase and Erk. Analysis of p38 MAP kinase, Erk, and JNK was performed according to manufacture's instruction. Briefly, after separating proteins from cell lysate by a 15% SDS-PAGE, the cell lysate containing 10 μg of protein was electrophoretically transferred to a

membrane, and the membrane was incubated with specific antibody to the phosphorylated threonine and tyrosine of p38 MAP kinase (affinity-purified rabbit poly-clonal IgG), specific antibody to the phosphorylated threonine and tyrosine of Erk (affinity-purified rabbit poly-clonal IgG), or specific antibody to the phosphorylated threonine and tyrosine of JNK (affinity-purified rabbit poly-clonal IgG) for analysis of JNK. Then it was incubated with the HRP-conjugated anti-rabbit IgG antibody and HRP-conjugated anti-biotin antibody to detect biotinylated protein markers. Blots were incubated with enhanced chemiluminescence (ECL) solution for 1 min and exposed on a Kodak (Rochester, NY) XAR film.

Transient transfection and luciferase assay: Cells were cultured in 12-well dish and transfected with pGL-B (1 μg well^{-1}), pSG5-lacZ (0.25 μg well^{-1}), and dominant negative mutant plasmid (DNRas, DNRaf-1, DNErk, DNJNK, DNp38) or vector control pBSSK^{+} (3 μg well^{-1}). The luciferase activities were determined 48 hr after dengue-2 virus infection by a Dual-light luciferase and β-galactosidase reporter gene assay system (Tropix, Bedford, MA). Briefly, equivalent amounts of protein lysates (10 μl) were mixed with buffer A. Light signal emitted from the luciferase enzyme in the extract was measured immediately by a luminometer (Minilumate LB 9506, Germany) after the addition of buffer B containing luciferin and Galacton-Plus. After 30 min incubation at room temperature, light signal from the accumulated product of β-galactosidase and Galacton-Plus reaction was elicited by adding of a light emission accelerator and measured by the same luminometer.

Construction of inducible plasmids: The dengue-2 virus prM, core, NS1 and E genes in the plasmids pCR-DVMP, pCR-DVCP, pCDNA-NS1 and pCR-DVEP were subcloned into the inducible plasmid vector pTREM2. The expression of pTREM2-DVCP and pTREM2-DVNS1 was confirmed by immunostaining 48 hr after transfection.

RESULTS

Dengue-2 virus infection induces phosphorylation of MAP kinases, Erk, p38 and JNK: To determine whether dengue-2 virus infection could induce the phosphorylation of three major MAP kinases, Erk, p38 and JNK, Chang liver cells were infected with dengue-2 virus. The levels of phosphorylated Erk (especially p42 Erk) in dengue-2 virus infected cells increased at 2 hr and reached the plateau at 12 hr postinfection (p.i.), and then declined at 24 hr, 48 hr p.i., respectively (Fig. 1). The level of phosphorylated p38 in dengue-2 virus infected cells was significantly increased at 12 hr p.i., and then slightly declined from 24 to 48 hr p.i. (Fig. 1). Differently, the levels of phosphorylated JNK in dengue-2 virus infected cells were slightly increased at 2 hr p.i. and remained at the same level until 48 hr p.i. (Fig. 1). In contrast, heat inactivated dengue viruses suppressed the expression levels of the three MAPKs. Our data demonstrate that active dengue-2 virus can induce phosphorylation of the three major MAPKs to various levels as well as at different time intervals compared to mock infected cells.

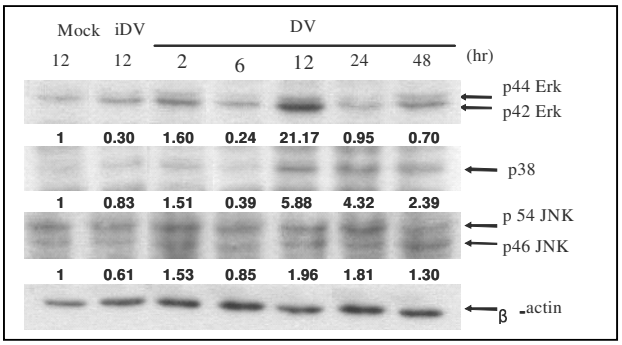

Fig. 1: Phosphorylation of MAPKs in dengue-2 virus infected Chang liver cells by Western blotting. Chang liver cells were infected by dengue-2 virus for the desired times. The lysates from Chang liver cells were separated by the 15% SDS-PAGE. The gel was transferred to the membrane and blotted with a specific antibody to phosphorylated JNK. The membrane was stripped and reprobed using a specific antibody to phosphorylated Erk. The membrane was stripped again and reprobed using a specific antibody to phosphorylated p38. Finally, the membrane was stripped and reporbed using a specific antibody of β -actin as the internal control. To quantify the band intensity, the intensity of MAPK was seated as 1, all the numbers shown are the comparison with mock

Dengue-2 virus activation of RANTES is through Erk, p38 and JNK signaling pathways: RANTES activation induced by dengue-2 virus infection has been reported[9]. The above data demonstrated that dengue-2

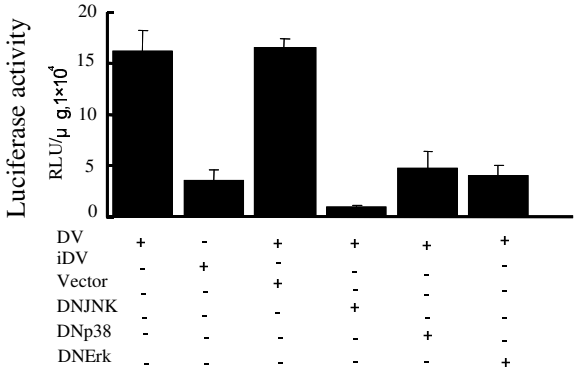

Fig. 2: The effects of dominant negative mutant DNA on RANTES expression in dengue-2 virus infected Chang liver cells by luciferase reporter assay. Chang liver cells were transfected with pGL-B (1 μ g well^{-1}), pSG5-lacZ (0.25 μ g/well), and dominant negative mutants (DNJNK, DNp38, and DNErk) or vector control pBSSK^{+} (3 μ g well^{-1}) in 12-well culture dish and followed by infection with dengue-2 virus or heat inactived dengue-2 virus (M.O.I.=100). The luciferase activity was measured at 48 hr p.i

virus infection could induce the phosphorylation of Erk, p38 and JNK. It is noteworthy to clarify which of the MAPKs is possibly involved in dengue-2 virus infection-induced RANTES expression. Dominant negative mutants (DN): DNErk, DNJNK, DNp38 for Erk, JNK, and p38, were co-transfected with RANTES reporter plasmid (pGL-B)[9] to block the specific MAPK signaling pathway. RANTES activity was measured by RANTES luciferase reporter analysis at 48 hr p.i.. (Fig. 2) shows that dengue-2 virus infection induced RANTES activity in Chang liver cells was suppressed to the basal level in the presence of DNp38, DNErk or DNJNK. However, cotransfection of RANTES luciferase reporter gene DNA with the empty vector (pBSSK^{+}), RANTES activity was not affected. This observation was also seen in Hep3B and HMEC-1 cells suggesting that dengue-2 virus infection-induced RANTES expression in Chang liver, Hep 3B and HMEC-1 cell lines is mediated through Erk-, p38- and JNK-dependent signaling pathways. Moreover, these three MAPKs are not dispensable for dengue virus induced RANTES activation.

Ras and Raf-1 are involved in dengue-2 virus infection-induced RANTES activation: To unveil the up-stream regulator of the MAPK signaling pathways,

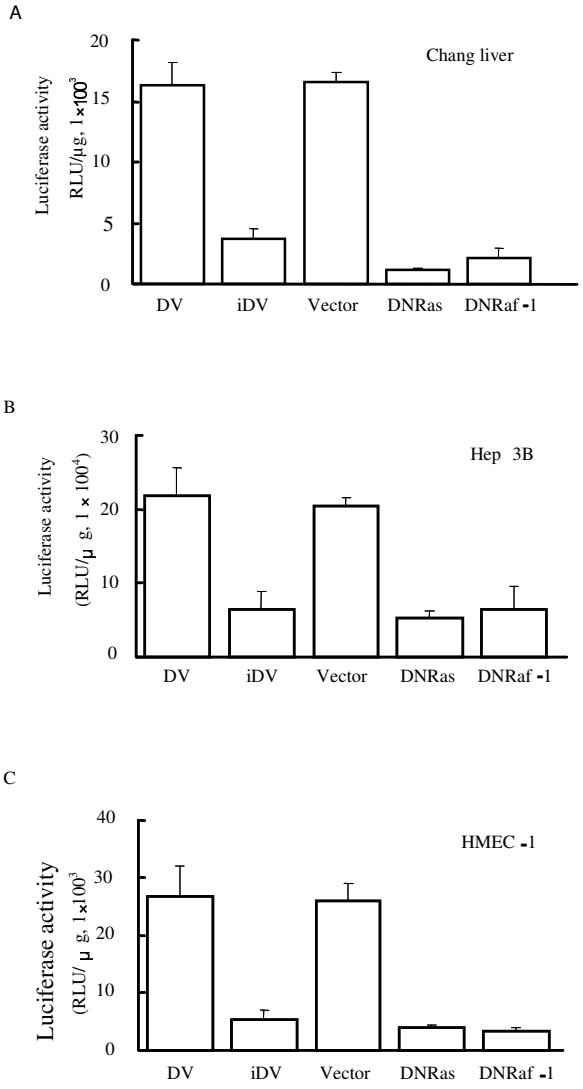

Fig. 3: The effect of dominant negative Ras and Raf-1 on RANTES expression in dengue-2 virus infected cells by luciferase reporter assay. Cells were transfected with pGL-B (1 μ g well^{-1}), pSG5-lacZ (0.25 μ g well^{-1}), and dominant negative mutants (DNRas and DNRaf-1) or vector control pBSSK^{+} (3 μ g well^{-1}) in 12-well culture dish, followed by infection with dengue-2 virus or heat inactived dengue-2 virus
(A): Chang liver cells
(B): Hep 3B cells
(C): HMEC-1 cells

Ras/Raf-1 were considered because they are the up-stream transducers of many MAPK signaling pathways. Dominant negative mutant of Ras or Raf-1 (DNRas or DNRaf-1, respectivity) was co-transfected with RANTES reporter plasmid (pGL-B) (Lin *et al*.,

2000) into Chang liver cells (A), Hep 3B (B) and HMEC-1 (C) cell lines. RANTES activity was measured by RANTES luciferase reporter analysis at 48 hr after dengue-2 virus infection. (Fig. 3) shows that dengue-2 virus induced RANTES activity was declined to the basal level while Ras, or Raf-1 was blocked by DNRas or DNRaf-1. Our data clearly indicate that dengue-2 virus infection-induced RANTES expression is mediated through Ras- and Raf-1-dependent signaling pathways.

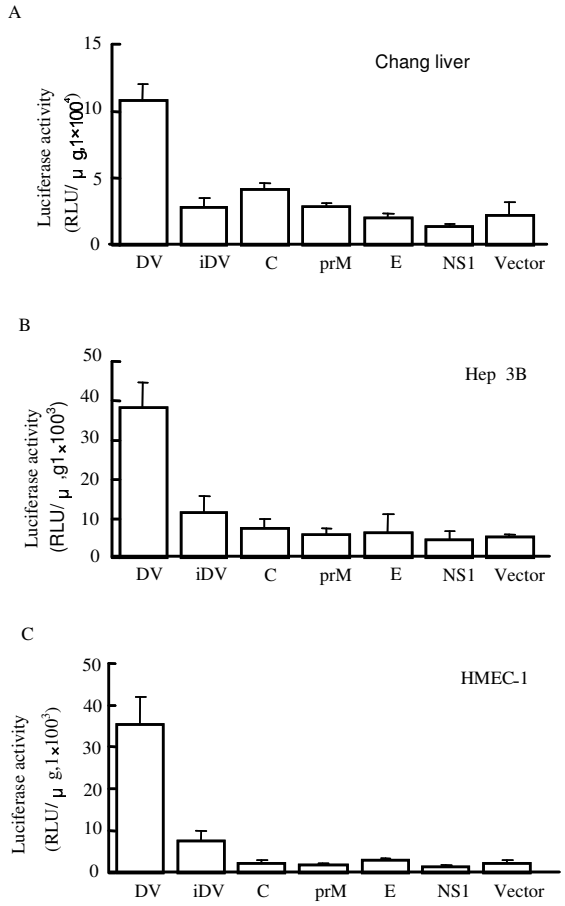

Fig. 4: The effect of single dengue-2 virus gene on RANTES activation in various cell lines. Cells were transfected with pGL-B (1 μg/well), pSG5-lacZ (0.25 μg/well), and dengue-2 virus single gene plasmid (pTREM2-DVCP, pTREM2-DVMP, pTREM2-DVEP, or pTREM2-DVNS1) or vector control pTREM2 (3 μg/well) in 12-well culture dish. The cells were infected with dengue-2 virus or heat inactived dengue-2 virus (M.O.I.=100). Luciferase activity was measured 48 hr p.i.. (A): Chang liver cells
(B): Hep 3B cells
(C): HMEC-1 cells. C: core protein

Dengue-2 virus prM, core, E and NS1 proteins are not involved in RANTES activation: To clarify the role of dengue-2 virus prM, core, E and NS1 protein in RANTES activation, the inducible plasmids harboring dengue-2 virus prM, core, E or NS1 gene were constructed. The expression of the transgenes was detected by immunhistochemistry analysis (data not shown). To determine whether these viral gene products participate in RANTES activation, plasmid: pTREM2-DVCP, pTREM2-DVMP, pTREM2-DVEP, or pTREM2-DVNS1 together with RANTES reporter plasmid (pGL-B) were cotransfected into Chang liver (A), Hep 3B (B) and HMEC-1 (C) cell lines and RANTES activity was measured at 48 hr post-transfection. The RANTES activity was not induced while the cells were transfected with each of the single genes prM, core, E and NS1 as compared to that of dengue-2 virus infected cells (Fig. 4). Our results show that dengue-2 virus single gene prM, core, E or NS1 has no effect on RANTES activation indicating multifactors may be necessary for RANTES activation.

The activation of RANTES expression by dengue-2 virus induced oxidative stress is not regulated by MAPK signaling pathways: According to our findings, we propose a model that dengue-2 virus may up-regulate RANTES expression through Ras/Raf-1/Erk, p38 and JNK signaling pathways (Fig. 6). We previously reported that oxidative stress is essential for dengue virus induced RANTES activation[9]. To reveal the relationship between oxidative stress and MAPK signaling pathways, Chang liver cells pretreated with the pharmaceutical inhibitors PD98059, SB203580 or JNK inhibitor II to block Erk, p38 and JNK-dependent pathways. The H_2O_2 expression after dengue-2 virus infection is not affected in pretreated or non-pretreated Chang liver cells by flow cytometry analysis (Fig. 5). Our data indicate that dengue-2 virus induced oxidative stress does not utilized Ras/Raf-1/Erk, p38 and JNK signal pathways to generate H_2O_2 and to active RANTES.

DISCUSSION

In this study, dengue-2 virus infection induced the phosphorylation of three major MAPKs Erk, p38 and JNK at various time points post infection as well as to various levels. Many extracellular stimuli induced maximal MAP kinase phosphorylation and activity within 60 min. These three MAPKs are all activated 2 hr p.i.. The maximal phosphorylation of p38 and Erk was observed at 12 hr after dengue-2 virus infection. The inconsistency of MAPK activation suggest that

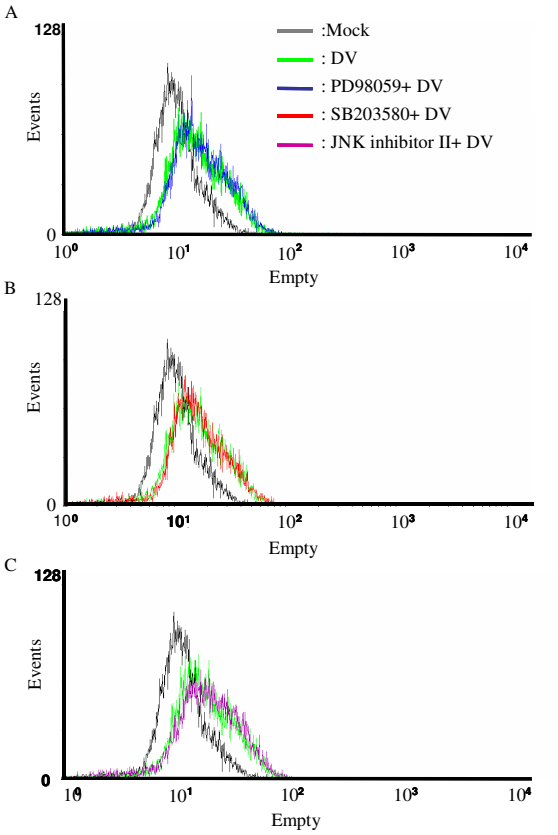

Fig. 5: Measurement of H_2O_2 production of Change liver cells after dengue-2 virus infection. Chang liver cells pre-treated with PD98059 (50 μ M), SB203580 (10 μ M), or JNK inhibitor II (10 μ M) for 2hr and inoculated with dengue-2 virus (M.O.I.=100). Forty eight hours p.i. 2',7'-dichlorofluorescin diacetate (DCFH-DA, 20 μ M) was used to measure H_2O_2 production by flow cytometer.
(A) Chang liver cells pretreated with PD98059 (B) Chang liver cells preated with SB203580 (C) Chang liver cells preated with JNK inhibitor II

each signaling pathway may regulate different transcriptional factors, which then act in combination to up-regulate RANTES gene. The RANTES luciferase reporter gene analysis demonstrates that only live dengue-2 virus could induce RANTES overexpression indicating that the entry and replication of the virus may be involved in RANTES activation (comparing of DV2 and iDV2 infection).

Dominant-negative mutants of Erk, p38 and JNK (DNErk, DNp 38 and DNJNK) further confirm that

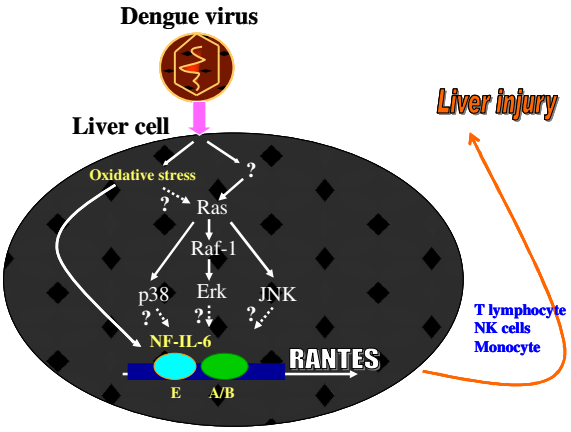

Fig. 6: Diagram of signal transduction of RANTES activation after dengue-2 virus infection. Some connections (the dotted lines) in the diagram are still unclear

dengue-2 virus infection induced RANTES activation is through Erk, p38 and JNK pathways. However, NF-IL6 is the major transcription factor to activate RANTES and the phosphorylation of a threonine residue located at N-terminal of the DNA-binding domain of NF-IL6 by a ras-dependent MAP kinase has been reported[41]. Moreover, dengue-2 virus induced RANTES expression is mediated by activation of NF-IL6 through an oxidative stress-dependent and an oxidative stress-independent pathway[9]. It is well known that one of the up-stream transducers of Erk signaling pathway is Ras/Raf-1. Consistently, we demonstrated that dengue-2 virus infection utilizes Ras/Raf-1 and Erk, p38 and JNK pathways to induce RANTES activation (Fig. 6).

The kinetics of MAPK phosphorylation is closely correlated with the kinetics of dengue virus replication. We are interested in unraveling whether the single viral gene in dengue-2 virus genome, may be responsible for MAP kinase phosphorylation.

However, none of the core, prM, E and NS1 gene expression could induce RANTES activity indicating the regulation of MAPK phosphorylation is not at signal gene level. Some possibilities can not be excluded in this study. 1. Dengue virus has a total of 10 gene products. Whether other gene products can induce RANTES up-regulation remaining unclear? 2. Other factors may be required together with core, prM, E or NS1 expression to activate RANTES expression. This possibility can be clarified by using dengue-2 virus infectious cDNA clone, together with site-directed mutagensis to abort the function of specific genes.

Infection of various viruses could also induce RANTES expression in a wide variety of cells[40, 42, 43]. RANTES representing by its chemotactic activity for

eosinophils plays a pivotal role in the induction of airway inflammation of asthmatics through the recruitment of eosinophils to the inflammation site of airway after influenza virus infection[38]. Whether RANTES secretion induced by dengue virus infection of liver cells can recruit immune cells deserves further clarification. Moreover, whether liver damage in dengue patients was caused by a direct effect of virus replication or an indirect effect of RANTES-mediated inflammation needs further investigation. In summary, this study unravels the signaling pathway for RANTES activation in dengue-2 virus infected liver cells and endothelial cells.

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