July 2005

Chemokine Induction by Dengue Virus Infection: Mechanisms and the Role of Viral Proteins: a Dissertation

Carey L. Medin

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A Thesis Presented

By

CAREY L. MEDIN

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY

JULY 26, 2005

PROGRAM IN IMMUNOLOGY AND VIROLOGY
CHEMOKINE INDUCTION BY DENGUE VIRUS INFECTION:
MECHANISMS AND THE ROLE OF VIRAL PROTEINS

A Dissertation
By
Carey L. Medin

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Program in Immunology and Virology

July 26, 2005
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ABSTRACT

The focus of this thesis is the role of dengue virus in the induction of chemokines. Dengue virus (DENV) occurs as four distinct serotypes, called DENV 1, 2, 3, and 4. Symptomatic DENV infection ranges from a self-limited febrile illness, dengue fever (DF), to a more severe disease, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). DHF is characterized by increased capillary permeability resulting in decreased plasma volume, which may be accompanied by hemorrhagic manifestations. Many factors including T cell cross-reactivity, viral burden, antibody-dependent enhancement and induction of chemokines and cytokines have been reported in DHF and may play a role in the pathogenesis of DENV infection.

Cytokines have been shown to modulate endothelial cell permeability [1-3]. Recent studies have shown that DENV-infected endothelial cells secrete the chemokine, interleukin (IL)-8 in vitro [4]. In addition, the permeability of an endothelial cell monolayer was found to be increased by interleukin-8 (IL-8) in vitro [5]. This thesis examines the effects of DEN2V infection on the induction of chemokines, and specifically, which DEN2V viral protein(s) are involved in the induction of IL-8.

The chemokine induction profile following DEN2V infection was initially assessed in various cell lines that may represent potential targets in vivo, including monocytes, liver cells, and endothelial cells. We hypothesized that distinct profiles of chemokine secretion can be induced by DEN2V infection of various cell types in vitro. We found RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted) and IL-8 were induced in two of the five cell lines. DEN2V infection of primary monocyte-derived dendritic cells induced RANTES and IL-8 along with macrophage
inflammatory protein-1α (MIP-1α), MIP-1β and monocyte chemoattractant protein-1 (MCP-1) but at an earlier time post infection than in the cell lines. These results showed that DEN2V infection induces distinct chemokine profiles in many cell types. In addition, monocytic-derived DCs can secrete chemokines upon infection with DEN2V.

Characterization of the signaling pathways induced by DEN2V revealed that DEN2V induction of chemokines in human embryonic kidney (HEK293A) cells is mainly through the nuclear factor kappaB (NFκB) pathway, as previously reported for endothelial cells and 293T cells [4, 6]. Alternatively, the liver cell line (HepG2) activated mainly activator protein (AP)-1. In addition, DENV infection can induce the activation of the interferon-stimulated response element (ISRE) driven promoter.

IL-8 has been shown to have multiple effects on the immune system ranging from recruiting cells to the site of infection to countering the antiviral effects of type I interferon (IFN) [7, 8]. Previous reports have shown that viral proteins can induce chemokines such as seen with IL-8 induction with the nonstructural protein 5A (NS5A) and core proteins from hepatitis C virus [9, 10]. We hypothesized that protein(s) from DENV could induce chemokine production. The expression of DENV proteins was analyzed for effects on IL-8 and RANTES production in HEK293A cells. The effects of viral replication on IL-8 and RANTES induction were also analyzed using a DENV replicon that contains genes for the capsid protein and the nonstructural proteins. Transfection of plasmids expressing NS5 or the DEN2V replicon induced the expression and secretion of IL-8 but not RANTES. We attributed the lack of RANTES induction to the inability of NS5 or the DEN2V replicon to induce transcription from the ISRE driven promoter. We also found that NS5 and the DEN2V replicon induced IL-8 mainly
through the CCAAT/enhancer binding protein (c/EBP) and AP-1 pathways. The profile of transcription factor activation is different from what was seen with DENV infection of HEK293A cells and suggests that the transient expression of the NS5 protein and the replication and/or translation of the DEN2V genome use different pathways than viral infection to induce IL-8.

In addition, we found that the expression of prM-E, known to produce virus-like particles, could induce IL-8 secretion and activate transcription from the IL-8 promoter. As with the expression of NS5, RANTES was not induced. Analysis of the transcription factors involved in IL-8 induction using luciferase reporter constructs indicated that expression of prM-E induced transcription of IL-8 through NFκB, AP-1 and c/EBP, similar to what was seen with DEN2V infection of HEK293A cells. These results suggest that production of virions or virus-like particles induce IL-8 but that another mechanism in the viral life cycle is responsible for the induction of RANTES expression by DEN2V infection.

We were also interested in the effects of drugs that have been used previously to inhibit cytokine or chemokine production on chemokine induction during DEN2V infection. We hypothesized that pharmacological inhibitors of cytokines will inhibit secretion of chemokines in DEN2V infected cells. We found that the pharmacological inhibitors SB203580 and rolipram enhanced chemokine production in a DEN2V infected liver cell line (HepG2), whereas dexamethasone had the same effect in a kidney epithelial cell line (HEK293A). We conclude that drugs that inhibit signaling pathways involved in cytokine production in other experimental systems can have variable effects on chemokine induction in different cell types during DEN2V infection.
The data generated in this thesis extend our understanding of how DEN2V manipulates the host cell during viral infection to produce chemokines and perhaps enhance viral propagation and dissemination through the induction of IL-8. In addition, this study provides insight into the variable effects pharmacological drug treatment may have on disease progression during DENV infection. These results increase our understanding of DENV pathogenesis and may be helpful in finding better strategies for treatment and prevention.
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<td>antibody dependent enhancement</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate transaminase</td>
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<tr>
<td>ALT</td>
<td>alanine transaminase</td>
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<tr>
<td>c/EBP</td>
<td>CCAAT/enhancer binding protein</td>
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<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T cell</td>
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<tr>
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<td>DF</td>
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<td>DHF</td>
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<td>dengue shock syndrome</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>Fc</td>
<td>constant fragment</td>
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<td>Fc receptor</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<td>JUN-N-terminal protein kinase</td>
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<td>mitogen activated protein kinase</td>
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<td>MOI</td>
<td>multiplicity of infection</td>
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<td>nuclear factor kappaB</td>
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<td>nuclear localization signal</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>RANTES</td>
<td>regulated upon activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-golgi network</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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<td>toll receptor</td>
</tr>
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CHAPTER I:
INTRODUCTION

A. EPIDEMIOLOGY

Dengue is one of the most important human viral diseases transmitted by arthropod vectors in terms of morbidity and mortality. Currently approximately 50–100 million dengue infections and 500,000 cases of DHF occur annually. Of these cases, around 24,000 deaths occur depending on the epidemic activity. Over half the world’s population lives in areas that are at risk for dengue transmission [11, 12].

Dengue viruses are transmitted to humans through the bite of infected female mosquitoes of the genus Aedes. The primary mosquito vector is Aedes aegypti [13]. Mosquitoes can acquire the virus while feeding on the blood of an infected person. After virus incubation for 8-10 days, an infected mosquito is capable of transmitting the virus to susceptible individuals for the rest of the mosquito’s life. A. aegypti feed on humans and breed in collections of clean water (storage jars, containers, etc.). Only the females seek blood meals, and they feed primarily during the day [14].

The geographical distribution of DENV is limited to regions where Aedes mosquitoes are present; these are generally tropical and subtropical regions [15] (Figure I-1). Most countries in central and south America had eradicated A. aegypti in the 1950s and 1960s due to efforts of the Pan American Health Organization. Unfortunately, these efforts were terminated, and by the late 1970s A. aegypti had reinfested many countries [11]. Along with the increase in the mosquito population was the introduction of new strains and serotypes of DENV followed by the emergence of DHF [11, 16]. Several
factors have been implicated in the global resurgence of DENV: failure to control the
*Aedes* mosquito population, increased airplane travel to dengue endemic areas,
World Distribution of Dengue - 2000

Figure I-1. World distribution of dengue and Aedes aegypti in 2000.

B. CLINICAL DISEASE IN HUMANS

1. Dengue Disease

There are four serotypes of DENV (1, 2, 3, and 4). Infection with one serotype will give the host life long immunity to that serotype, but only transient cross protective immunity to the other three serotypes. Dengue virus infections are often asymptomatic, but infection with any serotype can result in undifferentiated fever, dengue fever (DF) or dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) [12].

Classical DF symptoms include an abrupt onset of high fever lasting 5-7 days that can return to normal and then spike again (‘saddleback fever’), severe headache, retro-orbital pain, myalgia, nausea, vomiting, rash, neutropenia, leukopenia and thrombocytopenia [11, 12, 16]. Infants and young children may have undifferentiated fever with a maculopapular rash whereas older children and adults tend to have either a mild febrile illness or the classical DF [11].

DHF occurs in ~2% of individuals that are infected a second time with a different serotype of DENV (Figure I-2). Generally, symptoms are similar to DF initially. However, at the time of defervesence the patient can progress to the more severe disease, DHF. Clinical findings of DHF at the time of defervesence include plasma leakage, which results in hemoconcentration, and thrombocytopenia [11]. A system for grading the severity of DHF has been defined by the World Health Organization (WHO) [12]. Grade I DHF requires all of the following findings: fever, severe thrombocytopenia (platelet count <100,000 cells/µl), plasma leakage, and a positive tourniquet test. In addition to these minimal criteria for DHF, Grade II DHF includes spontaneous bleeding.
Grades III and IV are considered to be DSS in which hypovolemia and circulatory shock develop [12, 17].
Figure 1-2. Frequency of dengue fever and dengue hemorrhagic fever in children ages 1-14. DF is more prevalent than the more severe forms of DENV disease, DHF/DSS. However, the incidence of DHF/DSS increases during a secondary infection. Figure is based on data from reference [18] and the portrayal is adapted from reference [19].
2. Pathological findings

Although DHF is classified by plasma leakage, no destruction of vascular endothelial cells is evident in autopsy tissue samples [20]. Therefore, plasma leakage is thought to be likely due to altered vascular permeability rather than to structural destruction of endothelial cells [20]. The mechanism of DENV induced plasma leakage is still undefined, but the process is thought, based on in vitro studies, to be mediated by proinflammatory cytokines and chemokines such as IL-6, tumor necrosis factor α (TNFα), IL-8 and RANTES [21].

DHF is frequently associated with moderate degrees of hepatic functional abnormalities and can show characteristics typical of acute hepatitis. Most changes in the liver are focal and mild with fatty changes, formation of Councilman bodies and degeneration of hepatocytes and Kupffer cells [22, 23]. In addition, the levels of aspartate transaminase (AST) and alanine transaminase (ALT) are significantly higher in patients with more severe grades of DHF [24, 25].
C. PATHOGENESIS OF INFECTION

Approximately 80 – 90% of DHF cases occur during a second DENV infection [18, 26, 27]. DHF commonly occurs in areas where multiple serotypes of dengue virus are circulating. The hallmark sign of DHF is plasma leakage. Plasma leakage occurs near the time of defervesence and after peak viremia [28]. Generally, the plasma leakage is short-lived without tissue damage. These clinical features suggest that plasma leakage following DENV infections is due to an immunologic response rather than a direct viral effect. Some of the factors that have been associated with dengue pathogenesis include levels of viremia, antibody, cytokine and T-cell responses.

1. Virulence of viral strains

Rico-Hesse et al found an association of DHF in the American countries - Venezuela, Brazil, Colombia and Mexico - with the introduction of a Southeast Asian genotype of dengue type 2 virus (DEN2V) in the 1990s [29]. In addition, they found no evidence that the ‘American’ genotype which has only been associated with DF was still present in areas that experienced the introduction of strains of the Southeast Asian genotype [29]. Recently, the same research group reported a greater production of viral progeny from DCs that were infected with some of the Southeast Asian genotype viruses when compared to the American genotype viruses [30]. These results suggest that viruses with the potential to cause DHF (Southeast Asian genotype) can replicate more efficiently in DCs, which may give them an advantage over their more benign counterparts (American genotype) by increasing the levels of viremia in the human host.

2. Antibody Dependent Enhancement
Antibody dependent enhancement (ADE) is speculated to play a key part in the pathogenesis of severe infections during secondary DENV infections. Antibodies to DENV are present in the blood due to primary infection. ADE is thought to occur when circulating antibody is at subneutralizing concentrations [15, 31-37]. During secondary infection, subneutralizing antibodies recognize the DENV and form complexes. The constant fragment (Fc) portion of the antibody is recognized by cells expressing Fc receptors (FcR) such as monocytes [38]. The binding of antibody-virus complexes to the FcR allows enhanced uptake of virus, resulting in an increased number of cells being infected by the virus [39]. The antibodies that mediate ADE in dengue are primarily directed against the E protein [40].

3. Complement activation

A marked reduction of complement proteins and a concurrent increase in complement fragments was observed in DHF/DSS, and the degree of complement activation correlated with the severity of the disease [41]. Complement activation, assessed by an increase in complement fragments C3a and C5a, preceded onset of shock in DENV patients in another study [42]. The complement fragments C3a and C5a are proinflammatory molecules involved in activation and chemotaxis of myeloid cells expressing anaphylatoxin receptors C3aR and C5aR/CD88 [43]. C5a is a potent chemoattractant of neutrophils and monocytes. C3a is known to induce histamine release from mast cells, which enhances vascular permeability [6, 43]. It has also been demonstrated that the complex C5b-9 forms on the surface of endothelial cells and can create gaps in the endothelium without cytolysis [44]. Avirutnan et al. found that serotype cross-reactive Abs to DENV could deposit C3 and C5b-9 on dengue infected
endothelial cells *in vitro*, and that this occurred independent of DENV-induced cell death [6]. These mechanisms have been proposed to contribute to vascular leakage in DHF [6].

**4. T lymphocytes**

Cytotoxic T cells (CTLs) have been shown to play an important role in viral clearance from the host [45, 46]. Virus-specific CTLs directly lyse virus-infected antigen presenting cells. Additionally, these CTLs can release cytokines such as IFNγ upon activation. Initially, CD8+ T cells were thought to be the only cytotoxic T cells. However, some CD4+ T cells have also shown cytotoxic activity [47-49]. It is still thought that CD8+ T cells are the main mechanism for clearance and recovery from viral infections.

CTLs directly lyse cells through the perforin/granzyme pathway. Perforin forms pores in the target cell causing a loss of plasma membrane integrity and, subsequently, cell lysis. CTLs also express Fas ligand, which can interact with Fas that is expressed on antigen presenting cells [50]. This interaction induces apoptosis of the target cell.

A predominant Th1 response, stimulating CTL activity, is thought to occur during the initial stage of dengue infection, as characterized by higher serum concentrations of TNFα, IL-2, IL-6, and IFNγ [51]. A Th2 response, which is involved in antibody-mediated responses, tends to appear later in DENV infection and is characterized by an increase in IL-10, IL-5 and IL-4 levels [51]. Severe cases of DENV infections had increased levels of IL-10, IL-13, IL-18 and TGFβ in several studies, whereas high levels of IL-12 were found in patients with less severe dengue disease [52-54]. These results suggest that predominant Th2 response can increase the severity of DENV infection.
It has been proposed that T cells play an important role in the immunopathogenesis of DENV disease [55-58]. Primary DENV infection induces the development of DENV-specific memory CD4+ and CD8+ T cells. In response to *in vitro* stimulation with DENV antigens, a memory DENV-specific CD4+ and CD8+ CTL response are detected [57]. CD4+ T cells produce IFNγ, TNFα, and TNFβ upon stimulation *in vitro*. IFNγ has been shown to increase expression of Fcγ receptors on monocytes, which can further augment DENV infection of monocytes by virus-antibody complexes [59]. In addition, activated DENV specific CD4+ T cell clones were able to lyse non antigen presenting HepG2 hepatoma cells, suggesting a role for T cell immune responses in liver injury during DENV infections [57].

Kurane et al have shown that DENV-specific cytotoxic T cells can be detected in human volunteers who received live attenuated monovalent dengue vaccines as a primary infection [60]. Characterization of the DENV-specific CD8+ T cells found that some of the T cells were cross-reactive with other serotypes of dengue virus, indicating the potential for them to be activated during a secondary infection [28, 58, 61]. Studies looking at epitopes of DENV proteins from all four serotypes and their ability to activate memory CD8+ T cells found that the epitopes that elicited the predominant responses were contained within the NS3 and NS1/NS2A proteins [62]. Other epitopes that activated memory CD8+ T cells were found within the E and prM proteins [62]. These studies show that dengue specific cross-reactive T cells can be activated during a secondary infection.

Markers of T cell activation, including levels of soluble CD4, soluble CD8, soluble IL-2 and soluble TNFR, are elevated in DENV infected patients with acute
disease [63-66]. Increased production of cytokines including IL-10, IL-13, IL-18, IFN γ and TNFα have also been associated with more severe DENV disease [52, 53, 67]. These results suggest that immune activation contributes to the pathogenesis of DENV.

Based on the in vitro evidence previously mentioned regarding T cell activation, ADE and complement activation, our laboratory has proposed a model for DENV pathogenesis. It is proposed that antibody-dependent enhancement of DENV infection is likely to result in an increased number of DENV infected cells during a secondary infection. This could increase the number of antigen presenting cells and thereby increase the number of activated T cells. In secondary infections, cross-reactive memory T cells generated during the primary infection are reactivated earlier and more intensely than naive T cells. This contributes to the higher level of T cell activation seen during secondary DENV infections (Figure I-3).
Figure 1-3. Model for DHF pathogenesis. Memory DENV-specific T cells are activated following a secondary infection of the host with a different DENV serotype. The activated memory T cells rapidly express cytokines such as TNFα and IFNγ. In addition, DENV specific antibodies can increase the viral burden of virus-infected cells expressing Fcγ receptors by ADE. The increased number of viral antigen presenting cells can also activate memory T cells. The accumulated production of cytokines by memory and naïve T cells during a secondary infection along with complement activation are thought to enhance the effect on vascular endothelial cells and lead to plasma leakage. Figure reprinted from reference [68].
D. VIRUS STRUCTURE AND COMPOSITION

1. RNA

The viral family *Flaviviridae* is subdivided into the three genera - Flavivirus, Pestivirus and Hepacivirus; a separate group of viruses that includes GB virus C/hepatitis G viruses (GBV-C) also belongs to this viral family but has not been assigned a definite taxonomic classification [69]. Members of the genus Flavivirus have a type I cap structure (m7GpppAmp) at the 5’ end of the genome and a highly structured 3’ untranslated region (UTR) [70]. The flavivirus genomic RNA also is distinguished by the absence of a 3’-terminal poly(A) tract [71]. There is evidence that the 5’ and 3’ ends stack together to cause a cyclization of the genome which may be an important feature for RNA replication in Flaviviruses [72, 73]. The hepaciviruses (hepatitis C virus; HCV), pestiviruses and GBV-C control translation by an internal ribosomal entry site (IRES) in the 5’ UTR and have a shorter, less-structured 3’ UTR than flaviviruses [74].

The four serotypes of dengue virus are members of the genus Flavivirus, which also includes west nile, yellow fever and japanese encephalitis viruses [69]. Flaviviruses consist of a single stranded positive sense RNA genome that is approximately 11 kb. The viral genome is translated in the cytoplasm as a polyprotein (Figure I-4). The translocation of the polyprotein back and forth across the endoplasmic reticulum (ER) membrane is directed by signal and stop-transfer sequences (Figure I-5). The polyprotein is subsequently co- and post-translationally modified by viral and host-encoded proteases to produce three structural and seven nonstructural proteins. The mature virion contains the structural proteins: the capsid, C; a membrane associated protein (which is produced from the precursor prM), M; and the envelope protein, E. The nonstructural (NS) proteins
include large, highly conserved proteins NS1, NS3, and NS5 and four small hydrophobic proteins NS2A, NS2B, NS4A, and NS4B [71, 75, 76].

2. Structural proteins

The C protein is highly basic and forms a structural component of the nucleocapsid [76]. The terminal hydrophobic signal sequence is cleaved by the viral NS2B-NS3 protease before virion assembly resulting in the mature form of DENV C protein [77, 78]. The DENV C protein is necessary for encapsidation of the viral genome, although the mechanism is not known. Infected cells release subviral particles that do not contain either C or the viral genomic RNA, suggesting an important role for C in proper packaging of the infectious virion [79]. Recently, the structure of the DEN2V C dimer was elucidated by nuclear magnetic resonance (NMR) techniques [80, 81]. DEN2V C protein contains a hydrophobic cleft on one side where it is proposed to interact with the viral lipid bilayer, and a positively charged region on the opposing side that is thought to interact with the viral RNA [81].

The prM protein is a glycoprotein that forms heterodimers with E on the intracellular immature virion surface. The dimerization of prM and E is thought to prevent exposure of the fusion peptide within the cell [75]. The prM protein contains a stop transfer sequence and a signal sequence in two transmembrane helices. The stop transfer sequence localizes both prM and E to the lumen of the ER (Figure 1-5). prM and E dimerize within the lumen of the ER upon cleavage by a host signalase. Upon maturation and release of the virion from the cell, prM is further cleaved by the host enzyme, furin, to produce M. This process releases E from prM [82]. The E proteins
then reorganize into homodimers [83, 84]. The mature virion has a smooth appearance compared to the immature virion which contains spikes on the surface.

The structure of the E glycoprotein contains three domains. The amino terminus is located in the center of the folded protein (domain I). Domain II contains the dimerization domain and the fusion peptide. On the other side of domain I is domain III, which contains the immunoglobulin – like domain. This domain is thought to bind to the cellular receptor (reviewed in [85]).

Cells transfected with recombinant prM and E from tick borne encephalitis virus (TBE) or DENV release viral-like particles that do not contain C or genomic RNA [86, 87]. The relationship between prM and E have been studied using viral like particles, which undergo the same maturation process as the intact virion [86].

3. Nonstructural Proteins

The seven nonstructural proteins, NS1; NS2A, NS2B, NS3, NS4A, NS4B, and NS5, are encoded in the 3’ region of the viral genome (Figure 1-4). The NS1 protein contains a signal sequence that is located in the carboxy terminus of the E protein and is inserted into the ER [88]. NS1 is posttranslationally cleaved from NS2A by an unknown ER-resident host proteinase [89]. NS1 is a glycoprotein that forms homodimers and interacts with membranes [90-92]. It can be secreted from infected cells or localize to sites of RNA replication within the cell. Small amounts of the NS1 protein associate with the cell surface and are capable of signaling through a glycosyl-phosphatidylinositol (GPI) anchor in response to cross-linking by NS1-specific antibodies [93-95]. In addition, NS1 is an essential component of the viral replicase [96, 97].
The remaining nonstructural proteins are localized on the cytoplasmic side of the ER membrane [89, 98]. The NS2A protein was found to be involved in proteolytic cleavage of NS1 at its carboxy terminus [88]. The NS2B protein associates with NS3 to form the viral protease. The NS2B-NS3 protease mediates cleavage of the viral polyprotein at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B and NS4B/NS5 junctions [99-102].

The NS3 protein is part of the replicative complex and has several enzymatic functions. The serine protease domain of NS3 is in the amino terminus of the protein [71, 103-105] whereas the carboxy terminus of the protein functions as a helicase [106, 107], a nucleoside 5'-triphosphatase (NTPase) [107-109] [104]), and a RNA 5'-triphosphatase (RTPase) [110]. The small proteins NS4A and NS4B have not been studied extensively. However, it has recently been shown that NS4B, and to a lesser extent, NS2A and NS4A, are able to block type I interferon (IFN) signaling [111]. This effect is discussed further below (SECTION I. FLAVIVIRUS EVASION OF THE IMMUNE SYSTEM).

The NS5 protein is the largest of the dengue proteins (104 kDa) and is highly conserved among the flaviviruses [112]. NS5 contains a well characterized RNA-dependent RNA polymerase activity in the carboxy terminus (amino acids 455 - 900) [113-117]. Amino acids 1 - 296 of the amino terminus of DEN2V NS5 exhibits (nucleoside-2'-O-)-methyltransferase activity on capped RNA [118]. NS5 was found to interact with NS3 to form a replication complex [119]. In addition, NS5 can stimulate the NTPase activity of NS3, which is necessary for unwinding of dsRNA substrates by helicase activity during viral replication [120].
Figure 1-4. Schematic of flavivirus genome. Untranslated regions of the genome (top), with their 5’ and 3’ terminal structures, are indicated by black lines. The single open reading frame encodes a polyprotein (middle) that is processed by the viral NS2B-NS3 protease and host proteases to the mature viral proteins (bottom). Cleavage sites for host signalase (♦), the viral serine protease (†), furin or other Golgi-localized protease (♥), or unknown proteases (?) are indicated. Black bars represent hydrophobic regions. Shaded regions indicate mature structural proteins. The genome is not drawn to scale. Figure reprinted from reference [69] with permission from Lippincott Williams & Wilkins.
Figure I-5. Membrane topology of flavivirus proteins. The proposed orientation of the flavivirus polyprotein cleavage products with respect to the ER membrane is shown. The proteins are drawn to scale (areas are proportional to the number of amino acids) and arranged in order (left to right) of their appearance in the polyprotein. Mature structural proteins are shaded and carboxy-terminal membrane-spanning segments of M and E are indicated. Cleavage sites for host signalase (†), the viral serine protease (↓), furin or other Golgi-localized protease (♥), or unknown proteases (?) are indicated. Figure reprinted from [69] with permission from Lippincott Williams & Wilkins.
E. VIRAL LIFE CYCLE

Infection of the host cell with flaviviruses occurs by receptor-mediated endocytosis into clarithin-coated pits [32, 121-124]. Acidification of endosomes causes E protein rearrangement to form homotrimers on the surface of the virion [83, 84]. It has been proposed that flaviviruses follow class II membrane fusion [85]. The E protein in flaviviruses obtain their trimeric form after acidification and the fusion peptide is usually internally located [125]. The fusion peptide within domain II of flaviviruses are exposed by receptor binding and low pH. The hinge between domains I and II presumably allow E to insert the fusion peptide into the host’s lipid bilayer. Domain III is thought to fold back onto itself bringing the host bilayer and the viral bilayer into contact allowing hemifusion. The E proteins then form trimers on the virion surface [85].

After the virion has fused with the host cell membrane, the nucleocapsid core is released into the cytoplasm of the cell (Figure I-6) [32, 121, 123, 126]. The C protein disassociates from the viral RNA and translation of the viral RNA is initiated. Following translation and processing of the viral proteins, the nonstructural proteins form a viral replicase and bind to the viral RNA, initiating replication of the viral genome [127].

Cytoplasmic membranes in the perinuclear region of the cell undergo proliferation of the ER and subsequent appearance of smooth membrane vesicles during flavivirus infection [93, 94, 128-131]. These vesicles are sites of viral RNA replication.

Once the viral proteins are translated and the genome is replicated, the virion begins to assemble within the lumen of the ER. Immature viral particles are shuttled through the trans-golgi network (TGN). The immature virion contains a spiked surface which has been shown to be the prM protein capping the E protein (Figure I-7) [75].
During exocytosis, furin protease cleaves prM to M releasing E [132]. E then forms homodimers on the surface of the virion. The mature viral particle has been shown by cryoelectronmicroscopy to have a spikeless, smooth surface with sets of three parallel dimers containing E (Figure I-7) [75, 83]. Mature viral particles are exocytosed via secretory vesicles.
Figure I-6. Flavivirus life cycle. Virions enter the cell by receptor-mediated endocytosis (1,2). Low pH of the endosomal vesicle initiates conformational changes in the virion, fusion of the viral and cell membranes, and release of the nucleocapsid into the cytoplasm of the cell (3,4). The positive-sense RNA is translated into a single polyprotein that is co- and post-translationally modified by viral and host proteases (5). Genome replication occurs on intracellular membranes in the perinuclear region (6). Structural proteins and newly synthesized RNA bud into the lumen of the ER (7). Virus assembly occurs on the surface of the ER. The resultant non-infectious, immature viral
and subviral particles are transported through the TGN (8). The immature virion particles are cleaved by the host protease furin, resulting in mature, infectious particles. Mature virions and subviral particles are released from the cell by exocytosis (9). Figure was reprinted from reference [69] with permission from Lippincott Williams & Wilkins.
Figure 1-7. Structure of flavivirus virions. The intracellular (immature) virion depicted on the left shows the formation of prM and E heterodimers. Upon maturation, prM is cleaved to form M (right side of virion), allowing E to form E homodimers. This results in a smooth, spikeless surface. Figure reprinted from reference [69] with permission from Lippincott Williams & Wilkins.
F. CELLS SUSCEPTIBLE TO DENGUE INFECTION

It is unclear which cells are primary targets of DENV infection in vivo. Initial studies of tissue obtained at autopsy from DHF patients indicated that DENV was associated with mononuclear cells such as Kupffer cells in the liver, pulmonary macrophages, and mononuclear cells in the blood and skin [133]. Additionally, DENV has been isolated from peripheral blood leukocytes from patients with DHF [76, 134]. However, these cell types are not highly susceptible to DENV infection in vitro [135, 136]. Langerhans cells and monocyte-derived dendritic cells (DCs) have been shown to be more permissive to in vitro DENV infection than monocytes [135]. DENV-infected DCs express maturation markers and produce TNFα and IFNα [137, 138]. In vitro, B lymphocytes are also susceptible to DENV infection and produce TNFα and IFNα upon infection, similar to monocytes [139].

DENV antigen has also been found in hepatocytes in tissue obtained from autopsies of DHF patients [22, 23, 133, 140]. DENV infected patients were found to have high AST levels, suggesting liver involvement [141-143]. These results correlate with in vitro studies that show productive infection of five different liver cell lines and an increase in AST and ALT levels [143]. However, other immunological mechanisms can induce liver damage such as activated T cells and cytokines [57, 144].

In addition, DENV antigen has also been found in the sinusoidal endothelium from autopsies of DHF patients [22, 23, 133, 140]. In vitro, DENV can also productively infect endothelial cells [145, 146]. Other cell types such as T cell lines, monocytic cell lines and skin fibroblasts can be infected by DENV in vitro [147-149].

G. RECEPTOR FOR DENGUE INFECTION
The receptor for dengue viral infection is still undefined. Studies have shown that recombinant E protein binds to the highly sulfated form of the glycosaminoglycan (GAG) heparin sulfate (HS) [150, 151]. Treatment of cells with heparin, heparin-derived oligosaccharides, or suramin, a polyionic sulfate, inhibited dengue virus envelope protein binding to Vero cells and viral infectivity in vitro. HS is involved in many biological roles including cell attachment and migration, cell signaling, and virus infection [152]. HS is ubiquitously expressed on the surface of many cell types and is used by several microorganisms, including viruses, gram-positive and gram-negative bacteria, and parasites to gain entry into host cells [153-155]. Viruses that use HS GAGs include Sindbis virus [156, 157], Venezuelan equine encephalitis virus [158], Ross River virus [159] and human immunodeficiency virus [160].

It has been proposed that a coreceptor is involved for DENV infection to explain the limited cell tropism of DENV [161, 162]. Potential receptors/coreceptors include a 45 kDa glycoprotein in C6/36 cells [163], a 74-kDa protein present in Vero cells [162], two proteins of approximately 40 to 45 kDa and 70 to 75 kDa from a B-cell line and a myelomonocytic cell line [164], a 105 kDa protein in erythroleukemia cells [165], membrane proteins of 27, 45, 67, and 87 kDa in monocytes/macrophages [166] and proteins of 29 and 43 kDa from an endothelial cell line [145]. However, these proteins have not been definitively identified.

Recently, Reyes-del Valle et al used a His-tagged E protein of DENV as bait to pull down and analyze cellular proteins from neuroblastoma cells and U937, a myelomonocytic cell line, using affinity chromatography [161]. They found an 84 kDa molecule that interacted with E and identified it as heat shock protein 90 (Hsp90) by
mass spectrometry. Antibody to Hsp90 inhibited DENV infection of monocyte/macrophage cells, supporting its potential role as a cellular receptor for DENV.

DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN, CD209), a dendritic cell C-type lectin and its homologue L-SIGN have been shown to mediate DENV infection of primary dendritic cells or cells transfected with DC-SIGN or L-SIGN [167, 168]. Antibodies directed to DC-SIGN inhibited infection of DCs. Lozach et al found that interaction of E protein with DC-SIGN mediated DENV infection of monocytic-derived DCs but not Langerhans cells [169]. Furthermore, endocytosis through the interaction with DC-SIGN was not required for viral replication in HeLa cells, suggesting that DC-SIGN functions as an attachment receptor for enhanced infection through an unidentified receptor.

H. INNATE IMMUNITY

1. Viral induction of Type I IFN

The innate immune system is the first line of defense against an invading viral pathogen. Once the host recognizes the invading virus, signaling pathways are activated to induce expression of multiple transcription factors that bind to the interferon α and β promoters [170-172]. The transcription factors that bind to the IFNβ promoter positive regulatory domain I (PRDI), PRDII and PRDIV are interferon regulatory factor (IRF-3) and IRF-7, NFκB, and ATF-2/c-Jun, respectively. NFκB activation occurs by virus-induced phosphorylation and degradation of the IκB protein [173]. Activating transcription factor-2 (ATF-2) is also activated by phosphorylation through MAP kinase activation [170].
The RNA helicase, RIG-I, senses dsRNA in the cytoplasm of the infected cell through pattern recognition receptors (PRR) [174]. Toll receptor 7 (TLR7) and TLR8 recognize single stranded RNA (ssRNA) in the endosomes [175-177] whereas TLR3 and TLR9 recognize double stranded RNA (dsRNA) and unmethylated DNA, respectively [178, 179]. Activation of RIG-I and TLRs activates two IκB kinase (IKK)-related kinases, IKKε and TANK-binding kinase –1 (TBK-1) [180, 181]. These kinases phosphorylate and activate interferon regulatory factor 3 (IRF-3), which leads to IFNβ induction.

IRF-7 expression is induced by type I IFN and then activated by viral replication [182-184]. IRF-7 is also involved in promoting IFNα gene expression to elicit a full IFN response [172, 185-187].

2. Signaling from Type I IFN receptors

The receptor for IFNα and β is composed of two proteins, IFNAR-1 and IFNAR-2 [188]. Upon IFN binding to its receptor, the Janus tyrosine kinases (Jak) nonreceptor tyrosine kinases, Tyk2 and Jak1, which are constitutively bound to the subunits of the IFN receptor, are phosphorylated (Figure I-8). Phosphorylation of Tyk2 and Jak1 leads to phosphorylation of tyrosine residues within the cytoplasmic tail of the receptor and recruitment of signal transducers and activators of transcription (Stat) proteins [189]. The phosphorylated Janus kinases (Jaks) phosphorylate the receptor bound Stat proteins. The phosphorylated Stats form homo- and heterodimers that can translocate into the nucleus and bind in conjunction with a DNA binding adapter protein of the IFN regulatory factor (IRF) family (ISGF3) to DNA elements within IFN-sensitive genes (ISG) such as IFN-stimulated response elements (ISREs) [190]. Genes that contain ISREs
which include double-stranded RNA activated protein kinase (PKR), 2’ , 5’-oligoadenylate synthetase (OAS), myxovirus induced protein A (MxA), guanylate-binding protein (GBP), IRF7, chemokines and cytokines are activated by the Jak/Stat pathway [173]. In addition, Stat homo- and heterodimers can bind to gamma activated sequences (GAS) within promoters of other ISGs such as c-fos and IRF-1 [191]. Activation of the p38 MAPK pathway is important for full IFN-induced transcriptional activation through ISRE and GAS elements [192, 193].

3. DENV infection and innate immunity

As with other viruses, the innate immune response is activated by DENV infection and can inhibit DENV replication. Knockout mice that lack IFNα/β and IFNγ receptors develop limb paralysis and die after challenge with dengue virus [194, 195]. Pretreatment of cultured cells with IFNα/β dramatically reduced DENV replication through inhibition of translation of DENV RNA [196, 197]. In contrast, addition of IFNα/β after DENV replication has little effect on DENV replication [196, 197]. These data suggest that IFN signaling is not able to properly function in cells that are infected with DENV. In addition, DENV replicates to high titers (10^9 infectious doses per ml) in humans even though there are high levels of circulating IFNα [198-200]. These results suggest that there are mechanisms that DENV uses to counter the IFN response, as seen with other viruses [201, 202].
Figure I-8. Cellular response to viral infections. Viral dsRNA activates the transcription factors NFκB, AP-1, and IRF-3. Full activation of the IFNβ promoter requires cooperative action of these transcription factors, but, IRF3 is most important. Phosphorylation and activation of IRF-7 induces the expression of several IFNα subtypes which amplify the IFN response. Activation of both RIG-I, a dsRNA sensor, and toll receptors (TLRs) activates the kinases IKKe and TBK-1. These kinases phosphorylate and activate IRF-3 leading to IFNβ induction. IFNα/β can then bind to its receptor.
(IFNAR) and activates the expression of numerous ISGs through activation of the Jak/Stat pathway. Mx, OAS and PKR are enzymes with antiviral properties. Figure reprinted from reference [203].
I. FLAVIVIRUS EVASION OF THE IMMUNE SYSTEM

DNA viruses have evolved to acquire host genes and use these genes as decoys to evade the humoral or cellular antiviral response [204-206]. RNA viral proteins, on the other hand, tend to be multifunctional due to size constraints of the viral genome. Therefore, RNA viruses tend to use alternate mechanisms for immune evasion.

One mechanism that RNA viruses use to evade the immune system is to generate quasispecies due to replication with a low fidelity RNA polymerase [207, 208]. These viruses with random mutations in their genome can evade recognition by neutralizing antibodies and T cells. Changes in the hypervariable region of the immunodominant E2 protein in HCV, for example, have been associated with viral persistence and loss of humoral response [209-212]. In addition, the loss of antigen presentation and capacity to stimulate T cells to proliferate and produce cytokines in HCV correlates with changes in the immunodominant epitopes of NS3 protein [213-215]. Quasispecies of DENV with changes in C, E and NS2B proteins have been identified but have yet to be correlated with disease severity [216, 217].

The initial immune response to viral infection is IFN production [218]. Evidence has shown that flaviviruses are able to attenuate IFN antiviral effects [9, 196]. The distantly related member of the family Flaviviridae, HCV, has evolved multiple mechanisms to inhibit IFN antiviral activity. Expression of HCV proteins blocks the transcriptional response to IFNα [219]. HCV NS5A and E2 proteins prevent phosphorylation of eIF2α and the arrest of viral translation by inhibiting PKR activity [220-224]. In addition, the HCV core protein expressed in HepG2 and Huh-7 cells inhibited IFNα-induced nuclear STAT1 import [225]. This led to reduced MxA
expression and increase expression of suppressor of cytokine signaling-3 (SOCS3) and thereby preventing the activation of ISGs in response to IFN [225, 226].

RIG-I has recently been identified as a cytoplasmic dsRNA sensing mechanism and induces IFN expression. The RIG-I pathway elicits the activation of transcription factors such as NFκB and IRF-3, which are also involved in chemokine transcription [174]. The HCV NS3/4A protease inhibited IRF-3 phosphorylation by inhibiting RIG-I dependent signaling to the IFN pathway [227, 228]. In addition, Otsuka et al demonstrated that NS3 directly interacts with TBK1. This interaction prevents TBK1 from associating with IRF-3, thereby inhibiting activation of IRF-3 [229].

DENV has also been shown to counter the antiviral effects of IFNα. Diamond et al found that treatment of HepG2, Huh-7 or K562 cells with IFN as little as four hours after infection with DENV resulted in almost a complete loss of antiviral effect [196]. Munoz-Jordan et al reported that the DENV NS4B protein could block the IFN-induced signal transduction cascade by interfering with Stat1 function [111]. NS4A and NS2A proteins were also able to block IFN signaling but to a lesser extent. NS4B, NS2A and NS4A together were able to block IFN signaling completely. Recently, Jones et al used K562 cells stably transfected with a dengue replicon and found that Stat1 phosphorylation was inhibited during IFNα signaling but not during IFNγ signaling [230]. In addition, they report that DENV infection of K562 cells and cells containing the DENV replicon had reduced levels of phosphorylated Stat2, which is specific to the IFNα pathway. These results suggest that DENV specifically inhibits the IFNα pathway through the inhibition of Stat2. The evolution of independent mechanisms to inhibit the
antiviral activity of IFN by many viruses highlights its key role as one of the first antiviral defense mechanisms [218].
Chemokines have been implicated in many aspects of immunity including hemopoietic cell migration, Th1/Th2 development, angiogenesis/angiostasis, cell recruitment, lymphoid trafficking and lymphoid organ development [231]. Chemokines are small molecules whose primary role in immunity is to attract leukocytes to sites of inflammation. Chemokines can be divided into four classes – CC-, CXC-, C-, CX3C-chemokines. These four classes were based on the number and spacing of conserved cysteine residues found in the N-terminus of the polypeptide [232]. Cells attracted to chemokines express heterotrimeric, 7-transmembrane-spanning, G-protein coupled receptors [232]. Chemokine receptors have a short extracellular N-terminus that is essential for receptor activation and a short intracellular C-terminus [233]. Differential expression of chemokine receptors and the expression of specific chemokines from leukocytes determine the migration of particular cells during inflammation [204].

Chemotaxis of cells toward a chemokine gradient requires signaling from the short intracytoplasmic tails of the G-protein coupled receptors [234, 235]. After ligand binding, the receptor-bound heterotrimeric Gβγα subunits dissociate into Gαi and Gβγ subunits. Gβγ can directly activate phosphoinositol 3 kinase (PI3Kγ) and phospholipase C (PLC) [234, 235]. PLC yields inositol (1,4,5) triphosphate and diacylglycerol (DAG) [236]. Inositol triphosphate mobilizes the release of intracellular stores of calcium, which, in combination with DAG, activates many protein kinase C isoforms [237]. PI3Kγ activation is important for cytoskeletal rearrangement, chemotaxis and degranulation [238]. The Gαi subunit can induce Src family activation and the p85/p110
PI3K pathway, which are also involved in cell polarization/movement and the
degranulation response [232].

Chemokines regulate the migration of leukocytes into extravascular tissues [233, 239]. Leukocytes stop active rolling along the endothelium at sites of infection once specific vascular ligands of the immunoglobulin family, such as E-selectin, intercellular adhesion molecule (ICAM), vascular cell adhesion molecule I (VCAM-1), and mucosal addressin cell adhesion molecules 1 (MAdCAM-1), bind to their leukocyte integrin receptors [239, 240]. Chemokines expressed at the sites of infection produce a gradient of proteins. These chemokines increase the avidity of leukocyte receptor binding to endothelial ligands, thus creating a tight adhesion of the leukocyte to the vascular endothelium. This adhesion allows the leukocyte to transmigrate or undergo diapedesis at intercellular borders or junctions of endothelium [239, 241]. Leukocytes then move along the chemokine concentration gradient toward the site of infection.

Chemokines and chemokine receptors are important for defense against viruses [242]. Efficient migration of activated leukocytes to the sites of infection influences the outcome of disease. However, viruses can utilize the chemokine system to counteract the immune system. Examples include the use chemokine receptors CCR5 and CXCR4 for HIV infection of T lymphocytes and the expression of the respiratory syncytial viral glycoprotein, G, which can bind the fractalkine receptor, CX3CR1 and inhibit chemotaxis of natural killer cells and Th1 cells [243].

1. IL-8

Interleukin (IL)-8 (also known as CXCL8) is a member of the CXC chemokine family and interacts with two receptors, CXCR1 and CXCR2 [244]. IL-8 is produced by
many cell types, including monocytes/macrophages, T cells, neutrophils, fibroblasts, endothelial cells, keratinocytes, hepatocytes, astrocytes and chondrocytes. Moreover, IL-8 has chemoattractant effects on neutrophils and T cells [245, 246]. Expression of IL-8 is induced by a variety of stimuli such as LPS, TNF, IL-1 and viral infection [244]. IL-8 has been shown to promote adhesion of neutrophils to endothelial monolayers [247] and stimulate neutrophil migration across endothelium [248], pulmonary epithelium [249] and fibroblast monolayers [250].

The core IL-8 promoter contains AP-1, NFκB and CAAT/ enhancer binding protein (c/EBP) binding sites (Figure 1-9) [251]. In unstimulated cells, the IL-8 promoter is repressed by three mechanisms. NFκB-repressing factor (NRF) is bound to the negative regulatory element (NRE), which overlaps the NFκB binding site [252]. Octamer-1 (Oct-1) is bound to an octamer binding site that is on the complementary strand in the opposite direction of the c/EBP binding site [253]. Lastly, histone proteins are deacetylated by the action of histone deacetylase 1 (HDAC-1) [254].

Upon activation of the IL-8 promoter, the CREB-binding protein (CBP)/p300 hyperacetylates histones which results in chromatin remodeling [255]. In addition, c/EBP replaces OCT-1 and the p65 subunit of NFκB binds to its cognate site next to NRF. NRF then becomes a coactivator [252]. In addition, AP-1 and NFκB become phosphorylated [251]. Studies have shown that c/EBP binds weakly to the IL-8 promoter site and that strong cooperative binding occurs when NFκB is bound to its adjacent binding site [256].

Another level of control of IL-8 expression is post-transcriptional regulation by the p38 mitogen activated protein kinase (MAPK) pathway [251]. Activation of the
MAPK kinase 6 (MKK6) and MAPK-activated protein kinase-2 (MK-2), which are components of the p38 MAPK pathway, was shown to induce stabilization of the IL-8 transcript [257-259]. In addition, AP-1 is activated by MAPKs. There are three MAPK pathways that can activate AP-1 and contribute to IL-8 gene expression - the extracellular-regulated protein kinase (ERK), JUN-N-terminal protein kinase (JNK), and p38 MAPK cascades [251, 260].

2. RANTES

RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted), also known as CCL5, is a member of the CC family of chemokines. RANTES can bind to four receptors; CCR1, CCR3, CCR4, and CCR5 [261-264]. RANTES can attract many cell types, including CD4+ and CD8+ naïve and memory T cells, NK cells, dendritic cells and monocytes [265-269].

RANTES is induced in a variety of tissues in response to tissue damage, infection or foreign substances [270-272]. The promoter for RANTES has been characterized in T cells, monocytes, astrocytes and mesangial cells [272-275]. Six regions of the RANTES promoter, R(A-G), have been identified as important for RANTES expression, but these regions are not all functional in every cell type (Figure 1-9). For instance, in T cells the binding sites for R(C)Flat, (RANTES factor of late activated T lymphocytes) (RFLAT)-1, c/EBP and NFκB are important for RANTES transcription whereas Ets, CREB, AP-1, c/EBP, SP-1 and NFκB binding sites are important in monocytes [272]. In addition, three ISRE sites have been located between R(D) and R(E). The ISRE sites are important for binding of IRF-3, which is essential for virus- or IFN-stimulated RANTES expression [276].
3. MIP-1α and MIP-1β

The macrophage inflammatory protein-1 (MIP-1) family consists of proteins that are secreted by activated T cells, B cells, monocytes, mast cells, neutrophils, Langerhans cells, astrocytes, endothelial cells, fibroblasts and smooth muscle cells in response to many stimuli including LPS, viral infection, TNFα and IL-1α/β [277-281]. MIP-1 proteins are small (8–10 kDa) chemokines of the CC subfamily and are involved in recruiting proinflammatory cells to the site of injury or infection [282]. MIP-1 proteins are important chemoattractants of T cells [283], B cells [284], monocytes [285], NK cells [286], basophils [287] and eosinophils [288]. Two members of the MIP-1 family, MIP-1α (CCL3) and MIP-1β (CCL4), have been characterized and bind to the G-protein receptors, CCR1 and CCR5 [282].

The promoter for MIP-1 has not been well characterized but there is evidence that NFκB, AP-1 and c/EBP play a role in transcription (Figure 1-9) [289-291].

4. IP10

Interferon-gamma inducible protein 10 (IP10/CXCL10) is part of the CXC subfamily of chemokines and was first identified as an IFNγ inducible gene [292]. It is expressed by a variety of cells in response to IFNγ, viral infection and LPS including endothelial cells [293], macrophages [294] and astrocytes [295]. The receptor for IP10 is CXCR3, which also binds the chemokine Mig (monokine induced by IFN-γ) [292]. IP10 chemoattracts monocytes, T lymphocytes, and natural killer (NK) cells [286, 296]. In addition, IP10 can regulate maturation of T-cell and bone marrow progenitors, modulate adhesion molecule expression, and inhibit angiogenesis [297].
The IP10 promoter contains binding sites for ISRE binding proteins, GAS, NFκB, and AP-1 (Figure I-9). The NFκB and ISRE sites have been shown to be most important for full IP10 expression [298].
Figure 1-9. Schematic of chemokine promoters. Transcription factor binding sites for IL-8 [251], RANTES [272], IP10 [298] and MIP-1α [290] promoters. Note: not to scale.
K. TRANSCRIPTION FACTORS INVOLVED IN CHEMOKINE GENE EXPRESSION

1. AP-1

The transcription factor AP-1 contains variable combinations of basic-region leucine zipper (bZIP) domain proteins from Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) families, resulting in Jun homodimers and Jun-Fos heterodimers [299-302]. Jun–Jun and Jun–Fos dimers bind with highest affinity to the 12-O-tetradecanoylphorbol-13-acetate (TPA) response element (TRE) [TGA(C/G)TCA] [303].

Phosphorylation of c-Jun by c-Jun N-terminal kinase (JNK), a member of the MAPK, increases the transcriptional activity of AP-1. In response to various stimuli, the JNKs are activated by phosphorylation of specific serine and threonines by MKK 4 or 7, which are in turn activated by phosphorylation by MKKK (Figure 1-10) [304]. ERK1 and ERK2 can also contribute to the induction of AP-1 by phosphorylating proteins that increase c-Fos synthesis [305]. The p38 MAPK pathway has been shown to increase the half-life of cytokine and chemokine mRNA through the AU-rich elements and thereby enhance the effects of AP-1 induction of proinflammatory genes [304].

Jun and Fos proteins can also interact to form heterodimers with other bZIP proteins from subfamilies ATF, MAF, CNC and c/EBP allowing them to target DNA binding sites such as the cAMP response element (CRE), the antioxidant response elements (ARE), and half-sites composed of half of a TRE site and half of a MAF- or CNC-binding site [306]. AP-1 proteins can also interact with other proteins including NF-κB, CBP (CRE-binding-protein-binding protein) (p300), SMAD-3 and -4, and the retinoblastoma protein [306]. Specifically, the basic leucine zipper (bZIP) regions of c-
Fos and c-Jun have been shown to interact with the Rel homology domain in the p65 subunit of NFκB [307]. Unlike the interaction between NFκB and c/EBP, there is enhanced DNA binding of NFκB and Jun or Fos complexes to both the NFκB site and the AP-1 site [307, 308].
Figure 1-10. AP-1 activation pathway. Cytokines activate AP-1 through a cascade involving JNK phosphorylation and activation by MAP kinase kinase 4 or 7, and subsequent c-Jun phosphorylation. The ERK1/2 pathway is also activated and induces the synthesis and phosphorylation of c-Fos. c-Jun then dimerizes with c-Fos and binds to the AP-1 site in inflammatory gene promoters. In addition to JNK and ERK1/2, which regulate the activation of the AP-1 complex, the p38 MAPK pathway has been found to both stabilize proinflammatory gene mRNAs (such as cyclooxygenase (COX)-2, TNF-α, IL-6, and IL-8) and inhibit innate immune response genes (such as TLR2). Figure modified from reference [304] with permission.
2. c/EBP

CCAAT/enhancer binding protein (c/EBP, also known as NF-IL-6) was originally identified as a DNA-binding protein responsible for IL-1-stimulated IL-6 induction and includes c/EBPα, c/EBPβ, c/EBPδ, c/EBPε, c/EBPγ and c/EBPζ [309, 310]. c/EBPβ is expressed in various tissues such as the liver, adipose tissue, intestine, ovary, lung, skin, brain, kidney, heart, hematopoietic tissue, spleen, and mammary gland [310]. Many of the isoforms of c/EBP are expressed in liver and adipose tissues and regulate several hepatocyte- and adipocyte-specific genes [311].

The c/EBPs are a family of bZIP transcription factors that contain a leucine zipper motif involved in dimerization and an adjacent basic region involved in DNA binding in the carboxy terminus [310]. The amino terminus contains a transactivation domain. The c/EBP protein is normally repressed and is activated by phosphorylation of threonine residues by many kinases including the Ras activated MAPK pathway, PKA, and PKC (Figure I-11). Upon activation, c/EBPs can bind consensus palindromic DNA binding sites [a/gTTGCGc/tAAc/t] as homodimers or heterodimers with other c/EBP family members [312].

In addition, c/EBP proteins can heterodimerize with other bZIP family members such as ATF [313, 314], CREB [315], and AP-1 family members Fos and Jun [316]. The members of the NFκB family, p65, p50 and Rel are also able to interact with c/EBPα, c/EBPβ and c/EBδ to stimulate promoters with c/EBP binding sites but at the same time to inhibit promoters with NFκB sites [256]. Studies have also shown a direct interaction between the bZIP region of c/EBP and the Rel homology domain of NFκB [256].
Figure I-11. c/EBP activation pathway. The c/EBP transcription factor is activated by phosphorylation by the p38 MAPK pathway in response to stimuli such as cytokines and LPS. c/EBP binds to the C/CAAT consensus site in various promoters including iNOS, IL-8 and RANTES. Figure modified from reference [317].
3. NFκB

NFκB is an activator of a broad class of immune system genes including cytokines, immunoreceptors, antigen presentation proteins, regulators of apoptosis, and growth factors [318]. NFκB is present in virtually all cell types and was first described as a nuclear factor necessary for immunoglobulin kappa light chain transcription in B cells [319]. The NFκB family is composed of five structurally related DNA binding proteins; NFκB1/p50, NFκB2/p52, RelA/p65, Rel/c-Rel, and RelB [320]. All NFκB subunits contain a highly homologous Rel homology region located in the amino-terminus that mediates protein-DNA interactions. NFκB subunits also contain a dimerization domain located in the carboxy-terminus [321]. Although the most common NFκB complex is a heterodimer consisting of RelA/p65 and p50 subunits, various combinations of heterodimers and homodimers can occur [320]. RelA, Rel and RelB contain a transactivation domain in the carboxy-terminus whereas p50 and p52 lack a transactivating domain [322, 323]. Therefore, heterodimer combinations of NFκB proteins are transcriptionally active, whereas p50 homodimers and p52 homodimers are thought to be repressive [320].

NFκB activation is tightly regulated. Inactive NFκB is sequestered in the cytosol by IκB inhibitory subunits [324, 325]. Following stimulation, phosphorylation of two tandem serine residues on the N-terminal domain of the IκB molecule occurs by activation of two IκB-specific kinases, IKK1/α and IKK2/β (Figure 1-12) [324]. IκB is then ubiquinated and degraded by the proteasome, allowing nuclear translocation of NFκB. NFκB activity is further regulated by post translational modification such as phosphorylation and acetylation [326].
NFκB has been found to interact with other transcription factor families, including AP-1 [307] and c/EBP [308] as described above.
Figure I-12. NFκB activation pathway. Cytokine stimulation of cells activates IKK, which phosphorylates IκB. Phosphorylated IκB becomes ubiquitinated and is then degraded by the proteasome. NFκB, as depicted by p65/p50 heterodimer, translocates to the nucleus and activates transcription of inflammatory genes. Figure modified from reference [304] with permission.
L. PHARMACOLOGICAL INHIBITORS OF CYTOKINE AND CHEMOKINE PRODUCTION

Due to the important role of cytokines in inflammatory diseases, substantial efforts have been made to identify pharmacologic inhibitors of cytokine production. Pharmacological agents are commonly used to help define signaling pathways induced by various stimuli. Pharmacological inhibition of inflammatory gene expression is accomplished through various mechanisms including transcriptional control, particularly of NFκB and AP-1, as well as posttranscriptional and translational control [327-329]. These small molecule inhibitors can access signaling pathways within a cell and can have wide ranging anti-inflammatory therapeutic potential through inhibition of cytokines/chemokines [330].

1. SB203580

SB203580 is a pyridinyl imidazole compound that has been shown to inhibit p38 MAPK activation through competitive binding in the ATP pocket of p38 MAPK [331]. The p38 MAPK pathway consists of MAPK kinases (M KK)-3 and 6, p38 and downstream MAPK-activated protein kinases (MAPKAPK)-2, -3, and -5, MAPK-interacting kinases (Mnk)-1 and -2, and mitogen- and stress-activated protein kinases (Ms k)-1 and -2 [332]. It has been shown that p38 MAPK can stabilize mRNAs, including COX-2 [333], IL-1β [334], Gro-α [334], IL-6 [335], TNF-α [336], MIP-1α [336], and granulocyte/macrophage colony stimulating factor (GMCSF) [337]. It is been suggested that p38 MAPK stabilization occurs through AU-rich elements in the mRNA [338]. AU-rich elements destabilize mRNA by targeting it for deadenylation [258].
Although the precise mechanism is unknown, activation of p38 MAPK can inhibit this process and stabilize the mRNA.

There is evidence that MAPKs such as p38 can contribute to NFκB activation after binding to DNA elements, by facilitation of p65 subunit phosphorylation or interaction with the co-activator proteins CBP/p300 [339, 340]. Another study found that inhibition of p38 MAPK by SB203580 or another p38 MAPK inhibitor, SB202190, blocked IL-8 transcription and cytokine release in human bronchial epithelial (BEAS-2B) cells infected with *Streptococcus pneumoniae* [341]. The level of IL-8 promoter-bound p65-containing NFκB dimers was not inhibited with p38 MAPK inhibitors. However, phosphorylation on serine 536 of p65 that was bound to the IL-8 promoter was significantly reduced in these cells. In addition, RNA polymerase II recruitment, which is a measure of overall promoter activity, was also blocked. Recently, it was also shown that SB203580 could significantly suppress the release of IL-6, MIP-1α, IL-8 and MCP-1 in eosinophils stimulated with the Th2 cytokine IL-25 [342]. These studies suggest that SB203580 can inhibit cytokine/chemokine expression through inhibition of NFκB activation.

Inhibition of p38 MAPK can also have detrimental effects in inflammatory diseases. Tristetraproline (TTP), which is involved in regulating stability of TNFα and GM-CSF mRNAs, requires p38 MAPK for expression [343-345]. TTP deficient mice show an inflammatory phenotype due to high levels of TNFα protein and mRNA [346]. In addition, IL-10, which has the ability to down-regulate inflammatory responses [347], is suppressed by inhibition of p38 MAPK using SB203580 or SB202190 in LPS-stimulated monocytes [348, 349].
2. Rolipram

Rolipram (\((\pm)-4-(3'\text{-cyclopentyloxy}-4'\text{-methoxyphenyl})-2\text{-pyrrolidone})\) is a selective inhibitor of phosphodiesterase (PDE) IV. PDE IV is the predominant isoenzyme expressed in myeloid and lymphoid cells [350]. Inhibition of PDE IV leads to an increase in the intracellular concentration of cAMP [351]. cAMP is the product of synthesis by adenylyl cyclases and degradation by other phosphodiesterases (PDEs) [352]. cAMP binds to and activates protein kinase A (PKA), which allows phosphorylation of transcription factors that bind to the cAMP response elements (CRE) or CRE binding proteins (CREBs) [353].

Rolipram has been used in clinical trials safely as an antidepressant [354, 355]. Rolipram has also been shown to suppress activation of inflammatory cells [356]. Production of TNF\(\alpha\) was suppressed in LPS stimulated monocytes with treatment of rolipram [357]. It has also been shown that rolipram can significantly decrease IL-8 and TNF\(\alpha\) production and migration of eosinophils and neutrophils into bronchoalveolar lavage fluid in experimental antigen-induced asthma in monkeys [358]. Another study analyzing the effect of rolipram on HIV infected primary T cells showed that rolipram reduced the production of TNF\(\alpha\) and IL-10 through day 9 post infection [359]. Jimenez et al reported that rolipram reduced the production of TNF\(\alpha\), IL-5, IL-10, and IL-2 by activated human T cells [350]. Moreover, rolipram and pentoxifylline (a nonspecific phosphodiesterase inhibitor) inhibited the activation of NF\(\kappa\)B and NFAT and stimulated the activation of AP-1 and CREBs [350, 359].

3. Dexamethasone
Dexamethasone is a glucocorticoid that has been shown to play a role in suppressing inflammation. Glucocorticoids bind to a glucocorticoid receptor (GR) that is generally localized in the cytoplasm of many cell types [360]. Upon binding, the GR moves into the nucleus and binds as a homodimer to DNA at glucocorticoid response elements (GREs). Binding of GREs can lead to induction of expression of glucocorticoid-responsive genes such as the anti-inflammatory proteins IL-1 receptor antagonist, serum leukoproteinase inhibitor, clara cell protein 10 (CC10) and lipocortin-1 [361]. An increase in IκBα expression has also been shown to occur in lymphocytes in response to glucocortocoid treatment, thereby inhibiting NFκB [362].

Increased gene transcription occurs due to local unwinding of the DNA [363]. IL-1β induces chromatin remodeling, allowing p65 to bind to DNA, causing histone acetylation of CBP and the formation of a CBP-associated HAT complex [364]. In contrast, dexamethasone has been shown to inhibit IL-1β-induced gene expression [360, 365]. The mechanism of GR inhibition of gene transcription occurs by disrupting the CBP-associated HAT activity and recruiting HDAC to the p65/CBP complex, which further inhibits HAT activity and increases nucleosome compaction. This leads to repression of transcription.

Glucocorticoids are most noted for their ability to inhibit expression of inflammatory genes such as IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-13, TNF-α, GM-CSF and the chemokines IL-8, RANTES, Eotaxin, MIP1α, MCP-1 and MCP-3 (reviewed in [366]). The inhibitory effect of glucocorticoids is mediated by the interaction between nuclear GR and transcription factors involved in expression of inflammatory genes including AP-1, NFκB and c/EBPβ (reviewed in [367]).
Dexamethasone treatment in the presence of cotransfected GR inhibited activation of NFκB and AP-1 reporters [368]. The zinc finger domain of GR was found to be necessary for the inhibition of p65 subunit of NFκB and AP-1. However, dexamethasone treatment of cells transfected with fos, jun and NFκB expression plasmids did not inhibit transcription from the NFκB reporter construct. They conclude that synergistic NFκB/AP-1 activity was unaffected by dexamethasone.

Overall, studies indicate that dexamethasone treatment can inhibit NFκB activation through many mechanisms including synthesis of IkBα, nucleosome compaction or interaction of the p65 subunit of NFκB with the GR.

4. Indomethacin

Membrane phospholipids are converted into arachidonic acid by phospholipase A₂. Arachidonic acid is then converted into prostaglandin (PG) H₂ by COX, and then to prostanoids by specific synthases [369]. There are three COX isoforms; COX-1 constitutively produces housekeeping prostanoids, COX-2 is induced by inflammatory cytokines and COX-3 is a splice variant of COX-1 [369, 370].

Indomethacin is a nonselective COX inhibitor that has been shown to inhibit IL-8 secretion in airway smooth muscle cells stimulated with bradykinin [371], increase IL-6 in monocytes [372] and increase TNFα production in LPS stimulated monocytes and macrophages [373, 374]. Previous research analyzing the effects of multiple NSAIDs indicates that the anti-inflammatory effects of indomethacin do not occur through the inhibition of NFκB [375-377], AP-1[378, 379] or p42/p44 MAPK [380].

5. PDTC
PDTC is a derivative of DDTC (diethyldithiocarbamate, DDTC). DDTC has been used for the treatment of metal poisoning in humans [381]. In addition, DDTC has been used in patients enrolled in clinical human immunodeficiency virus (HIV) studies. Treatment of HIV-infected individuals with DDTC was associated with a delay in the onset of acquired immune deficiency syndrome (AIDS) [381-383]. This effect is thought to be due to inhibition of NFκB activation [382, 384, 385]. PDTC has also been shown to inhibit NFκB and has advantages over DDTC in that it can traverse the cell membrane and has prolonged stability at physiological pH [385, 386].

PDTC is an antioxidant that inhibits reactive oxidative species (ROS) [386, 387], inducible nitric oxide synthase (iNOS) activity and nitric oxide production [388]. Studies have shown that PDTC can inhibit induction of chemokines such as IL-8 from cells including human dermal microvascular endothelial cells in vitro [389], epithelial cells [390] eosinophils [391], human PBMC [392] and a lung cancer cell line [393] in parallel to its inhibition of NFκB.

**M. DENV INFECTION AND CHEMOKINES**

The pathogenesis of dengue disease is not completely understood but thought to be due to dysregulation of the immune response. Chemokines have been proposed to contribute to inflammation and disease pathogenesis by attracting memory T cells to the sites of viral replication during a secondary DENV infection [68]. DENV-specific memory T cells have a rapid response to DENV antigen and can increase the inflammatory response and plasma leakage during DENV infection.

In this regard, studies of chemokine induction by DENV have been done in various cells. Enhanced levels of chemokines such as MIP-1α, MIP-1β, IL-8 and
RANTES has been reported in PBMC, blood, serum and pleural fluids from DENV infected patients [394-397]. Spain-Santana et al detected an increase in the expression of MIP-1\(\alpha\) and MIP-1\(\beta\) mRNA in PBMC of dengue patients during acute illness [396]. Lin et al reported increased RANTES levels in plasma samples of DENV-infected patients when compared to patients with other febrile illnesses [397]. Raghupathy et al reported that levels of IL-8 protein in blood or pleural fluid were elevated in patients with DHF. In addition, they found higher levels of IL-8 mRNA by quantitative real time polymerase chain reaction (RT-PCR) in patients with severe disease when compared to DF patients [394]. Juffries et al assessed the levels of IL-8 in plasma samples from DENV infected children in relation to levels of neutrophil degranulation molecules, elastase and lactoferrin [395]. They found that levels of IL-8 and elastase were higher in patients with DSS than patients without DSS. Furthermore, Huang et al reported that IL-6 and IL-8 protein levels were higher in sera from patients with DHF than in DF patients or patients with other febrile illnesses [21]. These studies suggest that there is a correlation between chemokine production and disease severity in patients infected with DENV.

Due to the lack of an animal model for DHF, most of the work investigating primary target cells and potential mechanisms of pathogenesis has been done in vitro. Many cell types have been analyzed for chemokine secretion following DENV infection. In vitro infection of the human myeloid cell line, K562, has been reported to induce secretion of MIP-1\(\alpha\) and MIP-1\(\beta\) [396]. Bosch et al found that primary human monocytes secreted IL-8 upon infection with DENV [4]. Chen et al analyzed the kinetics of cytokine and chemokine levels in supernatants from DENV infected monocytes/macrophages [398]. They found secretion of MIP-1\(\alpha\), RANTES and IL-8
from DENV infected monocytes/macrophages within hours of DENV infection, with levels peaking on day 2, day 4 and day 7 post infection, respectively, whereas viral yields peaked at 42 hours post infection. In addition, Chen et al found that IL-1β, TNFα, IFNα and IL-12 production by DENV infected monocyte/macrophages peaked at day 2, day 3, day 4 and day 7 post infection, respectively. They proposed that chemokines were initially induced to attract cells to the sites of infection and that prolonged chemokine expression in combination with cytokines functionally matured the infiltrating cells.

Endothelial cells are important for maintaining vascular integrity and have been investigated for their role in plasma leakage during DENV infection. Talavera et al found an increase of permeability of HMEC-1 (a human microvascular endothelial cell line) monolayer infected with DENV for 48 hours, which correlated with actin cytoskeleton rearrangements [5]. Furthermore, addition of IL-8 showed effects similar to DENV infection on permeability and actin reorganization.

Endothelial cells have also been reported to support DENV infection and secrete chemokines. Avirutnan et al reported that primary endothelial cells derived from human umbilical cord veins (HUVEC) could be infected with DENV and induced RANTES expression [6]. Huang et al reported that DENV-infected HUVECs induced IL-6 and IL-8 but not IL-1β [21]. In addition, inhibition of DENV replication with ribavirin inhibited IL-6 and IL-8 secretion from DENV-infected HUVECs. It has also been reported that a DENV-infected endothelial cell line, ECV304, has an increase in IL-8 and RANTES protein and mRNA [4, 6]. Bosch et al reported that DENV-infected ECV304 cells induced IL-8 primarily through NFκB rather than NF-IL-6 (c/EBP) [4]. These studies indicate that DENV can directly infect endothelial cells. Furthermore, infection of
endothelial cells results in secretion of mediators that may be directly involved in altering the vascular wall. In addition, the production of chemoattractants by endothelial cells can attract cells to the site of infection, which may themselves produce mediators involved in vascular leakage.

Mast cells have also been analyzed for their role in the pathogenesis of DENV infection. Tuchinda et al reported an increase in histamine levels in urine from patients with DHF, suggesting activation of mast cells [399]. In addition, the mast cell line, KU812, expresses Fc receptors and was shown to be infected with DENV in the presence of nonneutralizing antibody and produce IL-6 and IL-1β [400]. In another study, King et al found that KU812 cells produce RANTES, MIP-1α and MIP-1β but not IL-8 upon antibody-enhanced DENV infection [401]. Furthermore, stimulation of KU812 cells with the inflammatory cytokines IL-1β and IL-6 did not significantly induce RANTES expression, indicating that RANTES expression is not due to induction of other cytokines. In the same study, mast cells cultured from cord blood expressed RANTES and MIP-1β during antibody-enhanced DENV infection. These results suggest that mast cells may also be involved in the pathogenesis of DENV infection.

As previously mentioned, liver involvement has been seen in patients with DHF. Lin et al have shown that liver cell lines can be productively infected with DENV and induce cytopathic effects [143]. In another study they reported that DENV induced RANTES protein and mRNA in liver cells by 36 hours post infection [397]. Analysis of the RANTES promoter by luciferase reporter assays and electrophoretic shift mobility assays identified protein binding to the c/EBP and NFκB sites within the RANTES promoter to be important for RANTES induction in liver cells [397].
In summary, many cell types have been analyzed for their ability to support DENV replication and induction of cytokines/chemokines. However, a comprehensive study of chemokine induction by DENV infection of multiple cell types and primary monocyte-derived DCs has not been done. In addition, the mechanism(s) of chemokine induction by DENV has not been fully defined.
Previous reports had shown that DENV infection could induce chemokines in vivo and in vitro. It had been suggested that chemokine production could contribute to DENV disease, implying that inhibition of chemokine production could be of therapeutic value. However, little was known about the mechanism of chemokine induction during DENV infection. Other viral systems such as HCV and HIV had shown that individual viral proteins could induce chemokine production. We speculated that DENV could also induce chemokine production through the action of specific viral proteins. The objective of this thesis was to analyze the mechanism by which DENV induces chemokine production. In particular, we hypothesized that an individual DENV protein(s) can induce chemokine expression.

The specific aims of this thesis are as follows:

1. Analyze chemokine induction by DENV in different human cell types.
   a. Characterize chemokine induction in both continuous cell lines and primary monocyte-derived dendritic cells.
   b. Identify transcription factors involved in DENV induced chemokine induction.

2. Analyze the effects of pharmacological inhibitors of cytokine pathways on chemokine induction during DENV infection.

3. Define the involvement of individual dengue viral protein(s) in chemokine induction.
   a. Identify DENV protein(s) involved in the induction of chemokines.
   b. Characterize transcription factors involved in the induction of chemokines by
viral proteins.
CHAPTER II:
MATERIALS AND METHODS

A. CELLS AND CELL LINES

HepG2 (ATCC, Manassas, VA), a human hepatocarcinoma cell line [402], and Vero cells (an African green monkey kidney cell line; ATCC, Manassas, VA) were maintained in Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, penicillin (100U/ml), and streptomycin (100μg/ml). HEK293 (ATCC, Manassas, VA) and HEK293A (Invitrogen, Carlsbad, CA), human embryonic kidney cell lines [403], were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS, 0.1mM nonessential amino acids, penicillin (100U/ml), and streptomycin (100μg/ml). ECV304 cells (transformed human large-vessel umbilical cord endothelial cells) were grown in M199 medium (Life Sciences) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). K562 (ATCC, Manassas, VA), a chronic myelogenous leukemia cell line, U937, a myelomonocytic cell line, and Thp-1, an acute monocytic leukemia cell line, were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml).

B. DENDRITIC CELLS

PBMC were obtained by centrifugation using Accuspin tubes (Sigma-Aldrich, St. Louis, MO). CD14+ monocytes were isolated by positive selection using magnetic cell sorting (MACS), according to the manufacturer’s protocol (Milteyl Biotec, Auburn, CA). The CD14 positive cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, rGM-CSF (800u/ml) and rIL-4 (500u/ml) for 5-7 days, changing half the
media and adding cytokines every other day. DCs were stained to assess purity with lineage markers CD3, CD14, CD16, CD56, and CD20 (FITC), CD1a (PE) and HLA-DR (PerCP) and analyzed by FACS. DCs were > 85% pure.

C. ANTIBODIES AND CYTOKINES

Fluorescein isothiocyanate (FITC)-conjugated DENV complex specific monoclonal antibody (clone M8051125) was obtained from Fitzgerald Industries International, Inc. DENV specific monoclonal antibody was derived from a hybridoma cell line, 3H5-1 (ATCC, Manassas, VA). LPS (1 μg/ml), PMA (100 ng/ml), ionomycin (1 μg/ml), and polyI:C (100 μg/ml) were obtained from Sigma-Aldrich, St. Louis, MO. IFNα2b (1000 U/ml) was obtained from R&D systems, Minneapolis, MN.

D. DENV PROPAGATION

DENV strain New Guinea C was used for infection. DENV New Guinea C was received from Dr. Walter E. Brandt of the Walter Reed Army Institute of Research, Washington, DC. The virus was propagated in Aedes albopictus cells (C6/36) as previously described [404]. The titers of the virus pools were determined by fluorescent focus assay in CV1 cells [405].

E. INFECTION WITH DENV

Cells were infected with DENV strain New Guinea C (NGC) as previously described [406] at a multiplicity of infection (MOI) of 1, 5 or 10 for 2 hours at 37°C. For assays assessing chemokine protein levels by ELISA, supernatants were harvested for each time point, and fresh media was added to the cells.

Cells were fixed with 1% paraformaldehyde and stained for DENV antigen by indirect immunofluorescence (IF) on various days post infection, using 3H5 as the
primary antibody and FITC-conjugated goat anti-mouse immunoglobulin G antibody (Sigma-Aldrich, St. Louis, MO) as the secondary antibody. For experiments using direct immunofluorescence, the DENV complex-FITC conjugate antibody was used. The percentage of infected cells was assessed using flow cytometry.

F. VIRUS INACTIVATION

DEN2V was inactivated by ultraviolet (UV) light. The UV light source was a Phillips TUV 30W G30T8 UV-C light bulb. The virus was exposed at a distance of 15 cm for 30 minutes at room temperature. Alternatively, DEN2V was heat inactivated at 56°C for 30 minutes.

G. RNA ISOLATION

Total RNA was prepared using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. For RT-PCR, RNA was treated with DNase according to the manufacturer’s protocol (Qiagen, Valencia, CA).

H. RIBONUCLEASE PROTECTION ASSAY

The human chemokine multiprobe template set (hCK-5) was used to make the radiolabeled probe according to the manufacturer’s protocol (Riboquant, Pharmingen, San Diego, CA). The RNase protection assay was performed according to the manufacturer’s protocol (Torrey Pines Biolabs, Inc., La Jolla, CA). Briefly, equal amounts of total cellular RNA were hybridized to the 32P-labeled hCK-5 riboprobe cocktail. The hybridized RNA was then digested with RNase A. The RNA was precipitated and then electrophoresed on a denaturing acrylamide gel and the bands were detected by autoradiography. Specific chemokine bands were identified on the basis of their individual migration patterns in comparison with the undigested probes. The bands
were quantified by densitometric analysis using a Storm 820 phosphoimager and Image Quant densitometric software (Amersham, Piscataway, NJ). GADPH and L32 were used as internal controls.

I. ELISAS

MIP-1α, MIP-1β, MCP-1, IL-8, and RANTES protein concentrations were determined in cell culture supernatants using commercially available ELISA kits according to the manufacturer's instructions (R&D systems, Minneapolis, MN).

J. QUANTITATIVE RT-PCR

Two hundred nanograms of total cellular RNA was used to synthesize cDNA using Omniscript RT and oligo dT primers following the manufacturer's protocol (Qiagen, Valencia, CA). PCR was performed in triplicate using TaqMan IL-8 and β-actin primers and probes (Applied Biosystems, Foster City, CA) and the Gene Amp 5700 Sequence Detection System. For quantitation of mRNA, a standard curve of control DNA was generated, and samples were normalized to the endogenous β-actin control. The ratios of gene expression in infected or transfected cells versus uninfected or untransfected cells, respectively, were calculated.

For quantitation of DEN2V RNA from supernatants of virus-infected cells, cDNA was synthesized from isolated total RNA using Multiscribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) and the reverse DEN2V primer (5'CATTCCATTCTTCTGGCGTCT3') following the manufacturer’s instructions. PCR was performed using the Taqman Universal PCR master mix and DEN2V forward (5'AAGGTGAGATGAAGCTGTAGTCTC3') and reverse primers and a DEN2V specific probe (6FAMCTGTCTCCTCAGCATCATTCCAGGCATAMRA) and the
GeneAmp 7300 sequence detection system (Applied Biosystems, Foster City, CA).

Quantitation of viral RNA in genome copies per milliliter was performed using a standard curve of DEN2V RNA.

K. PBMC SAMPLES FROM PATIENTS WITH ACUTE DENV VIRUS INFECTIONS

Serial PBMC samples were studied from 26 children who participated in a prospective study of acute DENV infection (16 with DF and 10 with DHF) in Thailand [407]. Patients were enrolled in the study if they presented within 72 hours of the onset of fever. Blood samples were collected daily until 1 day after defervescence. Convalescent samples were taken 8 to 10 days after enrollment. “Fever days” were assigned retrospectively as described, where fever day 0 refers to the day of defervescence. As described previously [396], PBMC (approximately 2×10^6 cells) isolated from whole blood using Histopaque (Sigma-Aldrich, St. Louis, MO) were resuspended in 1 ml of buffer (4M guanidine isothiocyanate, 40 mM Tris-HCl pH 6.4, 17 mM EDTA pH 8.0, 1% Triton X-100), frozen at -80°C and shipped on dry ice. Total RNA was prepared using the RNeasy kit (Qiagen, Valencia, CA) substituting the lysis buffer for Buffer RLT.

L. PHARMACOLOGICAL INHIBITORS

4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB203580) [408-410], rolipram [359, 411], ammonium pyrrolidinedithiocarbamate (PDTC) [412], indomethacin [413], and 9α-fluoro-16α-methyl-11β, 17α, 21-trihydroxy-1,4-pregnadiene-3,20-dione (dexamethasone) [414] were purchased from Sigma-Aldrich Co., St. Louis, MO. Cells were infected with DEN2V at an MOI of 5 for 2 hours at 37°C as above or in the presence of 10 μM SB203580, 100 μM rolipram, 250 nM
dexamethasone, 250 nM PDTC, or 30 μM indomethacin. These drug concentrations were selected based on their ability to inhibit cytokine production in previous studies cited above. The media was changed at time points specified with fresh media containing the same drugs. The cell monolayer in drug-treated wells was assessed by microscopy and compared with untreated DEN2V infected cells.

M. PLASMIDS

A plasmid expression the DEN2V replicon was kindly provided by Dr. Andrew Dayton [415]. This replicon contains the genes for expression of C and the nonstructural proteins and is expressed under the control of the CMV promoter. Four luciferase reporter constructs containing the IL-8 promoter were generously provided by Dr. Naofumi Mukaida (Kanazawa University, Japan): one with wild-type (WT) binding sites for NFκB, AP-1 and c/EBP, and the other three with mutant binding sites for NFκB (GGAATTTCCCT to TAACCTTCCT from -80 to -71), AP-1 (TGACTCA to TATCTCA from -126 to -120) or c/EBP (CAGTTGCAAATCGT to AGCTTGCAAATCGT from -94 to -81) [416]. Luciferase reporter constructs containing tandem repeat binding sites for NFκB, AP-1 and c/EBP were obtained from Stratagene (La Jolla, CA). Plasmids expressing signaling molecules Mal, MEKK1, or TBK1 and the luciferase reporter construct containing the ISRE of the IFIT2 gene were generously provided by Dr. Kate Fitzgerald (UMass Medical School, MA) [181, 417]. The pcDNA3.1 plasmid was obtained from Invitrogen, Carlsbad, CA.

Primers were designed for PCR amplification and cloning of each of the DEN2V genes (Table II-1). Each 5’ and 3’ primer contained attB1 and attB2 sequences, respectively, for homologous recombination into the Gateway entry vector pDONR201
(Invitrogen, Carlsbad, CA). The prM, E and NS1 forward primers included the putative leader sequences [88, 95, 98, 418].

PCR was performed using rTth DNA polymerase (Applied Biosystems, Foster City, CA) and DEN2V infectious clone as the template (kindly provided by Dr. Barry Falgout, FDA [419]). The PCR products were cloned into pDONR201 by homologous recombination using Gateway Technology following the manufacturer's instructions. Mammalian expression vectors were made by homologous recombination of each DEN2V gene into the pDEST40 vector, which contained a carboxy-terminal V5 epitope and 6XHis tag, or pDEST47, which contained a carboxy-terminal GFP tag, as described in the Gateway Technology manual (Invitrogen, Carlsbad, CA). The identities of the clones were confirmed by DNA sequencing.

**N. TRANSFECTIONS**

HEK293A cells were transfected using Effectene (Qiagen, Valencia, CA) following the manufacturer's instructions. Briefly, HEK293A cells were seeded onto 6 well plates at 2X10^5 cells per well 24 hours before transfection. To transfect, 0.6 μg of DENV protein expressing plasmid and 0.2 μg of lacZ expressing plasmid were diluted in EC buffer (Qiagen, Valencia, CA) for each condition. In addition, 6.4 μl of Enhancer (Qiagen, Valencia, CA) was added to the mixture and incubated for 2 minutes at room temperature. The mixture was spun down; 8 μl of Effectene (Qiagen, Valencia, CA) was added and incubated for 5 minutes at room temperature. Growth media was added to the mixture and the mixture was added to the cells. Transfection efficiency was approximately 30% as assessed by β-galactose staining following the manufacturer's protocol (Invitrogen, Carlsbad, CA).
O. WESTERN BLOT

Laemmli sample buffer containing 350mM DTT was added to DEN2V infected Vero cells collected 48 at hours post infection and prM-E transfected HEK293 cells and supernatants collected at 24 hours later. Samples were heated at 90°C for 10 minutes and run on a Criterion 10% Tris-HCL SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA) along with the MagicMark standard (Invitrogen, Carlsbad, CA) to detect band size. Samples on the gel were transferred to a nitrocellulose membrane following manufacturer’s recommendations (Bio-Rad Laboratories, Hercules, CA). The E protein was detected using the WesternBreeze chemiluminescent western blot Immunodetection kit (Invitrogen, Carlsbad, CA). The 3H5 antibody, which detects DENV E protein, was used as the primary antibody. X-ray film was exposed to the membrane to detect E specific bands.

P. LUCIFERASE REPORTER GENE ASSAY

HEK293A cells were plated in a 96 well plate at 2X10^4 cells per milliliter. After 24 hours, the cells were transfected with reporter plasmids and/or expression plasmids using Genejuice according to the manufacturer’s protocol (Novagen, EMD Biosciences, Inc, San Diego, CA). Twenty four hours (for plasmids) and 72 or 96 hours (for virus) later, cells were lysed, and the luciferase activity was determined. A Renilla reniformis luciferase reporter under the control of the herpes simplex virus thymidine kinase promoter, pRL-TK, was used as an internal control to normalize reporter gene activity. Luciferase activities were determined by a luminometer using the dual-luciferase reporter assay according to the instructions of the manufacturer (Promega Co., Madison, WI). All
conditions were tested in triplicate. At least two independent experiments were performed for each assay.

Q. CONFOCAL MICROSCOPY

The GFP tagged NS5 or NS3 expression plasmids were transfected into 293A cells using Genejuice following the manufacturer’s protocol (Novagen, EMD Biosciences, Inc, San Diego, CA). Cells were washed with 1X Phosphate Buffered Saline (PBS) and fixed in 100% cold ethanol for 5 minutes. The cells were air dried and mounted using VectaShield from Vecta Laboratories which contains the DAPI (4',6-diamidino-2-phenylindole) nuclear stain. Cells were analyzed using the Leica TCS SP2 AOBS confocal microscope and Leica Confocal Software (LCS; Leica Microsystems, Bannockburn, IL.)
Table II-1. Primers used for cloning of DEN2V genes.

<table>
<thead>
<tr>
<th>Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence (5' → 3')&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (forward)</td>
<td>GGGGACAAGTTTGTACAAGAAAAAGCAGGCTTCACCATTGAATAAACAAACGAAAAGGGCGAG</td>
</tr>
<tr>
<td>C (reverse)</td>
<td>GGGGACCACCTTTGTACAAAGAAAAAGCTGGGCTCCGCCATCACTGTGGGAATCGC</td>
</tr>
<tr>
<td>prM (forward)</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>prM (reverse)</td>
<td>GGGGACCACCTTTGTACAAAGAAAAAGCTGGGCTCCATGTCAATTGGAAGACGACAGCTG</td>
</tr>
<tr>
<td>E (forward)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAGCGCTTATTTACATATGACAG</td>
</tr>
<tr>
<td>E (reverse)</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS1 (forward)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS1 (reverse)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS2A (forward)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS2A (reverse)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS2B (forward)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS2B (reverse)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS3 (forward)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS3 (reverse)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS4A (forward)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS4A (reverse)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS4B (forward)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS4B (reverse)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS5 (forward)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
<tr>
<td>NS5 (reverse)</td>
<td>GGGGACAAGTTTGTACAAAAGCAAGCTTCACCATTGAAGAACACGCGATATCAATATGCTGATTC</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primer names indicate the gene amplified for cloning.

<sup>b</sup> Bold sequence is the DEN2V sequence and the underlined sequences are added stop codons.
CHAPTER III:

ANALYSIS OF CHEMOKINE INDUCTION BY DENGUE VIRUS

A. CHEMOKINE PRODUCTION DURING DEN2V INFECTION OF CELL LINES.

Previous studies had shown that endothelial cells, liver cells, mast cells, monocytes and dendritic cells can be productively infected with DENV. However, these studies did not analyze chemokine production from multiple target cell lines or primary monocyte-derived DCs. We hypothesized that distinct profiles of chemokine secretion can be induced by DEN2V infection of various cell types in vitro. In addition, we wanted to find a cell line(s) that would facilitate in vitro studies of DENV induced chemokine induction.

We analyzed chemokine induction by DEN2V infection in various cell lines, including myelomonocytic cell lines U937, K562 and Thp-1, the hepatoma cell line HepG2, the endothelial cell line ECV304, and the embryonic kidney cell line HEK293. Of the cell lines tested, HepG2, HEK293 and K562 cells were most susceptible to DEN2V infection as assessed by flow cytometry. HepG2 cells were infected 25% by day 1 and 50% by day 6 with DEN2V with an MOI of 1 (data not shown). Thirteen percent of ECV304 cells were infected by day 1 and 6% were infected by day 6 with an MOI of 1. HEK293 cells were infected 37% by day 1 with DEN2V with an MOI of 1. By day 4 post infection, HEK293 cells were infected only 3% with extensive destruction of the monolayer. K562 cells were infected 60% by day 1 and 25% by day 6 with an MOI of 10. Infection of Thp-1 and U937 cells with DEN2V at an MOI of 10 was undetectable by
flow cytometry with or without enhancing antibody. In comparison, DEN2V RNA levels in culture supernatants of HepG2 and HEK293 cells reached maximum values (~10^7 genome copies/ml) by 48 hours post infection (Figure III-1).

The secretion of chemokines in HepG2, HEK293 and ECV304 cell lines peaked late in infection, between days 3 and 5. DEN2V infected HepG2 cells secreted IL-8, RANTES, MIP-1α and MIP-1β (Table III-1). Only IL-8 and RANTES were secreted in DEN2V-infected HEK293 cells. Although the percentage of infected ECV304 cells was low, RANTES expression increased during DEN2V infection by day 2 when compared to mock infected cells. ECV304 cells have previously been reported to induce IL-8 upon DEN2V infection [4]. Although K562 cells were readily infected with DEN2V, none of the chemokines tested were detected. These results indicate a unique chemokine induction profile for each cell type during DEN2V infection. In addition, these data suggested that HepG2 and HEK293 cells were good candidate cell lines for *in vitro* studies of DEN2V induced chemokines because they were productively infected and produced multiple chemokines.

To assess whether protein expression of each chemokine was regulated at the transcriptional level or during translation, chemokine mRNA levels in HepG2 cells were analyzed by ribonuclease protection assay (RPA) (Figure III-2). DENV infection induced the expression of IL-8, RANTES, MIP-1α, MIP-1β and IP10 mRNAs. The levels of mRNA peaked between days 3 and 5, which was similar to the kinetics of protein expression (Figure III-2 and Table III-1). To quantify the induction of IL-8 mRNA by DEN2V infection, real time fluorogenic RT-PCR was performed. As seen in figure III-3, IL-8 mRNA levels peaked at day 4 post infection.
Table III-1. Chemokine production from DEN2V infected HepG2, HEK293 and ECV304 cell lines\(^a\).

<table>
<thead>
<tr>
<th></th>
<th>Day 1(^b)</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D2V Media</td>
<td>D2V Media</td>
<td>D2V Media</td>
<td>D2V Media</td>
<td>D2V Media</td>
</tr>
<tr>
<td>HepG2</td>
<td>IL-8(^c)</td>
<td>11</td>
<td>3</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RANTES(^c)</td>
<td>4</td>
<td>2</td>
<td>73</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MIP-1(\alpha)</td>
<td>48</td>
<td>4</td>
<td>480</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>MIP-1(\beta)</td>
<td>6</td>
<td>8</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>HEK293</td>
<td>IL-8(^c)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RANTES</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>ECV304</td>
<td>RANTES</td>
<td>3</td>
<td>1</td>
<td>13</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) Results are in pg/ml

\(^b\) Cell lines were infected with DEN2V NGC at an MOI of 1.

\(^c\) Representative result of three independent experiments.

\(^d\) ND, not determined
Figure III-1. Production of DEN2V progeny during *in vitro* infection of HepG2 and HEK293 cells. DEN2V RNA levels in supernatants of infected cell lines were assessed by quantitative real time RT-PCR. The data are expressed as mean ± standard deviation from a representative experiment done in triplicate.
Figure III-2. Chemokine mRNA expression in HepG2 cells infected with DEN2V.

HepG2 cells were infected with DEN2V at MOI of 1. Total RNA was isolated for each time point after viral infection. The figure is representative result for a minimum of two separate experiments. Detection and quantification of the indicated human chemokine mRNAs were analyzed by ribonuclease protection assay as outlined in the Materials and
Methods. P denotes probe lane, D denotes day, (+) indicates infection with DEN2V and (-) indicates mock infection.
Figure III-3. IL-8 mRNA induction in HepG2 cells infected with DEN2V. IL-8 mRNA levels in DEN2V infected HepG2 cells were assessed by quantitative fluorogenic RT-PCR. Results were normalized to endogenous β-actin RNA levels.
B. CHEMOKINE INDUCTION FOLLOWING IN VITRO DENV INFECTION OF PRIMARY HUMAN CELLS AND PBMC IN VIVO.

The above studies confirmed the induction of chemokine expression by DENV infection in human cell lines. However, there has been comparably little data on chemokine induction by DENV in non-transformed human cells. Chemokine induction has been shown in primary monocytes following DENV infection [4, 398]. Since DCs have also been proposed as an important target for DENV infection in vivo, we performed a single preliminary experiment to analyze chemokine induction from DEN2V infected dendritic cells. Monocytes were isolated from PBMC and were cultured for 7 days with IL-4 and GM-CSF to generate myeloid DCs [135, 137]. The cells were stained for HLA-DR, lineage markers (CD3, CD20, CD56 and CD14) and CD1a and sorted by flow cytometry. Approximately 85% of the cells were phenotypically dendritic cells (HLA-DR⁺, lineage⁻ and CD1a⁺) (Figure III-4). The DCs were infected with DEN2V at MOIs of 0.1, 1 and 10. The cells were harvested on day 5 post infection to determine the percentage of cells infected by flow cytometry. As seen in figure III-5 ~ 1% of the cells were infected by day 5 post infection for MOI of 0.1 whereas ~18% of the cells were infected for MOI of 10. Culture supernatants were collected days 1, 3 and 5 post infection for analysis of chemokine levels by ELISA. IL-8, RANTES, MIP-1α, and MIP-1β levels were significantly higher in cultures of infected DCs by day 1 post infection (Table III-2). The levels of chemokines decreased but remained higher than background through day 5. MCP-1 levels were higher in cultures of DEN2V infected cells only at day 1 post infection. A substantial amount of cell death occurred by day 5 in DEN2V-infected cultures and may explain the decrease in chemokine levels in the
supernatants. Although the analysis of chemokine secretion from DEN2V-infected dendritic cells was obtained from a single PBMC donor, the results indicate that chemokine induction by DEN2V also occurs in primary cells that are potential targets of DENV infection \textit{in vivo}.

To determine whether IL-8 gene expression also occurs \textit{in vivo} during DENV infections, we analyzed IL-8 mRNA levels in PBMC collected from 26 subjects with acute DENV infections using quantitative RT-PCR. All subjects had DENV viremia detected by RT-PCR at the time of entry into the study [396]. IL-8 mRNA levels were numerically expressed as \( \Delta C \), values relative to \( \beta \)-actin mRNA levels. As shown in figure III-6, IL-8 mRNA was detected in PBMC from all subjects tested. Mean IL-8 mRNA levels were highest later in infection towards the end of the febrile period (fever day -1 to 0), particularly in subjects with DHF. However, there were no statistically significant differences in IL-8 mRNA levels between subjects with DF and those with DHF.
Figure III-4. FACS staining for phenotypic markers expressed on dendritic cells.

CD14+ monocytes isolated from PBMC were cultured for 7 days with rIL-4 and GM-CSF. DCs were infected at an MOI of DCs as described in the MATERIALS AND METHODS. After 7 days, DCs were harvested and stained for HLA-DR, lineage markers and CD1a to assess the percentage of cells that have a DC phenotype.
Figure III-5. DEN2V infection of monocyte-derived dendritic cells. DCs were infected at A. MOI of 0.1 B. MOI of 1 C. MOI of 10 D. C6/36 supernatant treated cells (ctrl). Day 5 post infection, DCs were harvested and stained with DEN2V specific antibody as described in MATERIALS AND METHODS.
Table III-2. Chemokine production from DEN2V-infected DCs.

<table>
<thead>
<tr>
<th>Day</th>
<th>Condition</th>
<th>IL-8*</th>
<th>RANTES*</th>
<th>MIP-1α*</th>
<th>MIP-1β*</th>
<th>MCP-1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>media</td>
<td>400</td>
<td>6.2</td>
<td>70</td>
<td>180</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>C6/36</td>
<td>220</td>
<td>11.2</td>
<td>70</td>
<td>300</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>DEN2V 0.1**</td>
<td>13920</td>
<td>11960</td>
<td>8000</td>
<td>16060</td>
<td>2180</td>
</tr>
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*concentration in pg/ml

** 0.1, 1 and 10 refer to MOIs
Figure III-6. IL-8 gene expression in PBMC of patients with acute DENV infection.

Total RNA was isolated from serial PBMC samples obtained from 16 subjects with dengue fever (DF, ○) and 10 subjects with dengue hemorrhagic fever (DHF, ◯). IL-8 and β-actin mRNA levels were measured by quantitative RT-PCR using TaqMan primers and probes. IL-8 mRNA levels are expressed as the difference in Ct between β-actin and IL-8. The mean levels of IL-8 mRNA in each group are indicated by the (—).
C. IL-8 INDUCTION REQUIRES REPLICATION COMPETENT VIRUS AND IS NOT DUE TO IFNα.

To assess whether the IL-8 expression in DEN2V infected HepG2 cells was due to DEN2V antigen or replicating virus, DEN2V was heat inactivated or UV inactivated. The virus was added to HepG2 cells at a (preinactivation) MOI of 1 for 2 hrs at 37°C and supernatants were collected daily for analysis of IL-8 secretion by ELISA. Viral progeny was also measured in the supernatants by indirect fluorescent antibody staining. Supernatants from live DEN2V infected HepG2 cells contained approximately 10^6 fluorescent focus units per ml (ffu/ml) by Day 3, whereas infectious DEN2V was not detected in the supernatants from HepG2 cells treated with UV or heat inactivated virus. As seen in figure III-7, IL-8 secretion was induced only in cells infected with live DEN2V, suggesting that replication competent virus is necessary for IL-8 expression.

We considered the possibility that induction of IL-8 in cells infected with DEN2V was due indirectly to induction of IFNα production. Lipopolysaccharide (LPS), polyI:C or IFNα treatment of HepG2 cells did not induce RANTES, MIP-1α or MCP-1 expression (Figure III-8). In addition, there was no IL-8 induction in HepG2 and HEK293 cells treated with LPS, polyI:C or IFNα (Figure III-9). However, PMA/Ionomycin treatment induced IL-8 secretion by 3 hours post stimulation in HepG2 cells and by 8 hrs in HEK293 cells (Figure III-9). Moreover, IFNα was not produced in either HepG2 or HEK293 cells in response to DEN2V infection or stimulation with polyI:C (data not shown). These results suggest that activation of the innate immune system is not involved in IL-8 secretion during DEN2V infection. Overall, the results indicate that IL-8 secretion during DEN2V infection is directly due to replication competent virus.
Figure III-7. IL-8 expression in HepG2 cells requires replication competent DENV.

UV and heat inactivated virus, untreated virus (MOI of 1) and C6/36 supernatants were added to HepG2 cells for 2 hours at 37°C. Supernatants were analyzed for IL-8 protein by ELISA for days 3, 4 and 5 post infection.
Figure III-8. Chemokine secretion from HepG2 cells stimulated with LPS, polyI:C, DEN2V or IFNa. HepG2 cells were infected with DEN2V at an MOI of 1 or stimulated
with 1 \( \mu \text{g/ml} \) LPS, 100 \( \mu \text{g/ml} \) polyI:C or 1000 U/ml IFN\( \alpha \)2b. Supernatants were collected each day and analyzed for levels of A. MIP-1\( \alpha \), B. RANTES or C. MCP-1 by ELISA.
Figure III-9. IL-8 secretion from HepG2 and HEK293 cells stimulated with LPS, polyI:C, PMA and ionomycin or IFNα. A. HepG2 and B. HEK293 cells were stimulated with 1 μg/ml of LPS, 100 μg/ml of polyI:C, 100 ng/ml PMA and 1μg/ml of ionomycin, 1000 U/ml of IFNα2b or no stimulation (media) for 1, 3, 8 or 24 hrs. Supernatants were analyzed for IL-8 secretion at each time point by ELISA.
D. TRANSCRIPTION FACTORS INVOLVED IN CHEMOKINE INDUCTION BY DEN2V

To assess transcription factors involved in the induction of IL-8 by DEN2V, we transfected HEK293A and HepG2 cells with luciferase reporter constructs containing tandem repeats of the consensus binding sites for the transcription factors c/EBP, AP-1 and NFκB, a construct containing a promoter driven by an ISRE site, or constructs containing the RANTES or IL-8 promoters. In addition to the wt IL-8 promoter, we also obtained reporter constructs containing mutations in the binding sites for c/EBP (mtc/EBP), AP-1 (mtAP-1) or NFκB (mtNFκB) [416] as described in the MATERIALS AND METHODS. We tested whether DEN2V infection would activate transcription from these reporters using a luciferase assay system.

DEN2V infection of HEK293A cells induced transcription from the IL-8 promoter 15-fold over uninfected cells (Figure III-10). Transcription from the ISRE driven promoter was induced by DEN2V 13-fold, and the RANTES promoter was induced 5-fold at 72 hours post infection. The IKK-related kinase TBK1 has recently been shown to phosphorylate and activate IRF-3; overexpression of TBK1 induced transcription from both the ISRE and RANTES promoters (positive control), consistent with published reports [180, 181]. Similarly, overexpression of Mal, the TLR4 adaptor molecule, induced transcription from the IL-8 promoter (positive control), as previously reported [417, 420].

DEN2V infection of HEK293A cells activated NFκB three fold, AP-1 two fold and c/EBP one and half fold over the control (Figure III-11). In contrast, DEN2V infection of HepG2 cells activated AP-1 approximately three fold over control whereas
there was no detectable activation of the c/EBP or NFκB reporters (Figure III-12). These results suggest that NFκB, AP-1 and c/EBP are involved in IL-8 induction during DEN2V infection of HEK293A cells; whereas AP-1 may play the predominant role in DEN2V infected HepG2 cells. Activation of transcription factors by DEN2V, therefore, appears to be dependent on the cell type that is infected.

Transcription factors have been reported to cooperate with each other to synergize activation of a promoter [256, 307, 308]. Therefore, we wanted to assess the importance of each of the transcription factor binding sites within the context of the IL-8 promoter. In HEK293A cells, activation of each of the mtc/EBP, mtAP-1, and mtNFκB IL-8 promoters by DEN2V infection of HEK293A cells showed a two fold reduction compared to the wildtype IL-8 promoter by day 4 post infection (Figure III-13). These results suggest that activation of all three transcription factors is needed for full IL-8 induction in DEN2V-infected HEK293A cells. In contrast, none of the mutations in the transcription binding sites within the IL-8 promoter showed inhibition of IL-8 induction with DEN2V infection of HepG2 cells (Figure III-14). These results suggest that AP-1 may be important but not necessary for IL-8 induction during DEN2V infection of HepG2 cells. AP-1 has been shown to cooperate with NFκB and c/EBP to induce gene expression such as IL-8 [307]. Therefore, the level of activation of each of the transcription factors may not be as important for IL-8 induction as the ability to interact with each other.

None of the conditions tested in HepG2 cells activated NFκB, yet studies have implicated NFκB as the predominant transcription factor involved in chemokine induction during DENV infection [4]. Therefore, to verify that our reporters were able to
be induced we treated HepG2 cells with PMA (phorbol myristate acetate) and ionomycin. PMA is a potent inducer of protein kinase C (PKC) which catalyzes the phosphorylation of other proteins within the cell. PMA has been shown to induce IL-8 in keratinocytes, mononuclear cells, osteoblasts, HEp-2 cells (a human epidermal larynx carcinoma cell line) and Thp-1 cells (a human myelomonocytic cell line) [421-424]. We found induction of transcription from RANTES, IL-8, c/EBP, AP-1 and NFκB promoters with PMA and ionomycin treatment, which indicates that HepG2 cells are capable of activating NFκB (Figure III-15).

In summary, the results suggest that the pattern of c/EBP, AP-1 and NFκB activation due to DEN2V infection is dependent on the cell line infected. DEN2V infection of HEK293A activates mainly NFκB, and to a lesser extent, AP-1 and c/EBP, whereas DEN2V infection of HepG2 activates predominantly AP-1. Furthermore, the lack of NFκB activation in DEN2V-infected HepG2 cells was not due to a defect in the cell line.
Figure III-10. DEN2V activates the ISRE binding site, RANTES and IL-8 promoters. HEK293A cells were transfected with luciferase reporter constructs containing the RANTES or IL-8 promoter or tandem repeat binding sites for ISRE, and infected 24 hours later with DEN2V at an MOI of 1, UV inactivated DEN2V or C6/36 supernatants. At 72 hours post infection, luciferase reporter gene activity was measured. Data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to C6/36 treated cells for each construct. There is a statistically significant difference (*P* < 0.02) between DEN2V and C6/36 treated cells for ISRE, RANTES and IL-8 reporters using the student t-test. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate.
Figure III-11. Activation of transcription factors by DEN2V infection of HEK293A.

HEK293A cells were transfected with luciferase reporter constructs containing the RANTES or IL-8 promoter or tandem repeat binding sites for c/EBP, AP-1 or NFκB and infected 24 hours later with DEN2V at an MOI of 1, UV inactivated DEN2V or C6/36 supernatants. At 96 hours post infection, luciferase gene activity was measured. Data were normalized for transfection efficiency based on R. reniformis luciferase activity and expressed as fold induction relative to C6/36 treated cells for each construct. There is a statistically significant difference (P ≤ 0.02) between DEN2V and C6/36 treated cells for IL-8, c/EBP, AP-1 and NFκB reporters using the student t-test. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate.
Figure III-12. Activation of transcription factors by DEN2V infection of HepG2 cells. HepG2 cells were transfected with luciferase reporter constructs containing the RANTES or IL-8 promoter or tandem repeat binding sites for c/EBP, AP-1 or NFκB and infected after 24 hours with DEN2V at an MOI of 1. At 72 hours post infection, luciferase gene activity was measured. Data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to media for each construct. There is a statistically significant difference (*P* ≤ 0.05) between DEN2V and media treated cells for IL-8 and AP-1 reporters using the student t-test. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate.
Figure III-13. Effect of DEN2V infection on transcription from mutated IL-8 promoters in HEK293A cells. HEK293A cells were transfected with luciferase reporter constructs containing the IL-8, mtc/EBP, mtAP-1, and mtNFκB promoters and infected after 24 hours with DEN2V at an MOI of 1. At 96 hours post infection, luciferase gene activity was measured. Data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to C6/36 treated cells for each construct. There is a statistically significant difference (*P* ≤ 0.01) between DEN2V and C6/36 treated cells for IL-8, mtc/EBP, mtAP-1 and mtNFκB reporters using the student t-test. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate. Mtc/EBP, mtAP-1, and mtNFκB represent the 133-luc plasmid with point mutations in the NF–IL-6, AP-1, and NFκB binding sites, respectively.
Figure III-14. Effect of DEN2V infection on transcription from mutated IL-8 promoters in HepG2 cells. HepG2 cells were transfected with luciferase reporter constructs containing the IL-8, mtc/EBP, mtAP-1, and mtNFκB promoters and infected after 24 hours with DEN2V at an MOI of 1. At 72 hours post infection, luciferase gene activity was measured. Data were normalized for transfection efficiency based on R. reniformis luciferase activity and expressed as fold induction relative to media for each construct. There is a statistically significant difference ($P \leq 0.05$) between DEN2V and media treated cells for IL-8 and AP-1 reporters using the student t-test. The data are expressed as mean ± standard deviation from a representative experiment done in triplicate.
Figure III-15. PMA and ionomycin stimulation of HepG2 cells activated transcription from chemokine promoters and transcription factor reporters. HepG2 cells were transfected with luciferase reporter constructs containing the RANTES or IL-8 promoter or tandem repeat binding sites for c/EBP, AP-1 or NFκB. After 24 hours, cells were stimulated with 100 ng/ml PMA and 1 μg/ml ionomycin. Cell lysates were harvested for analysis of luciferase activity at 24 hours post stimulation. The data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to media for each construct. The data are expressed as mean ± standard deviation from a representative experiment done in triplicate.
E. ROLE OF NFκB IN DENV-INDUCED GENE EXPRESSION.

DENV infection induces transcription factors NFκB, AP-1 and c/EBP, which are involved in the induction of many genes including chemokines [4, 6, 397, 425-427]. It has been reported that NFκB plays a predominant role in IL-8 induction during DENV infection [4, 251]. The above data suggested that NFκB plays a role in IL-8 and RANTES expression in HEK293A cells. To further assess the role of NFκB activation in induction of IL-8 and RANTES expression by DENV in HEK293A cells, we used a plasmid that expresses an IkBα dominant negative mutant (IkB super repressor (SR), IkBα-S32/36A) [181] to block NFκB activation and nuclear localization. As seen in figure III-16, expression of the IkBSR substantially inhibited both IL-8 and RANTES transcription on days 3 and 4 post infection with DEN2V. PMA and Ionomycin treatment also induces NFκB activation which stimulates the production of many cytokines such as IL-1, IL-6, TNFα and IFNγ [320]. As shown in figure III-17, IkBSR also significantly inhibited PMA and ionomycin induced IL-8 secretion when compared to the pcDNA control. These results confirm the importance of NFκB activation for IL-8 secretion due to PMA and Ionomycin stimulation in HEK293A cells. In addition, we show that NFκB is required for IL-8 and RANTES transcription in DEN2V-infected HEK293A cells.
Figure III-16. Activation of IL-8 and RANTES promoters in DEN2V-infected HEK293A cells transfected with IκBSR. HEK293A cells were cotransfected with a luciferase reporter gene for IL-8 or RANTES and an expression plasmid containing IκBSR or pcDNA3.1 control. 24 hours later, cells were infected with DEN2V at an MOI of 1. Luciferase activity was assessed at A. 72 hours or B. 96 hours post infection. The data were normalized for transfection efficiency based on R. reniformis luciferase activity.
and expressed as fold induction relative to uninfected cells transfected with pcDNA3.1. The data are expressed as mean ± standard deviation from a representative experiment done in triplicate.
**Figure III-17.** IL-8 production from PMA and ionomycin stimulated HEK293A cells transfected with IκBSR plasmid. HEK293A cells were transfected with an expression plasmid containing IκBSR or pcDNA3.1 control. Cells were stimulated after 24 hours with 100 ng/ml PMA and 1 μg/ml ionomycin (P/I). At 24 hours supernatants were collected and analyzed for IL-8 protein levels by ELISA. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in duplicate.
Cytokines play an important role in inflammatory diseases and much effort has been made to identify pharmacologic inhibitors of proinflammatory cytokine production. Pharmacological agents are also commonly used to help define signaling pathways induced by various stimuli. The benefit of these small molecule inhibitors is that they can access signaling pathways within a cell producing a wide range of anti-inflammatory theurapeutic potential through inhibition of cytokines/chemokines [330]. We wanted to assess the effect of pharmacological inhibitors of cytokine expression from previous studies on DEN2V-induced transcription of chemokines in HEK293A. Previously, we have shown that the embryonic kidney cell line, HEK293A, which is relatively easy to transfect, induces IL-8 and RANTES upon DEN2V infection by day 3 (Figure IV-1). We hypothesized that pharmacological inhibitors of cytokines will inhibit secretion of chemokines in DEN2V-infected HEK293A cells.

To analyze the effects of pharmacological inhibitors, we transfected HEK293A cells with RANTES and IL-8 promoter constructs (Figure IV-2). After 24 hours, the cells were infected with DEN2V at an MOI of 5 in the presence of the pharmacological inhibitor. Supernatants were taken on days 3 and 4 post infection for chemokine analysis.
by ELISA (Figure IV-1) and new media was added to the well. The inhibitors were added to the culture medium each time the media was replaced. In addition, cells were harvested on day 2 post infection to determine the percent of infected cells by flow cytometry. As seen in table IV-1, DEN2V infection rates in SB203580, rolipram and dexamethasone treated HEK293A cells were similar to untreated DEN2V-infected cells. In addition, the cell monolayer remained intact through day 4 post infection in each condition (data not shown).

To characterize the effects these inhibitors had on transcription of IL-8 during DEN2V infection, we transfected HEK293A with constructs containing tandem repeats of c/EBP, AP-1 and NFKB binding sites (Figure IV-3) and reporters that contain the IL-8 promoter with mutations in transcription factor binding sites (Figure IV-4).

1. Treatment of DEN2V-infected cells with SB203580

The production of IL-8 by DEN2V-infected cells was ~50% lower in the presence of SB203580, an inhibitor of the p38 MAPK pathway, when compared to untreated DEN2V-infected HEK293A cells (Figure IV-1). RANTES production, on the other hand, was not affected by SB203580 treatment in DEN2V-infected HEK293A cells. There was a similar inhibition of transcription from the IL-8 reporter in DENV-infected cells in the presence of SB203580 treatment on day 3 post infection but not on day 4 post infection (Figure IV-2). There was ~50% increase in the activation of the AP-1 promoter with SB203580 treatment of DEN2V-infected cells compared to untreated DEN2V-infected cells on day 3 post infection (Figure IV-3 A). However, there was no difference in the transactivation of c/EBP, AP-1 and NFκB in DEN2V infected cells in the presence or absence of SB203580 on day 4 post infection (Figure IV-3 B). SB203580 treatment
reduced transcription ~80% from the NFκB mutant IL-8 promoter in DEN2V-infected cells when compared to untreated DEN2V infected cells. Similarly, transcription from the mutant AP-1 IL-8 promoter was reduced ~40% when compared to DEN2V infection without treatment (Figure IV-4). The mutant IL-8 promoter results suggest that inhibition of NFκB and, to a lesser extent, AP-1 binding to the IL-8 promoter is not the mechanism by which SB203580 inhibits IL-8 transcription during DEN2V infection of HEK293A cells.

Overall, these results indicate that inhibition of the p38 MAPK pathway by SB203580 reduces the induction of IL-8 transcription and secretion by DEN2V infection. This inhibition of IL-8 transcription was not due to a decrease in activation of c/EBP, AP-1 and NFκB. Inhibition of the p38 MAPK pathway due to SB203580 treatment during DEN2V infection may activate pathways that can induce the inhibitory forms of transcription factors such as Fra-1, which binds to AP-1 sites [426] or inhibit cooperative interactions between transcription factors.

2. Treatment of DEN2V-infected cells with rolipram

Treatment of HEK293A cells with rolipram, a PDEIV inhibitor, decreased IL-8 and RANTES secretion by ~30% when compared to untreated DEN2V-infected cells on days 3 and 4 (Figure IV-1). Induction of transcription from the IL-8 and RANTES promoters was also reduced (Figure IV-2). Transcription from promoters containing tandem repeats of c/EBP, AP-1 or NFκB in rolipram treated DEN2V-infected cells was not significantly different than untreated DEN2V-infected cells (Figure IV-3).

In contrast, transcription from the mt NFκB IL-8 promoter was significantly inhibited in rolipram-treated DEN2V-infected HEK293A cells when compared to
untreated DEN2V-infected cells. No significant difference in activation of the mtc/EBP or mAP-1 reporters were seen in DEN2V-infected HEK293A cells treated with rolipram versus untreated DEN2V-infected cells (Figure IV-3). These results suggest that NFKB activity is not affected by rolipram treatment of DEN2V-infected HEK293A cells.

In summary, these results show that inhibition of PDEIV suppresses expression of IL-8 and RANTES protein as well as the induction of transcription from the IL-8 and RANTES promoter. Transcription from the c/EBP, AP-1 or NFKB promoters were not inhibited by rolipram treatment of DEN2V-infected HEK293A cells although transcription from the mutant NFKB IL-8 promoter was decreased. These results suggest that inhibition of PDEIV does not inhibit IL-8 and RANTES transcription and protein expression from DEN2V-infected cells by decreasing activation of these transcription factors. Increases in levels of cAMP due to rolipram treatment may activate pathways that induce inhibitory forms of transcription factors or inhibit cooperative interactions as previously mentioned above in DEN2V-infected cells treated with SB203580.

3. Treatment of DEN2V-infected cells with dexamethasone

Analysis of chemokine protein levels in the culture supernatants showed an increase in IL-8 (~35%) and RANTES (~75%) in DEN2V-infected HEK293A cells treated with the glucocorticoid, dexamethasone when compared to untreated DEN2V-infected cells by day 4 (Figure IV-1). In contrast, dexamethasone treatment reduced IL-8 transcription in DEN2V-infected cells ~60% by day 4 post infection (Figure IV-2). There was no significant difference in transcription from the RANTES promoter between dexamethasone-treated or untreated DEN2V-infected HEK293A cells on day 4.
There was no significant difference in the activation of c/EBP- or AP-1-driven promoters with dexamethasone treatment of DEN2V-infected HEK293A cells when compared to untreated DEN2V-infected cells on day 4 (Figure IV-3). However, the activation of the NFκB driven promoter was not above the media control with dexamethasone treatment (Figure IV-4). However, induction of transcription from the mutant NFκB IL-8 promoter was inhibited on day 4 with dexamethasone treatment of DEN2V-infected HEK293A cells compared to untreated DEN2V-infected cells. These results suggest that dexamethasone treatment of DEN2V-infected HEK293A cells can inhibit NFκB activation but had no significant effect on AP-1 or c/EBP activation.

Overall, these results show that dexamethasone treatment of DEN2V-infected HEK293A cells increases IL-8 and RANTES secretion. This increase in protein production was not due to an increase in transcription from the chemokine promoters or activation of the transcription factors c/EBP, AP-1 or NFκB. In fact, transcription from the NFκB promoter and the chemokine promoters was inhibited with dexamethasone treatment of DEN2V-infected cells. These results suggest that the increase in IL-8 and RANTES production with dexamethasone treatment of DEN2V infected HEK293A cells is due to a mechanism other than an increase in transcription, for example, stabilization of the chemokine mRNA.

4. Treatment of DEN2V-infected cells with PDTC

PDTC treatment of DEN2V infected HEK293A cells decreased IL-8 and RANTES protein secretion (Figure IV-1) and transcription from the IL-8 promoter (Figure IV-2) when compared to DEN2V infection without treatment on days 3 and 4. Transcription from the RANTES promoter was not studied. There was no inhibition of
transcription from AP-1 or c/EBP promoters, but, there was inhibition of transcription from the NFκB promoter (Figure IV-3). These results suggest that the inhibition of DEN2V-induced IL-8 transcription by PDTC treatment is mediated through inhibition of NFκB binding. (Note: Analysis of the mutant IL-8 reporter constructs were not done with PDTC treatment.)

5. Treatment of DEN2V-infected cells with indomethacin

Indomethacin treatment of DEN2V-infected cells substantially decreased IL-8 (~85%) and RANTES (~80%) secretion compared to untreated DEN2V-infected cells (Figure IV-1). In addition, the activation of the IL-8 promoter and c/EBP, AP-1 and NFκB reporters by DEN2V infection was inhibited with indomethacin treatment when compared to untreated DEN2V-infected HEK293A cells (Figures IV-2 and IV-3). This suggests that multiple pathways are involved in the inhibition of IL-8 and RANTES induction with indomethacin treatment during DEN2V infection. (Note: Analysis of the mutant IL-8 reporter constructs were not done with indomethacin treatment.)
Table IV-1. Effects of pharmacological inhibitors on rate of DEN2V infection in HEK293A cells.

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</tr>
<tr>
<td>Rolipram (100 μM)</td>
<td>90</td>
</tr>
<tr>
<td>Dexamethasone (250 nM)</td>
<td>81</td>
</tr>
<tr>
<td>Untreated</td>
<td>90</td>
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</table>

<sup>a</sup> % infection was assessed by flow cytometry on day 2 post infection as described in the MATERIALS AND METHODS.

<sup>b</sup> HEK293A cells were infected with an MOI of 5 with or without inhibitors as described in the MATERIALS AND METHODS.
A.

![Graph A](image)

B.

![Graph B](image)

**Figure IV-1.** IL-8 and RANTES secretion from DEN2V-infected HEK293A cells treated with pharmacological inhibitors. HEK293A cells were infected with DEN2V at an MOI of 5 for 2 hours at 37° C with or without addition of inhibitors to culture medium as described in the MATERIALS AND METHODS. Supernatants were taken 72 or 96 hours post infection for detection of A. IL-8 or B. RANTES protein by ELISA. The data shown is a representative result from two independent experiments. RP, SB, Dex and Indo represent rolipram, SB203580, dexamethasone and indomethacin treated cells, respectively.
Figure IV-2. Activation of the IL-8 and RANTES promoters in DEN2V infected HEK293A cells treated with pharmacological inhibitors. HEK293A cells were transfected with reporter constructs containing the IL-8 or RANTES promoter. After 24
hours, cells were infected with DEN2V at an MOI of 5 for 2 hours at 37° C with or without addition of inhibitors as described in MATERIALS AND METHODS. Cell lysates were harvested for analysis of luciferase activity at A. 72 or B. 96 hours post infection. The data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to media for untreated cells. The data are expressed as mean ± standard deviation for a representative experiment from a minimum of two separate experiments, each done in triplicate. RP, SB, Dex and Indo represent rolipram, SB203580, dexamethasone and indomethacin treated cells, respectively.
Figure IV-3. Activation of the transcription factors c/EBP, AP-1 and NFκB in DEN2V-infected HEK293A cells treated with pharmacological inhibitors.

HEK293A cells were transfected with reporter constructs containing the IL-8 promoter or
tandem repeat binding sites for transcription factors c/EBP, AP-1 or NFκB. After 24 hours, cells were infected with DEN2V at an MOI of 5 for 2 hours at 37°C with or without addition of inhibitors as described in MATERIALS AND METHODS. Cell lysates were harvested for analysis of luciferase activity at A. 72 hours B. 96 hours post infection. The data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to media for untreated cells. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate. RP, SB, Dex and Indo represent rolipram, SB203580, dexamethasone and indomethacin treated cells, respectively.
Figure IV-4. Activation of mutant IL-8 promoters in DEN2V-infected HEK293A cells treated with pharmacological inhibitors. HEK293A cells were transfected with vectors encoding a reporter gene for wild type, mtc/EBP, mtAP-1 or mtNFκB IL-8 promoters and infected with DEN2V at an MOI of 5 after 24 hours. At A. 72 or B. 96 hours post infection, luciferase gene activity was measured. Data were normalized for
transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to media for untreated cells. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate. RP, SB, Dex and Indo represent rolipram, SB203580, dexamethasone and indomethacin treated cells, respectively.
B. EFFECTS OF INHIBITORS ON IL-8 AND RANTES INDUCTION BY DEN2V IN A HEPATOCARCINOMA CELL LINE.

Since the studies described above (Chapter III) showed differences in the activation of transcription factors by DEN2V infection in HEK293A and HepG2 cells, we wanted to determine if the effects of the pharmacologic inhibitors on the induction of chemokines and transcription factors we found with HEK293A cells would also occur in HepG2 cells. We hypothesized that inhibitors of cytokines will inhibit secretion of chemokines in DEN2V infected HepG2 cells. We infected HepG2 with DEN2V alone or in the presence of drugs at an MOI of 5. Supernatants were taken on various days post infection for chemokine analysis by ELISA (Figures IV-5 and IV-6), cell death was assessed by microscopy in comparison with untreated cells and cells were harvested to determine the percent of infected cells by flow cytometry on day 6 post infection (Table IV-2). In addition, cells were transfected with reporter constructs and infected with DEN2V at an MOI of 5 24 hours later either in the presence or absence of the inhibitors (Figures IV-7, IV-8 and IV-9). As seen in table IV-2, treatment of DEN2V-infected HepG2 cells with inhibitors did not significantly affect the rate of infection on day 6 post infection.

1. Treatment of DEN2V-infected cells with SB203580.

SB203580 treatment of DEN2V infected HepG2 cells did not inhibit IL-8 or RANTES secretion by day 4 post infection (Figures IV-5 and IV-6). In fact, a significant increase in IL-8 and RANTES secretion occurred in drug-treated DEN2V-infected cultures by day 6 post infection when compared to untreated DEN2V infected cells (Figure IV-5). Furthermore, there was an increase in transcription from the IL-8 and
RANTES promoters in SB203580 treated cells when compared to untreated DEN2V-infected HepG2 cells by day 4 post infection (Figure IV-7). In addition, there was little difference in cell death of the monolayer with SB203580 treatment of HepG2 cells when compared to untreated cells by day 6 post infection with DEN2V (Table IV-2).

Analysis of transcription factors showed enhanced activation of transcription from the AP-1 promoter in DEN2V-infected HepG2 cells treated with SB203580 when compared to untreated DEN2V-infected cells, whereas there was no significant change in the activation of the c/EBP or NFκB reporter (Figure IV-8). SB203580 treatment of DEN2V-infected HepG2 cells showed moderate inhibition from the mutant AP-1 IL-8 promoter on day 3, whereas on day 4 there was a moderate inhibition of transcription from the mutant c/EBP IL-8 promoter (Figure IV-9). These results suggest that AP-1 may play a role in the enhanced induction of transcription of IL-8 and RANTES in DEN2V-infected HepG2 cells treated with SB203580 when compared to untreated DEN2V-infected cells.

Overall, the results show that SB203580 treatment of DEN2V-infected cells increased IL-8 and RANTES secretion, and this was due, in part, to an increase in transcription from the IL-8 and RANTES promoter. In addition, the results suggest that AP-1 is important for induction of transcription in DEN2V-infected cells treated with SB203580. The enhanced transcription from mutant AP-1 reporter suggests cooperative interaction of AP-1 with c/EBP or NFκB.

2. Treatment of DEN2V-infected cells with rolipram.

Rolipram treatment of DEN2V-infected HepG2 cells did not inhibit IL-8 or RANTES secretion on day 4 post infection when compared to untreated DEN2V-infected
cells (Figures IV-5 and IV-6). As seen with SB203580, rolipram treatment enhanced IL-8 and RANTES secretion by day 6 when compared to untreated DEN2V-infected HepG2 cells (Figure IV-5). In contrast, rolipram treatment inhibited transcription from the IL-8 promoter but not the RANTES promoter in DEN2V-infected HepG2 cells (Figure IV-7). In addition, there was an increase in cell death by day 6 (Table IV-2).

Activation of the AP-1 reporter was enhanced with rolipram treatment of DEN2V-infected HepG2 cells on day 3 post infection when compared to untreated DEN2V-infected cells (Figure IV-8). However, there was no significant difference in activation of AP-1 promoter on day 4 in DEN2V-infected HepG2 cells treated with rolipram when compared to untreated DEN2V-infected cells. Furthermore, there was no significant difference in activation of NFκB and c/EBP promoters in DEN2V-infected HepG2 cells treated with rolipram versus untreated. The IL-8 promoters containing a mutation in the c/EBP, AP-1 or NFκB site showed no significant change in transcription in rolipram-treated DEN2V-infected HepG2 cells on day 3 when compared to untreated cells (Figure IV-9). However, on day 4 post infection, mutant c/EBP and NFκB promoters showed a decrease in transcription in DEN2V-infected HepG2 cells treated with rolipram.

In summary, these data show that enhanced secretion of IL-8 and RANTES in DEN2V infected HepG2 cells treated with rolipram was not due to enhanced transcription of mRNA. There was, however, transient enhanced AP-1 activation with rolipram treatment of DEN2V-infected HepG2 cells. This may be an indirect effect from activation of an upstream factor in the MAPK pathways that can lead to activation of the p38 MAPK pathway and JNK pathway (see Figure I-10). The p38 MAPK has been
shown to induce factors that can increase mRNA stability. Furthermore, the p38 MAPK pathway can be activated through the same MAPK pathway as the activation of AP-1 [304]. The results suggest that inhibition of PDEIV in DEN2V infected HepG2 cells may enhance protein expression, perhaps by increasing chemokine mRNA stability or an increase translation efficiency.

3. Treatment of DEN2V-infected cells with dexamethasone.

Dexamethasone treatment of DEN2V-infected HepG2 cells failed to inhibit IL-8 protein expression (Figures IV-5 and IV-6). In addition, there was no significant difference in the activation of the IL-8 promoter with dexamethasone treatment of DEN2V-infected HepG2 cells when compared to untreated cells (Figure IV-7). Both RANTES protein expression and activation of the RANTES promoter were increased with dexamethasone treatment of DEN2V-infected HepG2 cells in comparison with untreated DEN2V cells on day 3 (Figures IV-6 and IV-7). However, there was no significant difference in RANTES protein expression or transcription from the RANTES promoter in DEN2V-infected cells treated with dexamethasone by day 4.

Dexamethasone treatment of HepG2 cells inhibited cell death of the monolayer due to DEN2V infection on day 6 (Table IV-2).

Analysis of transcription factors c/EBP, AP-1 and NFκB also showed minimal effect with dexamethasone treatment when compared to untreated DEN2V-infected cells (Figure IV-8). In addition, induction of transcription from the IL-8 promoters containing mutations within the transcription factor binding sites had no significant difference with dexamethasone treatment of DEN2V-infected HepG2 cells when compared with untreated DEN2V-infected cells (Figure IV-9).
These results showed that dexamethasone treatment has no significant effect on IL-8 expression or activation of c/EBP, AP-1 and NFκB from DEN2V-infected HepG2 cells. In contrast, both RANTES secretion and transcription were induced in DEN2V-infected cells treated with dexamethasone. These results suggest that activation of other transcription factors that are specific for RANTES expression such as IRFs may be increased in DEN2V-infected HepG2 cells treated with dexamethasone.

4. Treatment of DEN2V-infected cells with PDTC.

PDTC treatment of DEN2V-infected HepG2 cells had no significant effect on IL-8 secretion when compared to untreated cells (Figure IV-5). In addition, there was no virus-induced cell death with PDTC treatment of DEN2V-infected HepG2 cells (Table IV-2). The effect of PDTC treatment of DEN2V-infected cells on IL-8 transcription or activation of transcription factors was not assessed.

5. Treatment of DEN2V-infected cells with indomethacin.

Indomethacin treatment of HepG2 cells induced IL-8 production in both infected and uninfected cells (Figure IV-5). This result suggests a nonspecific effect on IL-8 expression with indomethacin treatment. In contrast to IL-8 production, indomethacin inhibited RANTES secretion in DEN2V-infected HepG2 cells when compared to untreated cells. These results suggest that indomethacin can inhibit pathways that are specific to RANTES production, such as factors that can bind to ISRE in the RANTES promoter.

One caveat to these assays is that HepG2 cells tend to clump in culture. This made it difficult to obtain high transfection efficiencies. We, therefore, used the reporter system which allows normalization of the luciferase expression by normalizing values for
background expression of *Renilla reniformis*. Although this helped to assess induction of specific genes, we were unable to continue the assays past day 4 since the luciferase readings were very low. This was possibly due to multiple divisions of the cells, which would dilute the signal from the luciferase in cells containing the reporters.
Table IV-2. Effects of pharmacological inhibitors on cell death and rate of DEN2V infection in HepG2 cells.

<table>
<thead>
<tr>
<th>Pharmacological Inhibitor</th>
<th>Cell death&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Infection&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEN2V&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Uninfected</td>
</tr>
<tr>
<td>SB203580 (10 μM)</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Rolipram (100 μM)</td>
<td>++++</td>
<td>+/-</td>
</tr>
<tr>
<td>Dexamethasone (250 nM)</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>PDTC (250 nM)</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Indomethacin (30 μM)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Untreated</td>
<td>++</td>
<td>+/-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cell death was assessed microscopically on day 3 post infection.

<sup>b</sup> Cells were infected at an MOI of 5 with or without inhibitors as described in the MATERIALS AND METHODS.

<sup>c</sup> % infection was assessed by flow cytometry on day 6 post infection as described in the MATERIALS AND METHODS.

+/- equals ≤ 10% of the monolayer of cells were disrupted.

+ equals 20 - 40% of the monolayer of cells were disrupted.

++ equals 50 - 70% of the monolayer of cells were disrupted.

+++ equals 80 – 100% of the monolayer of cells were disrupted.
Figure IV-5. IL-8 and RANTES production from DEN2V-infected HepG2 cells treated with pharmacological inhibitors. HepG2 cells were infected with DEN2V at an MOI of 5 for 2 hours at 37° C with or without addition of inhibitors as described in MATERIALS AND METHODS. Supernatants were taken 24, 72 or 144 hours post infection for detection of A. IL-8 or B. RANTES protein by ELISA. The data shown is a representative result from two independent experiments. RP, SB, Dex and Indo represents rolipram, SB203580, dexamethasone and indomethacin treated cells, respectively.
Figure IV-6. IL-8 and RANTES secretion from DEN2V-infected HepG2 cells treated with pharmacological inhibitors. HepG2 cells were infected with DEN2V at an MOI of 5 for 2 hours at 37°C with or without addition of inhibitors as described in MATERIALS AND METHODS. Supernatants were collected 72 or 96 hours post infection for detection of A. IL-8 or B. RANTES protein by ELISA. RP, SB and Dex represent rolipram, SB203580 and dexamethasone treated cells, respectively.
Figure IV-7. Activation of the IL-8 and RANTES promoters in DEN2V-infected HepG2 cells treated with pharmacological inhibitors. HepG2 cells were transfected with reporter constructs containing the IL-8 or RANTES promoter. After 24 hours, cells were infected with DEN2V at an MOI of 5 for 2 hours at 37°C with or without addition of inhibitors as described in MATERIALS AND METHODS. Cell lysates were
harvested for analysis of luciferase activity at A. 72 or B. 96 hours post infection. The data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to media for untreated cells. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate. RP, SB and Dex represent rolipram, SB203580 and dexamethasone treated cells, respectively.
Figure IV-8. Activation of the transcription factors c/EBP, AP-1 and NFκB in DEN2V-infected HepG2 cells treated with pharmacological inhibitors. HepG2 cells were transfected with reporter constructs containing the IL-8 promoter or tandem repeat binding sites for transcription factors c/EBP, AP-1 or NFκB. After 24 hours, cells were infected with DEN2V at an MOI of 5 for 2 hours at 37°C with or without addition of
inhibitors as described in MATERIALS AND METHODS. Cell lysates were harvested for analysis of luciferase activity at A. 72 hours B. 96 hours post infection. The data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to media for untreated cells. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate. RP, SB and Dex represent rolipram, SB203580 and dexamethasone treated cells, respectively.
Figure IV-9. Activation of mutant IL-8 promoters in DEN2V-infected HepG2 cells treated with pharmacological inhibitors. HepG2 cells were transfected with vector encoding a reporter gene for wild type, mtc/EBP, mtAP-1 or mtNFαB IL-8 promoters and infected with DEN2V at an MOI of 5 after 24 hours. At A. 72 or B. 96 hours post infection, luciferase gene activity was measured and data were normalized for
transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to media for untreated cells. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate. RP, SB and Dex represent rolipram, SB203580 and dexamethasone treated cells, respectively.
C. SUMMARY OF EFFECTS OF INHIBITORS ON IL-8 AND RANTES INDUCTION
BY DEN2V

Table IV-3 summarized the effects of pharmacological inhibitors on DEN2V-infected HEK293A cells and HepG2 cells. With the exception of indomethacin, we found only moderate inhibition of IL-8 and RANTES production with the pharmacological inhibitors tested. However, dexamethasone treatment of DEN2V-infected HEK293A cells and SB203580 or rolipram treatment of HepG2 cells enhanced IL-8 and RANTES secretion in DEN2V-infected cells.

Dexamethasone (HEK293A) and rolipram (HepG2) treatment of DEN2V-infected cells did not increase transcription from the IL-8 or RANTES promoters when compared to untreated DEN2V-infected cells. Therefore, post transcriptional mechanisms such as mRNA stability may be involved in the enhanced secretion of chemokines. In contrast, SB203580 treatment of DEN2V-infected HepG2 cells also showed an increase in transcription from the IL-8 and RANTES promoters when compared to untreated DEN2V-infected cells. This suggests that an increase in transcription resulted in the enhanced secretion of chemokine proteins in SB203580 treated DEN2V-infected cells.

Most of the pharmacological inhibitors tested have been reported to have an effect on NFkB activation. However, only dexamethasone, PDTC and indomethacin treatment of DEN2V-infected HEK293A cells inhibited NFkB activation. There was no effect on NFkB activation in DEN2V-infected HepG2 cells with or without treatment of inhibitors. In contrast, an increase in activation of AP-1 was seen in DEN2V-infected HepG2 cells treated with SB203580.
We found significant activation of the wt and mutant IL-8 promoters in DEN2V-infected HEK293A and HepG2 cells. However, the activation of reporters containing tandem repeat consensus binding sites for each transcription factor was significantly lower. The higher level of luciferase activity with the IL-8 reporters could reflect the contribution of cooperativity between transcription factors within the IL-8 promoter to enhance transcription from the IL-8 promoter. Previous reports have shown that binding of NFκB to its cognate site within the IL-8 promoter cooperatively enhances binding of weakly bound c/EBP [256]. In addition, AP-1 and NFκB have been reported to interact with each other and enhance binding to their cognate sites [307]. Therefore, the results from the wt and mutant IL-8 promoters reflect the contribution of multiple transcription factors for transcription from the IL-8 promoters. In contrast, the results from cells transfected with the c/EBP, AP-1 and NFκB reporters reflect activation of individual transcription factors and not their effects on neighboring transcription factors during DEN2V infection with or without pharmacological inhibitors. Although the transcription factor reporters may suggest activation of a specific transcription factor, it is important to analyze the effect of the transcription factor within the context of the full IL-8 promoter.

The inhibitors used in this study were added to cells for a relatively longer period than what has been used in other systems (up to 6 days versus less than 24 hours, respectively). A limitation of this study may be the rate at which the inhibitors are metabolized in culture.

Overall, these results suggest that the enhanced secretion of chemokines in DEN2V-infected cells treated with inhibitors may depend on the activation of pathways that can activate specific transcription factors or post transcriptional mechanisms.
Table IV-3. Effects of pharmacological inhibitors on DEN2V-infected HepG2 and HEK293A cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inhibitor</th>
<th>chemokine secretion</th>
<th>chemokine transcription</th>
<th>transcription factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IL-8</td>
<td>RANTES</td>
<td>IL-8</td>
</tr>
<tr>
<td>HEK293A</td>
<td>SB203580</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>rolipram</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>dexamethasone</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>PDTC</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>indomethacin</td>
<td>↓↓</td>
<td>↓↓</td>
<td>ND</td>
</tr>
<tr>
<td>HepG2</td>
<td>SB203580</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td></td>
<td>rolipram</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↓↓</td>
</tr>
<tr>
<td></td>
<td>dexamethasone</td>
<td>-</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PDTC</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>indomethacin</td>
<td>-</td>
<td>↓</td>
<td>ND</td>
</tr>
</tbody>
</table>

↑ or ↓ = 1.5 to 2 fold difference compared to untreated DEN2V infection
↑↑ or ↓↓ = 2 fold or more difference compared to untreated DEN2V infection
- = no difference when compared to untreated DEN2V infection
ND = not done
CHAPTER V:

ANALYSIS OF DENGUE VIRAL PROTEINS AND CHEMOKINE INDUCTION

A. EXPRESSION OF DEN2V NS5 IS SUFFICIENT TO INDUCE EXPRESSION OF IL-8 BUT NOT RANTES.

Viral proteins such as NS5A and core protein from HCV can induce chemokines such as IL-8 [9, 10]. We hypothesized that DEN2V protein(s) can induce chemokine expression in transfected cells.

In order to study the effects of DEN2V proteins on chemokine induction, it was necessary to clone each of the DEN2V genes into expression vectors. Dengue viral genes were first cloned into an entry vector, pDONR201, by homologous recombination using the Gateway system (Invitrogen) (Figure V-1). All 10 clones were sequenced to verify that no mutations were introduced within the inserted genes by PCR. Several mutations were detected. The NS1 clone contained a nucleotide deletion at 2784 (g). The NS3 clone contained a silent mutation at nucleotide 4545 from t→c (Ile to Ile) and a nonconservative mutation at nucleotide 5793 from a→g (Asp to Gly). All nonconservative mutations were corrected by site-directed mutagenesis and the resulting plasmids were resequenced to verify that the genes contained the correct sequences.

All 10 DEN2V genes were transferred to a CMV driven mammalian expression vector (pDEST 40 or pDEST47) by homologous recombination. Due to the availability of antibodies for detection of protein production, the prM and E genes were engineered with a stop codon at the 3' end. No stop codons were introduced into the remaining
genes; therefore, these clones would express the V5 epitope and 6X His tag or GFP that is engineered into the vector.

The plasmids were transfected into HEK293 cells and analyzed by flow cytometry, microscopy or western blot for expression of the appropriate protein. E protein expression was detected by flow cytometry using the 3H5 monoclonal antibody. His-tagged NS1, and NS5 proteins were detected by flow cytometry using an anti-His antibody. Expression of V5 epitope tagged or GFP-tagged NS5 protein was also detected by western blot using an anti-V5 antibody or by confocal microscopy, respectively. GFP-tagged NS3 protein expression was detected by GFP using flow cytometry or confocal microscopy. V5- and His-tagged C, prM, NS2A, NS2B, NS4A and NS4B were not detected at high levels using an anti-V5 or His antibody and flow cytometry. This could be due to low transfection efficiencies, low expression levels of the protein, or protein folding that blocked antibody binding to the V5 or His tag.

To define further the mechanism for induction of chemokine production by DEN2V infection, we studied the effects of expression of individual DEN2V proteins on chemokine expression. To also test for the possibility that multiple viral proteins and/or RNA replication might also be required for IL-8 induction, we used a plasmid expressing a DEN2V replicon under the control of a CMV promoter [415]. The plasmids were transfected into HEK293A cells, and supernatants were collected on day 2. As shown in figure V-2A, the replicon induced IL-8 secretion 6-fold over the control plasmid. NS5 also induced secretion of IL-8, with levels 3-fold over the lacZ control plasmid. The gene containing the DEN2V replicon is at least three times the size of the NS5 gene. Thus, the amount of plasmid transfected into the cells may be significantly different for the
DEN2V replicon versus NS5 and therefore, could affect the level of IL-8 expression. The 3-fold increase of IL-8 secretion from HEK293A cells transfected with DEN2V replicon when compared to cells transfected with the NS5 expression plasmid may be due to an increase in the level of expression from the DEN2V replicon, resulting in an increase NS5 expression. Alternatively, cooperative interaction of multiple viral proteins may be involved in enhanced IL-8 secretion with the DEN2V replicon. In addition, replication and/or translation of the DEN2V genome may also activate pathways that induce IL-8 expression. In contrast, there was no RANTES secretion from cells transfected with either the NS5- or the replicon-expressing plasmid (Figure not shown).

Increases in the levels of IL-8 mRNA in cells transfected with the plasmids expressing NS5 or the replicon, measured by quantitative RT-PCR, correlated with the increases in IL-8 protein secretion (Figure V-2B). These results show that IL-8 expression can be induced by expression of the DEN2V NS5 protein.
Entry vector

Mammalian expression vector

Figure V-1. Gateway cloning of DEN2V genes (Invitrogen). A. Entry clones are engineered using homologous recombination to insert attB-flanked PCR product into the entry vector pDONR201 (donor vector) containing attP sites. This replaces the ccdB gene, which encodes a protein that interferes with E. coli DNA gyrase, thereby inhibiting
the growth of standard *E. coli* hosts. B. Homologous recombination between attL sites in the entry clone and the attR sites in the mammalian expression vector pcDNA-DEST40 (destination vector) produce the mammalian expression clones (expression clone) which express the gene of interest with a V5 epitope and a 6XHis tag at the C-terminus of the protein.
Figure V-2. NS5 and DEN2V replicon induce IL-8 expression from HEK293A cells.

A. Plasmids expressing DEN2V proteins or a DEN2V replicon were transfected into HEK293A cells. Supernatants were harvested day 2 post transfection and analyzed for IL-8 protein by ELISA. The data are an average from three independent experiments and the error bars represent the standard deviation. There is a statistically significant difference for fold induction ($P < 0.02$) between NS5 and LacZ and ($P < 0.03$) between the replicon and LacZ using the paired student t-test. B. IL-8 mRNA levels from
HEK293A cells transfected with NS5 plasmid, replicon, or untransfected cells were assessed by Taqman PCR on Day 2 post transfection. Results are normalized to untransfected cells = 100.
B. DEN2V NS5 AND REPLICON DO NOT ACTIVATE TRANSCRIPTION FROM ISRE OR THE RANTES PROMOTER.

The RANTES promoter contains binding elements for transcription factors NFκB and IRF-3 [276, 428]. IRF-3 has been shown to bind to the ISRE domain in the RANTES promoter to induce RANTES expression during viral infection [276]. We wanted to analyze the ability of NS5 and replicon to activate the interferon-stimulated regulatory element (ISRE), as well as RANTES and IL-8 promoters (Figure V-3). As previously shown, overexpression of the IKK-related kinase TBK1 induced transcription from both the ISRE and RANTES promoters (positive control). Similarly, overexpression of Mal, the TLR4 adaptor molecule, induced transcription from the IL-8 promoter (positive control). NS5 and the DEN2V replicon induced transcription from the IL-8 promoter. However, neither NS5 nor the dengue replicon activated ISRE or induced the RANTES promoter.
Figure V-3. NS5 and DEN2V replicon do not activate the ISRE or RANTES promoters. HEK293A cells were transfected with a vector containing a reporter gene for ISRE, RANTES or IL-8 promoters and cotransfected with an expression vector for NS5 or replicon. After 24 hrs, luciferase reporter gene activity was measured and data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to cells transfected with pcDNA3.1 for each construct. There is a statistically significant difference (*P* ≤ 0.01) between NS5 (100 ng) or the replicon (100 ng) and pcDNA3.1 transfected cells for IL-8 reporters using the student t-test. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate.
C. ACTIVATION OF TRANSCRIPTION FACTORS BY DEN2V NS5 AND REPLICON.

The transcription factors involved in IL-8 mRNA induction include c/EBP, AP-1 and NFκB [251]. To identify the transcription factors that are induced by NS5 or the replicon, we used luciferase reporter constructs with promoters that contain multiple copies of binding sites for c/EBP, AP-1 or NFκB. NS5 induced c/EBP driven transcription 7-fold and AP-1 5-fold; NFκB was induced less than 2-fold over the control plasmid (Figure V-4). The replicon had a similar profile in the induction of c/EBP (7 fold), AP-1 (3 fold) and NFκB (less than 2). These results suggest that c/EBP and AP-1 are activated by expression of NS5 or the replicon.

However, as previously mentioned in CHAPTER IV, there is cooperativity among transcription factors within the IL-8 promoter. Therefore, the activation of transcription factors can have a significant effect on neighboring transcription factors, resulting in enhanced IL-8 expression. To further assess the importance of each of these transcription factors for the induction of IL-8 by NS5, reporter constructs containing mutations in the IL-8 promoter binding sites for c/EBP (mtc/EBP), AP-1 (mtAP-1) or NFκB (mtNFκB) [416] were studied. As shown in figure V-5, a mutation in the c/EBP binding site within the IL-8 promoter reduced activation by NS5 expression ~50% when compared with the wild type (wt) IL-8 promoter. A mutation in the NFκB binding site had minimal effect (~20 % reduction) on the induction of IL-8 transcription by NS5 when compared to the wt IL-8 promoter. However, a mutation in the AP-1 binding site enhanced NS5 driven transcription from the IL-8 promoter by ~20%. These results suggest that c/EBP is a
dominant component in the induction of the IL-8 promoter by NS5. In contrast, AP-1 did not have a predominant effect on IL-8 induction by NS5 and may even be inhibitory.

It has previously been reported that NS5 contains a nuclear localization signal and localizes to the nucleus during the later stages of DEN2V infection [119]. We wanted to assess whether the NS5 in this study localized to the nucleus at the time of IL-8 induction. NS3, on the other hand, is not known to localize to the nucleus and was used as a negative control. We used constructs that expressed either NS3 or NS5 with a GFP tag. As seen in figure V-6, NS5 localized to the nucleus at 24 hours post transfection, whereas NS3 remained in the cytoplasm. It is thought that binding of NS3 to NS5 as part of the replication complex retains NS5 in the cytoplasm. Upon hyperphosphorylation of NS5, NS3 is released exposing the nuclear localization signal on NS5 and allowing NS5 to localize to the nucleus [119]. Since the NS5 expression plasmid was not cotransfected into cells with other components of the replication complex, it is possible that the nuclear localization signal within NS5 is able to bind the the importin receptors immediately. Thus, no cytoplasmic NS5 was seen by confocal microscopy in this experiment as has been seen in cells infected with DEN2V.
Figure V-4. Transcription factors activated by NS5 and DEN2V replicon. HEK293A cells were cotransfected with vector encoding a reporter gene for IL-8, c/EBP, AP-1, or NFκB and an expression vector for NS5 or DEN2V replicon. After 24 hours, luciferase gene activity was measured. Data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to cells transfected with pcDNA3.1 for each construct. There is a statistically significant difference (*P* ≤ 0.05) between NS5 (100 ng) or the replicon and pcDNA3.1 transfected cells for IL-8, c/EBP and AP-1 reporters using the student t-test. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate.
Figure V-5. Effect of NS5 expression on transcription from mutated IL-8 promoter reporter constructs. HEK293A cells were cotransfected with vector encoding a reporter gene for wild type, mtc/EBP, mtAP-1, and mtNFKB IL-8 promoters and an expression vector for NS5. After 24 hours, luciferase gene activity was measured. Data are normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to cells transfected with pcDNA3.1 for each construct. Mtc/EBP, mtAP-1, and mtNFKB represent the 133-luc IL-8 reporter plasmid with point mutations in the c/EBP, AP-1, and NFkB binding sites. There is a statistically
significant difference \((P \leq 0.05)\) between NS8 (80 ng) and pcDNA3.1 transfected cells for IL-8, mtc/EBP, mtAP-1 and mtNFkB reporters using the student t-test. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate.
Figure V-6. Cellular localization of DEN2V NS3 and NS5 proteins. HEK293A cells were transfected with plasmids expressing GFP-tagged NS3 (A-C) or NS5 (D-G) proteins. Cells were fixed on a glass coverslip with ethanol and nuclear structures were
stained with DAPI. A and D show DAPI staining (blue), B and E show GFP expression (green), and C and G show the overlay of DAPI stain and GFP expression for NS3 and NS5 transfected HEK293A cells, respectively. The center image, F, is the cell seen in phase contrast microscopy.
D. ANALYSIS OF TRANSCRIPTION FACTORS INDUCED BY EXPRESSION OF DEN2V PRM-E.

We did not detect induction of IL-8 expression in cells transfected with the DEN2V prM or DEN2V E proteins separately (Figure V-2A). We considered the possibility that expression of both proteins together, which leads to the formation of virus-like particles [86], might have effects on chemokine expression not seen with either protein alone. Therefore, we transfected HEK293A cells with a plasmid containing the DEN2V prM-E genome segment. DEN2V E protein was detected in both cell lysates and supernatants of HEK293A cells transfected with the prM-E expression plasmid by western blot (Figure V-7). Since the E protein is not generally secreted from the cell, detection of E in the supernatants suggests that viral-like particles were released from the cell. As mentioned in the section D: Virus Structure and Composition, the E protein is a glycoprotein. The double band seen in figure V-7 in the prM-E lysates may be due to different levels glycosylation of E.

Transfection of prM-E induced IL-8 secretion in HEK293A cells but was not significantly different than media (data not shown). To analyze the effect prM-E expression would have on IL-8 transcription, we cotransfected the prM, E and prM-E expression plasmids into HEK293A cells along with the IL-8 reporter construct. The expression of prM-E activated transcription from the IL-8 promoter 2 fold over prM or E transfected HEK293A cells (Figure V-8). To characterize the effect of prM-E expression on activation of transcription factors constructs, the prM-E expression plasmid was cotransfected with reporters containing tandem repeat binding sites for the transcription
factors c/EBP, AP-1 and NFκB. The c/EBP, AP-1 and NFκB luciferase reporters were activated approximately 2, 2.5 and 3 fold over the control, respectively (Figure V-9).

To analyze the importance of c/EBP, AP-1 and NFκB within the context of the IL-8 promoter, we cotransfected the prM-E expression plasmid with mutant IL-8 promoter constructs used in previous experiments. Analysis of mutant IL-8 promoters showed a slight decrease in IL-8 transcription by prM-E expression when the c/EBP site is mutated (Figure V-10). In addition, activation of IL-8 transcription by prM-E expression was not significantly different in the IL-8 reporter construct containing a mutation in the AP-1 binding site when compared to the wt IL-8 promoter. In contrast, the mtNFκB reporter had a ~50% increase in luciferase activity when compared with the wt IL-8 promoter suggesting an inhibitory effect for NFκB. This suggests that NFκB may have an inhibitory role in transcription from the IL-8 promoter. Overall, these results show a role for c/EBP, AP-1 and NFκB in the induction of IL-8 by prM-E expression.

It is important to note that the level of activation from the wt and mutant IL-8 reporters and the c/EBP, AP-1 and NFκB reporters with the prM-E expression plasmid was lower than what was seen with NS5 expression plasmid. This could be due to a lower level prM-E expression from the plasmid or a lower amount of prM-E that is retained in the cell. As seen in figure V-7, prM-E is released into the supernatants of transfected cells by 24 hours post transfection, which is when transcription from the IL-8 promoter is activated.

We wanted to know whether formation of virus-like particles could also induce the transcription from promoters containing an ISRE site. We cotransfected the prM-E expression plasmid with the IP10 or RANTES promoter constructs or an ISRE driven
promoter. We found that prM-E expression did not induce any constructs containing an ISRE binding site (Figure V-11). These results suggest that virus-like particle formation and release did not induce transcription factors such as IRFs that bind and activate transcription through the ISRE.
Figure V-7. Detection of E expression by western blot. Vero cells were infected with DEN2V at an MOI of 1 and cells were harvested 48 hours post infection. HEK293 cells were transfected with the prM-E expression plasmid. After 24 hours, supernatants and cells were harvested for western blot analysis. The E protein was detected using the 3H5 antibody as described in MATERIALS AND METHODS.
Figure V-8. **prM-E induces transcription from the IL-8 promoter.** HEK293A cells were cotransfected with a vector encoding a reporter gene for IL-8 and plasmids expressing DEN2V prM, E or prM-E. After 24 hours, luciferase gene activity was measured. Data are normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to cells transfected with pcDNA3.1 for each construct. There is a statistically significant difference for fold induction (*P* < 0.01) of luciferase activity between prM-E and pcDNA3.1 transfected cells using the paired student t-test. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate.
**Figure V-9. Transcription factors activated by prM-E.** HEK293A cells were cotransfected with vector encoding a reporter gene for IL-8, c/EBP, AP-1, or NFκB and the plasmid expressing DEN2V prM-E. After 24 hours, luciferase gene activity was measured. Data are normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to cells transfected with pcDNA3.1 for each construct. There is a statistically significant difference for fold induction ($P < 0.05$) of luciferase activity between prM-E and pcDNA3.1 transfected cells for AP-1 and NFκB reporters using the paired student t-test. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate.
Figure V-10. Effect of prM-E expression on transcription from mutated IL-8 promoter reporter constructs. HEK293A cells were cotransfected with vector encoding a reporter gene for IL-8, mtc/EBP, mtAP-1, and mtNFkB IL-8 promoters and an expression vector for DEN2V prM-E. After 24 hours, luciferase gene activity was measured. Data are normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to cells transfected with pcDNA3.1 for each construct. Mtc/EBP, mtAP-1, and mtNFkB represent the 133-luc IL-8 promoter plasmid with point mutations in the c/EBP, AP-1, and NFkB binding sites. There is a statistically significant difference for fold induction (*P* < 0.02) of luciferase activity between prM-E and pcDNA3.1 transfected cells for mtc/EBP, mtAP-1 and
mtNFκB reporters using the paired student t-test. In addition, there is a statistical significant for fold induction \((P < 0.04)\) of luciferase activity for prM-E transfected cells between wt IL-8 and mtc/EBP or mtNFκB reporters. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate.
Figure V-11. prM-E does not activate the ISRE, RANTES or IP10 promoters.

HEK293A cells were cotransfected with a vector containing a reporter gene for ISRE, RANTES or IP10 and the prM-E expression plasmid. After 24 hours, luciferase reporter gene activity was measured. Data were normalized for transfection efficiency based on *R. reniformis* luciferase activity and expressed as fold induction relative to cells transfected with pcDNA3.1 for each construct. The data are expressed as mean ± standard deviation from a representative experiment for a minimum of two separate experiments, each done in triplicate.
CHAPTER VI:
DISCUSSION

A. CHEMOKINE PROFILE OF DENGUE INFECTED CELL LINES AND PRIMARY CELLS

Our results demonstrate that dengue virus infection of diverse human cell lines can induce the production of multiple chemokines in vitro. This finding is similar to previous results from other investigators [4, 6, 396, 398, 401]. However, our study indicates that DEN2V infection can induce a unique profile of chemokine expression in each cell type tested. The differences in chemokine expression in different cell types may be partly explained by variable types of transcription factors that are expressed [429-431]. The chemokine expression peaked late, three to five days after dengue virus infection, in all the continuous cell lines we tested (Table III-1). Possible explanations for the delay in chemokine expression could be a need for the virus to reach a threshold of viral load to induce chemokines, a need for transcription factors involved in chemokine induction to be induced, or a time delay in viral protein translocation into the nucleus (discussed further below).

A similar pattern of late chemokine induction was reported in DENV infection of primary monocytes/macrophages, which showed peak levels at least 48 hours post infection [398]. In contrast, we have shown that dendritic cells expressed IL-8, RANTES, MCP-1, MIP-1α and MIP-1β as early as 24 hours post infection (Tables III-2-6). The earlier chemokine production in dendritic cells could reflect their role as sentinels of the immune system [431].
We also showed an increase in steady-state chemokine mRNA levels over time during DEN2V infection of cell lines (Figure III-2). Although we have not excluded the possibility that increased chemokine secretion is due to mRNA stabilization, as has been reported with respiratory syncytial virus-induced RANTES expression or adenovirus-induced IL-8 expression [432, 433], we have shown that transcription from the IL-8 promoter was induced by DEN2V infection.

B. ROLE OF TRANSCRIPTION FACTORS IN CHEMOKINE INDUCTION DURING DEN2V INFECTION

DEN2V infection of HEK293A cells induced NFκB-driven transcription (Figure III-11). Interestingly, NFκB was not activated in HepG2 cells by DEN2V infection. We were able to induce NFκB using PMA and ionomycin treatment in HepG2 cells, suggesting that these cells are not deficient in NFκB. Therefore the results suggest that DEN2V does not activate NFκB in HepG2 cells. Similarly, a recent paper looked at the activation of NFκB by analyzing levels of IκB Ser32 phosphorylation in another hepatic cell line, Huh-7, infected with DENV [427]. The authors found no IκB phosphorylation in Huh-7 cells infected with DENV at 48 hours post infection. These results suggest that NFκB is not activated in liver cells infected with DENV.

In contrast, this study shows that transcription from the AP-1 promoter was induced in DEN2V-infected HepG2 cells (Figure III-11). AP-1 activation was also seen in DENV infection of Huh-7 cells [427]. Studies have shown that AP-1 can interact with NFκB or c/EBP to enhance transcription of genes such as TNFα and IL-8 [426, 434]. Therefore, AP-1 activation could cooperatively interact with c/EBP to enhance
transcription factor binding to the chemokine promoter and induce chemokines in DENV infected HepG2 cells without activation of NFκB.

These findings with DENV infection of hepatic cell lines can be compared with the results of similar studies with HCV, a distantly related member of the family Flaviviridae. HCV coinfection is found in approximately one third of HIV-infected individuals in the United States [435], and these co-infected patients suffer from accelerated HCV disease progression [436]. An analysis was done of IL-8 secretion in HepG2 cells costimulated with HCV E2 and HIV gp120 to simulate coinfection of HIV and HCV in vivo [437]. These authors found that IL-8 production was induced through an NFκB independent mechanism. Additionally, AP-1 binding to DNA was enhanced. Another study found that expression of the HCV core protein activated AP-1, NFκB, RANTES and MCP-1 reporters in HeLa cells but inhibited transcription from these reporters in Huh-7 and HepG2 cells, suggesting that there are differences in the transcriptional response to viral proteins in various cell types [438]. In contrast to chronic infection with HCV, liver involvement during DENV infection occurs during the febrile period and is transient [143]. However, DENV shows many similarities to HCV in the induction of chemokines using viral proteins and may use similar pathways for induction of IL-8 in hepatic cells that do not require NFκB.

It has been established that TNF can induce IL-8 through an NFκB-dependent pathway in many cell types including liver cells [439]. However, a study looking at the effects of a proteasome inhibitor (MG132) on TNF induced IL-8 production in HepG2 cells and fetal hepatocytes found an increase in IL-8 protein and mRNA levels that occurred independent of NFκB [440]. The increase in IL-8 correlated with enhancement
of AP-1 binding to the DNA, an increase in JNK activation and inhibition of NFκB.

Another study found that ligation through the Fcγ receptor on Thp-1 cells induced IL-8, MIP-1α and MIP-1β [441]. This effect occurred mainly through activation of c/EBP.

These results indicate that IL-8 induction can occur by other pathways than NFκB activation such as c/EBP and/or AP-1 activation.

C. PHARMACOLOGICAL INHIBITORS

NFκB plays a predominant role in chemokine and cytokine induction in many experimental systems [304]. Similarly, studies reported that NFκB played a major role in DENV induced IL-8 production [4, 6] and our data in HEK293A cells yielded similar conclusions. We, therefore, wanted to assess the effects of pharmacological drugs that have been shown to inhibit NFκB and cytokines on IL-8 and RANTES production during DEN2V infection. The results from these studies were surprising. In a number of cases drug treatment enhanced IL-8 and RANTES secretion during DENV infection (e.g. dexamethasone treatment in HEK293A cells or SB203580 and rolipram treatment in HepG2 cells, Figures IV-1 and IV-5, respectively).

In HEK293A cells, all of the drugs tested, with the exception of dexamethasone, inhibited IL-8 and RANTES secretion (Figure IV-1). Interestingly, dexamethasone enhanced secretion of IL-8 and RANTES on day 3 and 4 post infection. In contrast, transcription from the IL-8 and RANTES promoter was inhibited when compared to untreated DEN2V infected HEK293A cells (Figure IV-2). Dexamethasone has been reported to inhibit both the JNK pathway and NFκB [362, 442]. Our results also showed an inhibition of NFκB activation in dexamethasone treated HEK293A cells infected with
DEN2V when compared to untreated DEN2V-infected cells. In contrast, transcription from the AP-1 and c/EBP promoters was unaffected in DEN2V-infected HEK293A cells treated with dexamethasone. These results suggest that the increase in IL-8 secretion in DEN2V-infected HEK293A cells treated with dexamethasone may be due to an increase in mRNA stability. An analysis of mRNA decay in the presence of actinomycin D could show whether the half life of chemokine mRNA has changed with dexamethasone treatment in comparison with untreated DEN2V infected cells.

Although SB203580 and rolipram enhanced IL-8 and RANTES secretion by day 6 post infection in HepG2 cells, they appeared to use different pathways to achieve this effect. The p38 MAPK pathway, inhibited by SB203580, has been implicated in increasing mRNA stability [304]. Therefore, inhibiting p38 MAPK would be expected to decrease the half life of IL-8 and RANTES mRNAs resulting in a decrease in protein levels. However, we found an increase in IL-8 and RANTES secretion. In addition, we found an increase in IL-8 transcription by day 3 post infection with treatment of SB203580 in DEN2V-infected HepG2 cells (Figure IV-2). This indicates that the increase in protein production in DEN2V-infected HepG2 cells treated with SB203580 was likely due to an increase in the transcription of IL-8.

Rolipram has been found to specifically inhibit PDEIV, which results in an increase in intracellular levels of cAMP [443]. High levels of cAMP in the cell can activate many pathways including induction of protein kinase A (PKA) and cAMP response elements (CRE) within promoters [443]. CRE-binding proteins have been found to play an important role in the physiology of the pituitary gland, in regulating spermatogenesis, in the response to circadian rhythms, and in the molecular basis of
memory [444]. Therefore, rolipram may have a more global effect on cell function, and it is, therefore, more difficult to delineate individual pathways involved in the induction of chemokines. A previous study showed that treatment of HIV-infected T cells with rolipram decreased TNFα and IL-10 production [359]. The authors attributed the cytokine suppression to the inhibition of NFκB and NFAT. The results from our study show that treatment with rolipram enhanced IL-8 and RANTES protein expression in DEN2V-infected HepG2 cells but not in HEK293A cells. In contrast, transcription from the IL-8 promoter was inhibited by rolipram (Figure IV-7). There was little difference in the activation of c/EBP, AP-1 or NFκB reporters between rolipram treatment of DEN2V infected HepG2 cells and untreated cells (Figure IV-8). These results suggest that post-transcriptional mechanisms, e.g. mRNA stability or translation, are responsible for the increase of chemokine protein levels.

The results of our experiments with pharmacological inhibitors in DEN2V infected HepG2 and HEK293A cells raise questions about how these drugs would potentially affect proinflammatory chemokines over an extended period of time. Most other studies using the pharmacological inhibitors tested here have been performed over relatively short term incubation times (< 24 hours). Although these drugs may inhibit certain pathways initially, the results from the present study indicate that prolonged use during DENV infection may lead to the activation of pathways that could increase the inflammatory response via chemokine induction in certain tissues such as the liver. If suppression of DENV-induced chemokine secretion is a desirable goal of therapy in DENV infections, and if our results with continuous cell lines are representative of the effects that could be expected in vivo, then treatment of dengue patients with the drugs
tested in this study could have detrimental consequences and perhaps enhance disease severity.

D. NS5 AND IL-8 INDUCTION

The observation that individual proteins of HCV, HIV and other viruses can induce chemokine production led us to study whether individual DEN2V proteins have a similar function. Our findings of IL-8 induction by NS5 reveal a novel mechanism for induction of chemokine production by DEN2V. The DENV NS5 is a large multifunctional protein containing an S-adenosyl-methionine(SAM)transferase domain in the N-terminal region [445] and a RNA dependent RNA polymerase domain in the C-terminal region [113, 446]. NS5 has been shown to be differentially phosphorylated at serine and threonine residues and located in both the cytosol and the nucleus [119, 447, 448]. Two nuclear localization signals, bNLS and a/bNLS, have been found in the interdomain region of NS5, and are recognized by importin b and a/b, respectively [449, 450]. The bNLS region of NS5 was found to overlap with the binding site of NS3. In the late stage of infection, NS5 becomes hyperphosphorylated and it is thought to dissociate from NS3, exposing the bNLS [119], [450]. The function of nuclear localized NS5 has not been identified. However, the timing of IL-8 secretion late in infection correlates with movement of NS5 to the nucleus [119]. It is possible that nuclear NS5 can bind directly or indirectly to the IL-8 promoter to induce gene expression. Chromatin immunoprecipitation (ChIP) may be useful in determining whether NS5 can interact directly with the IL-8 promoter.

It has been shown that different strains of DEN2V (e.g. American versus Asian genotypes) are associated with varying degrees of disease severity [29]. Previous
research compared sequences of the ‘Asian’ genotype of DEN2V, which is associated with DHF, and the ‘American’ genotype, which appears to be incapable of causing DHF [451]. Five of the eight consistent amino acid differences between these two genotypes in the nonstructural proteins were located in the N terminus of NS5. We constructed the NS5 expression plasmid from the infectious cDNA clone of Polo et al [419], derived from the DEN2V strain New Guinea C, a member of the ‘Asian’ genotype. It will be interesting to determine whether sequence differences within NS5 could change the ability of the protein to induce IL-8.

E. EFFECTS OF VIRAL REPLICATION AND VIRAL-LIKE PARTICLES ON GENE EXPRESSION

We also found that expression of the DEN2V prM-E induced IL-8 secretion and transcription. Our analysis of prM-E indicated that activation of c/EBP, AP-1 and NFκB were equally important for IL-8 induction (Figure V-10). This pattern was different from that seen with expression of NS5 or the DEN2V replicon. These results suggest that packaging of the virus may be one of the DENV-induced pathways that require NFκB activation to induce IL-8 expression.

RANTES gene expression requires binding of IRFs to the ISRE element along with activation of c/EBP, AP-1 and NFκB [276]. DEN2V infection induced activation of the ISRE element whereas expression of NS5, prM-E or the replicon did not. IRFs are important in regulating expression of Type I IFN genes, IFN-stimulated genes (ISGs) and other cytokines and chemokines such as RANTES and IP10 [452]. Reports have shown that replication of RNA viruses generates dsRNA intermediates that are recognized by TLR3 and non TLR3 mechanisms [453]. TLR3 activation results in nuclear localization
Recently, Collins et al reported that enveloped RNA viruses (Newcastle Disease virus and Sendai virus) were able to induce IFN stimulated genes (ISGs) such as ISG56 and IP10 without viral replication [455]. In our study, the RANTES and IP10 promoters were not induced by DEN2V prM-E expression or the replicon but induction did occur with DEN2V infection. In addition, UV-inactivated DEN2V did not induce IP10 or RANTES induction. These results suggest that a different stage of the DENV life cycle other than replication and/or translation of the genome or virion packaging and release are involved in activation of ISGs during DENV infection. Alternatively, both viral entry and replication may be required to induce genes containing ISRE elements during DENV infection.

F. MODEL OF IL-8 INDUCTION BY DEN2V

Expression of the core and NS5A proteins of HCV were able to activate transcription from the IL-8 promoter [9, 10]. Deletion experiments found that the C terminus region of NS5A, which contains the NLS, was responsible for the induction of IL-8. Polyak et al also found that NS5A activated IL-8 transcription through AP-1 and NFκB, whereas, the c/EBP site was inhibitory [9]. Qadri et al reported that HCV NS5A induction of AP-1 occurred through an increase in the expression of c-jun subunits while there was no effect on c-fos protein levels [456]. Similarly, the induction of AP-1 has been reported for other viruses. HIV Tat protein can induce c-Jun through an oxidant dependent mechanism [457] and hepatitis B protein HBx can induce AP-1 through the ERK and JNK pathway [458]. The present study has shown that several steps in the DEN2V viral life cycle - RNA replication, production of virus particles and the expression of individual viral proteins - activate a unique profile of transcription factors.
that can induce the expression of IL-8 by a DEN2V-infected cell. The NS5 protein can induce IL-8 by c/EBP and AP-1 activation. In addition, production of viral like particles can induce IL-8 mainly through NFκB and AP-1. DEN2V infection can induce IL-8 mainly through activation of NFκB and AP-1. These multiple pathways for the induction of IL-8 can ensure that IL-8 is expressed throughout the viral life cycle (Figure VI-1).

Previous studies had reported that NFκB plays a major role in DENV-induced IL-8 production [4, 6]. However, the inhibitors that enhanced IL-8 and RANTES protein levels in the supernatants of DEN2V-infected cells activated AP-1 and possibly c/EBP driven transcription. NFκB activation was not enhanced when compared to untreated DEN2V-infected cells in these same conditions suggesting that it has a minor role in IL-8 and RANTES induction with drug treatment. As noted earlier, this temporal pattern of IL-8 induction correlates with the movement of NS5 into the nucleus. In addition, the predominant role of AP-1 and possibly c/EBP in IL-8 induction by DEN2V in the presence of rolipram, SB203580 and dexamethasone was also seen when HEK293A cells were transfected with NS5. It is possible that nuclear NS5 plays a role in the increase in IL-8 expression in the presence of these drugs by activating c/EBP and AP-1.

G. IL-8 EFFECTS ON INNATE IMMUNITY

IL-8 induction has been found to counteract the antiviral effects of IFNα and enhance viral replication of many viruses including EMCV, poliovirus, CMV and HIV [8, 9, 459, 460]. HCV core protein, E1 and NS5A protein have been shown to counteract the antiviral IFN response [9, 461]. One of these mechanisms is the induction of IL-8 by the NS5A protein [9]. The induction of IL-8 by NS5A inhibited the effect of IFNα on EMCV viral replication.
DENV proteins have recently shown similar effects on innate immunity as seen with HCV. DENV NS4B and, to a lesser extent, NS2A and NS4A, inhibited IFN-induced signal-transduction by interfering with STAT1 function in a monkey kidney cell line [111]. Another study using a myeloid cell line (K562) that stably expresses the dengue replicon found that DENV nonstructural proteins reduced intracellular STAT2 levels [230]. Our data indicate an indirect mechanism to counter antiviral effects of IFNα through the induction of IL-8 by NS5. IL-8 can inhibit the induction of an antiviral state by IFNα in neighboring uninfected cells, thereby allowing viral progeny to infect these cells [8]. DENV may use this mechanism to counteract antiviral effects of innate immunity in conjunction with previous reported pathways, thus allowing further dissemination of the virus in neighboring uninfected cells.
Figure VI-1. Model for IL-8 induction by DEN2V and its effect on virus propagation. Multiple pathways for IL-8 expression are induced during DEN2V infection (left). The NS5 protein can induce IL-8 by c/EBP and AP-1 activation, whereas production of viral like particles (prM-E) can induce IL-8 mainly through NFκB and AP-1. DENV infection induces IL-8 mainly through activation of NFκB and AP-1. Binding of IL-8 to its receptor activates signaling through G proteins, Gα and Gβγ (right). Activation of IL-8 receptor signaling inhibits activation of the Jak/Stat pathway and thus, counteracts the antiviral effects of IFNα. The inhibition of the IFNα pathway creates a less hostile environment for viral infection, which will facilitate infection of neighboring cells with progeny virus.
H. IL-8 EFFECTS DURING SECONDARY DENV INFECTIONS AND DISEASE PATHOGENESIS

IL-8 has been shown to be elevated in sera and PBMC of patients with more severe dengue disease, DHF [394, 395]. In addition, we have shown that the mean levels of IL-8 mRNA in PBMC are higher later during DENV infection, although there were no statistically significant differences between DF and DHF patients. This may be due to the lack of higher mean viremia in the DHF patients compared to DF patients in this sample of subjects [199].

IL-8 can be induced by cytokines, which include IL-1 and TNF [251]. TNFα and IL-1β are induced in DF and DHF patients and may induce low levels of IL-8 [51, 67]. Recently, a study showed that stable transfections with HCV NS5A and core protein induced RANTES in Chang cells, a liver cell line [462]. When these HCV protein-transfected cells were stimulated with IL-1β, TNFα and IFNγ, they induced significant levels of levels of IP10 and Mig protein and mRNA when compared to mock transfected cells. By analogy, stimulation of DEN2V-infected cells by cytokines in vivo could further enhance the induction of chemokines such as IL-8 by NS5 or other viral proteins and contribute to the pathogenesis of DEN2V infection.

We have shown that NS5 can induce activation of c/EBP and that c/EBP plays an important role in the induction of IL-8. c/EBP was originally identified as a DNA-binding protein responsible for IL-1-stimulated IL-6 induction [309]. The consensus binding site for c/EBP has been found in promoter regions of several proteins involved in acute phase reactions, which includes IL-8 and IL-6 [309]. It would be interesting to
determine if NS5 could induce IL-6 expression through the c/EBP consensus site in addition to IL-8.

A recent paper has shown that IL-8 can alter the cytoskeleton and tight junctions of microvascular endothelium and change the permeability of the endothelial monolayer [5]. Moreover, elevated levels of IL-6 can also increase permeability of the endothelial barrier \textit{in vitro} [3, 5]. The combined expression of IL-6 and IL-8 due to activation c/EBP by DEN2V NS5 may increase the likelihood of plasma leakage in DENV infected patients.

Primary DENV infections can induce T cells that respond to antigens specific to the infecting serotype as well as T cells that respond to heterologous serotypes [58]. During secondary DENV infections, these memory T cells would have a lower threshold for activation than naïve T cells and the potential to respond more rapidly. This effector memory T cell response to the secondary DENV infection is thought to produce inflammatory cytokines that can induce plasma leakage [463].

Recently, the receptor for IL-8, CXCR1, was shown to be expressed on terminally differentiated effector memory CD8+ T cells, which express high levels of IFN\(\gamma\) and cytotoxic molecules upon activation [464, 465]. Incorporating this finding to the laboratory’s model on DHF pathogenesis that was mentioned previously, it is possible that expression of IL-8 from infected cells can recruit DENV-specific memory CD8+ T cells that express CXCR1 to the sites of infection. These T cells would rapidly produce inflammatory cytokines and thus, increase the chances of more severe dengue disease (Figure VI-2).
Previously, it has been reported that DHF patients tend to have a higher viral burden early in infection which may correlate with increased NS5 expression [144, 466]. The increase in NS5 may elevate levels of IL-8 and contribute to the pathogenesis seen in patients with DHF.

Overall, IL-8 is a chemoattractant for cells expressing CXCR1 such as neutrophils and cytotoxic memory CD8+ T cells. IL-8 has been shown to increase the permeability of an endothelial monolayer directly. Both of these mechanisms may play a role in plasma leakage that occurs in DHF. Furthermore, IL-8 can subvert the innate immune defense by inhibiting the antiviral effects of IFNα, thereby, potentially enhancing viral dissemination. Through a combination of these effects, enhanced IL-8 production by DENV may increase the likelihood of more severe disease particularly during secondary DENV infection.
Figure VI-2. Modified model for DHF pathogenesis. This is a model of a previous figure (Figure I-3) showing effects of IL-8 on DENV pathogenesis. Production of IL-8 from DENV-infected cells can directly increase the permeability of the vascular endothelium (orange arrow). In addition, DENV-specific CD8+ T cells that express CXCR1 can be recruited to the site of infection by IL-8 (green arrow). These mechanisms can increase the pathogenesis during DENV infection.
I. SUMMARY

The results described in this thesis have provided a better understanding of the mechanisms by which DENV induces chemokines. The important observations made in this thesis can be summarized as follows:

1. DEN2V can infect many cell lines including liver cells, kidney cells, monocytic cells, endothelial cells and as well as monocyte-derived dendritic cells in vitro.

2. DEN2V infection can induce unique chemokine profiles for each cell type.

3. DEN2V can activate different transcription factors for HepG2 cells and HEK293A cells.

4. The use of pharmacological inhibitors of cytokine production on DEN2V infected cells can paradoxically enhance the production of chemokines depending on the cell type infected.

5. DEN2V protein NS5 can induce expression of IL-8 through activation of c/EBP, and AP-1.

6. Production of DEN2V virus-like particles and replication and/or translation of nonstructural proteins can also induce IL-8 expression.
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