

**Activation of TNF  $\alpha$ , IL1- $\beta$  and Type-I IFN Pathways in human  
umbilical vein endothelial cells During Dengue 2 Virus Infection**

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By

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## ABSTRACT

Differential Display technique was used for gene profiling in transformed human umbilical vein endothelial cell line (ECV 304) and primary human umbilical vein endothelial cells (HUVECs) to study the cellular response to viral infection. After screening the mRNA from uninfected and infected HUVECs and ECV 304 cells with 16 different random primers we identified 8 gene targets. These genes included the human inhibitor of apoptosis-1 (h-IAP1), 2-5 oligoadenylate synthetase (2-5 OAS), 2-5 oligoadenylate synthetase –like (2-5 OAS-like), Galectin-9 (Gal-9), MxA, Mx1, Regulator of G-protein signaling (RGS2) and endothelial and smooth muscle cell-derived neuropilin-like protein (ESDN). We have found that HUVECs were a better model to study gene expression during dengue 2 virus infection but not the transformed cell lines, ECV 304. Of the 41 primer combinations utilized in ECV 304 cells detected only one up-regulated gene, h-IAP1 and 8 out of the 16 primer combinations tried for HUVECs. We hypothesize the activation of two novel signaling pathways (Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), Interleukin1- $\beta$  (IL1- $\beta$ ) in endothelial cells during D2V infection. Also, our data detected genes that are activated in the Type-I IFN (IFN- $\alpha/\beta$ ) signaling pathway during dengue 2 virus infection in HUVEC.

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## BACKGROUND

### Dengue Virus

#### *Virus Taxonomy*

Dengue virus belongs to the family *Flaviviridae*, genus *Flavivirus* which includes over 60 known pathogens for humans. These include Yellow Fever Virus ('flavi' derived from Latin for yellow), Japanese Encephalitis Virus, a major cause of encephalitis in Asia, Tick-Borne Encephalitis Virus, which is present in central and eastern Europe, and West Nile Virus, which has attracted attention because of its recent introduction into the Western Hemisphere. However, dengue virus (DV) is the most important flavivirus from the standpoint of worldwide morbidity and mortality (Rothman and Ennis, 2000). The term dengue probably means prudishness or affection, which refers to "the particular tortuous, affected gait, which patients adopt in consequence of the pain" (Burke, 1988).

#### *History*

Tracing the history of dengue has been difficult because the clinical features of classic dengue fever are not pathognomonic. One of the earliest descriptions of an outbreak compatible with classical dengue fever (DF) is that of (Rush, 1789) who saw patients with this syndrome in Philadelphia, USA in 1780. But descriptions of dengue like illnesses have been found as far back as the tenth century A.D. (Hayes and Gubler, 1992). Graham first demonstrated that dengue fever was transmissible by mosquitoes in 1903. The period from 1960 to the present has been characterized as the "twentieth-century pandemic of dengue" (Halstead, 1992) with constant simultaneous circulation of multiple dengue virus serotypes. Dengue hemorrhagic fever (DHF) was first recognized in Manila in 1956 (Rothman and Ennis, 2000).

### *Types of Dengue Virus*

Dengue virus is classified into four different serotypes namely, type 1, 2, 3 and 4. Infection from any of the four serotypes of dengue virus can result in dengue fever or dengue hemorrhagic fever when heterologous infection has occurred. Within the dengue virus complex, sequence homology is in the range of 65% to 70% between viruses of different serotypes, and >90% between viruses within the same serotype (Burke, 1988; Rothman and Ennis, 2000).

### *Genome, Structure and Morphology*

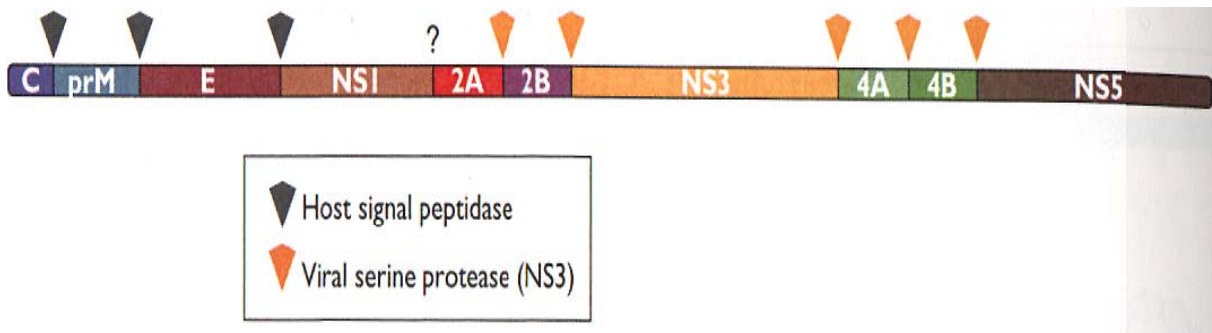
The dengue virion is a sphere with a diameter of 48 to 52 nanometers. The genome is 11,000 base pairs in length. The dengue genomic RNA is translated into a continuous single polyprotein, which is cleaved into individual proteins. Dengue virus encodes 3 structural and 7 non-structural proteins. The 3 structural proteins are located at the 5' end of the reading frame and 7 non-structural proteins are located at the 3' end. The virus replicates by translating into a polyprotein initially, which is cleaved into individual proteins by using the host proteases along with NS3 which encodes the viral protease (Fig. 1). It is a positive strand RNA virus that is 11kb in length. The virus preferentially infects macrophages, endothelial, epithelial and hepatic cells.

Among the structural proteins, the virus encodes 2 different proteins, a 58-kDa to 60-kDa glycoprotein (E) and an 8-kDa protein (M); E is the major envelope protein and M the membrane protein. A 22-kDa protein called the pre-membrane protein (pre-M) is processed during maturation to form M. The 3<sup>rd</sup> protein that the virus codes is the capsid

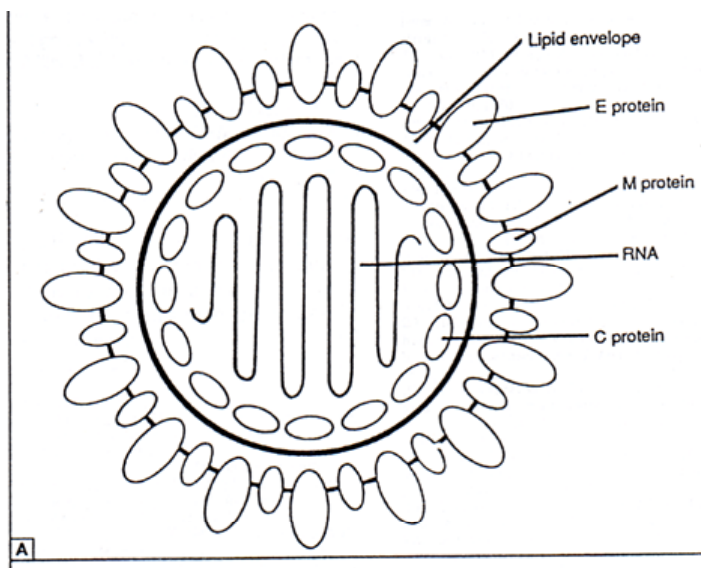


(C) protein, which associates with the viral nucleic acid. The non-structural proteins are NS1, 2A, 2B, 3,4A, 4B and NS5. NS5 codes for the viral RNA-dependent RNA polymerase responsible for the replication of the genome through a negative-stranded RNA intermediate. NS3 is the viral protease that along with the host proteases cleaves the long single polypeptide after it is translated. NS1 is essential for viral replication (Fig. 2) (Fields, 1996).

## Dengue Virus Genome Map



**Fig. 1** Map of dengue virus genome. The structural proteins (C, prM and E are encoded to the 5' end and the non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are encoded towards the 3' end of the genome.

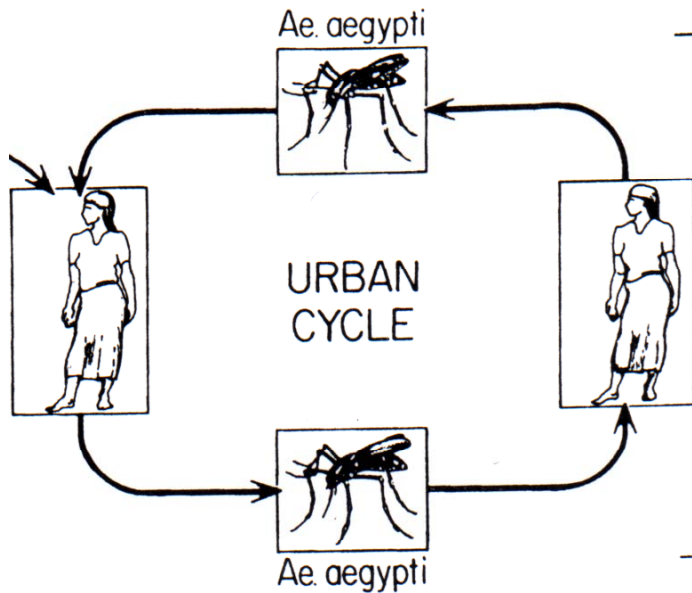


**Fig. 2** Structure of dengue virus. From Burke, D.S., 1988

### *Life Cycle*

The dengue life cycle involves an arthropod vector, a female *Aedes aegypti* adult mosquito, *A. albopitius* and *A. taylori* also can serve as vector for dengue viral disease transmission. Humans are the only known vertebrate hosts for dengue virus infection (Fig. 3). The virus is secreted into the mosquito saliva and the viral particles are transmitted through the saliva to the blood stream. The host develops dengue viremia after ~ 7 days of incubation. When this infected host is bitten by another *A. aegypti* mosquito, the virus disseminates and replicates throughout the mosquito and is secreted

into the mosquito's saliva completing the cycle (Burke, 1988; Rothman and Ennis, 2000). The prevalence of Dengue in the world is intimately related to the mosquito population (Fig. 4).



**Fig. 3** Life cycle of dengue virus (Burke, 1988)

### *Clinical Manifestations*

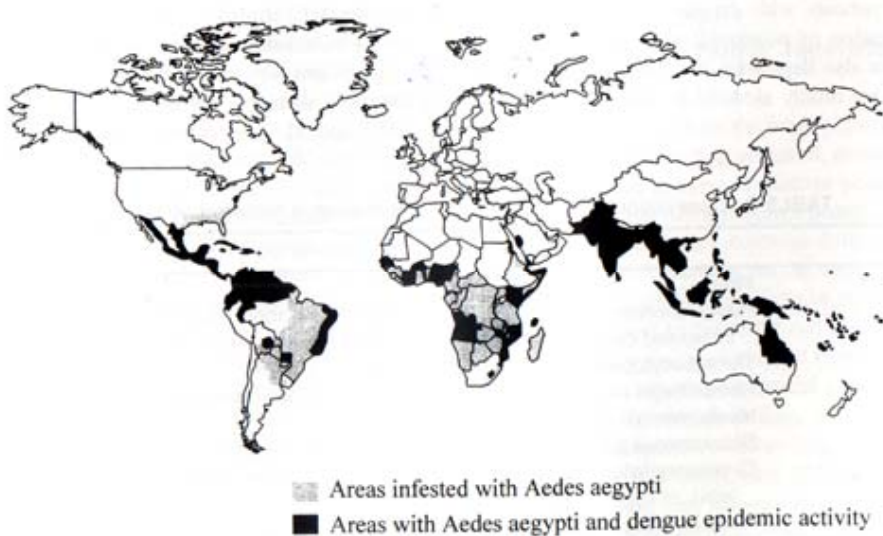
Dengue fever is an acute debilitating self-limited febrile illness while dengue hemorrhagic fever is an acute, potentially life threatening, capillary leak syndrome. During dengue hemorrhagic fever if the lost fluid is not replenished, circulating blood volume drops and leads to shock syndrome. Dengue fever is observed more frequently during primary infections while studies indicate that dengue hemorrhagic fever associates with secondary dengue infections. Marked leucopenia is observed in patients with DF along with depression of neutrophil and monocyte counts. The features

of DHF are hepatic damage, hemorrhagic manifestations detected by positive tourniquet test and marked thrombocytopenia. The four serotypes cannot be distinguished on clinical grounds alone and hence confirmation by serological tests or virus detection is necessary for accurate diagnosis of dengue virus infection (Hayes and Gubler, 1992; Rothman and Ennis, 1999; Rothman and Ennis, 2000).

### *Mechanism for Immunopathogenesis*

The cellular receptor that the E protein from dengue virus adheres to during primary infections is still not known nor is it known if other host components present in the circulation contribute to such binding. Dengue fever, which is seen mainly in primary infections lasts for 5- 7 days. Dengue hemorrhagic fever, which correlates with secondary infections, is categorized into 4 grades depending on the condition of the infected host by WHO. Grade 1, 2 and 3 for DHF and grade 4 for Dengue Shock Syndrome (DSS) (Burke, 1988). The theory of immune enhancement, developed extensively by Halstead, predicts that individuals who have been immunologically sensitized to one dengue virus serotype may develop nonneutralizing antibodies that actually enhance the entry of different serotype dengue viruses into mononuclear phagocytes, resulting in the release of mediators of vascular permeability (Halstead and O'Rourke, 1977). Hence, there are preexisting antibodies induced by the previous dengue infection that bind to the new virus serotype but do not neutralize infection instead lead to a more severe infection. This is known as Antibody Dependent Enhancement (ADE) of infection. Fc $\gamma$  receptors I and II are believed to participate in viral entry during ADE. Immunological studies of patients infected with dengue virus suggest that many tissues may be involved, as viral antigens are expressed in liver, lymph node, spleen, and bone marrow. It is yet not known how the same virus can produce a mild versus outcomes. The progression of DHF likely reflects a complex

interplay between host and viral factors and the production of inflammatory cytokines levels has shown to be implicated in the outcome (Rothman and Ennis, 2000).  $TNF\ \alpha$  has been shown to correlate plasma leakage and shock in animal models of dengue infection as well as in humans (Diamond et al., 2000; Halstead, 1988; Halstead, 1992; Rothman and Ennis, 1999; Tracey and Cerami, 1993).



**Fig. 4** World Distribution of Dengue in 1999. From The Center for Disease Control Public Health Image Library. (<http://phil.cdc.gov/Phil/default.asp>)

### *Differential Display technique*

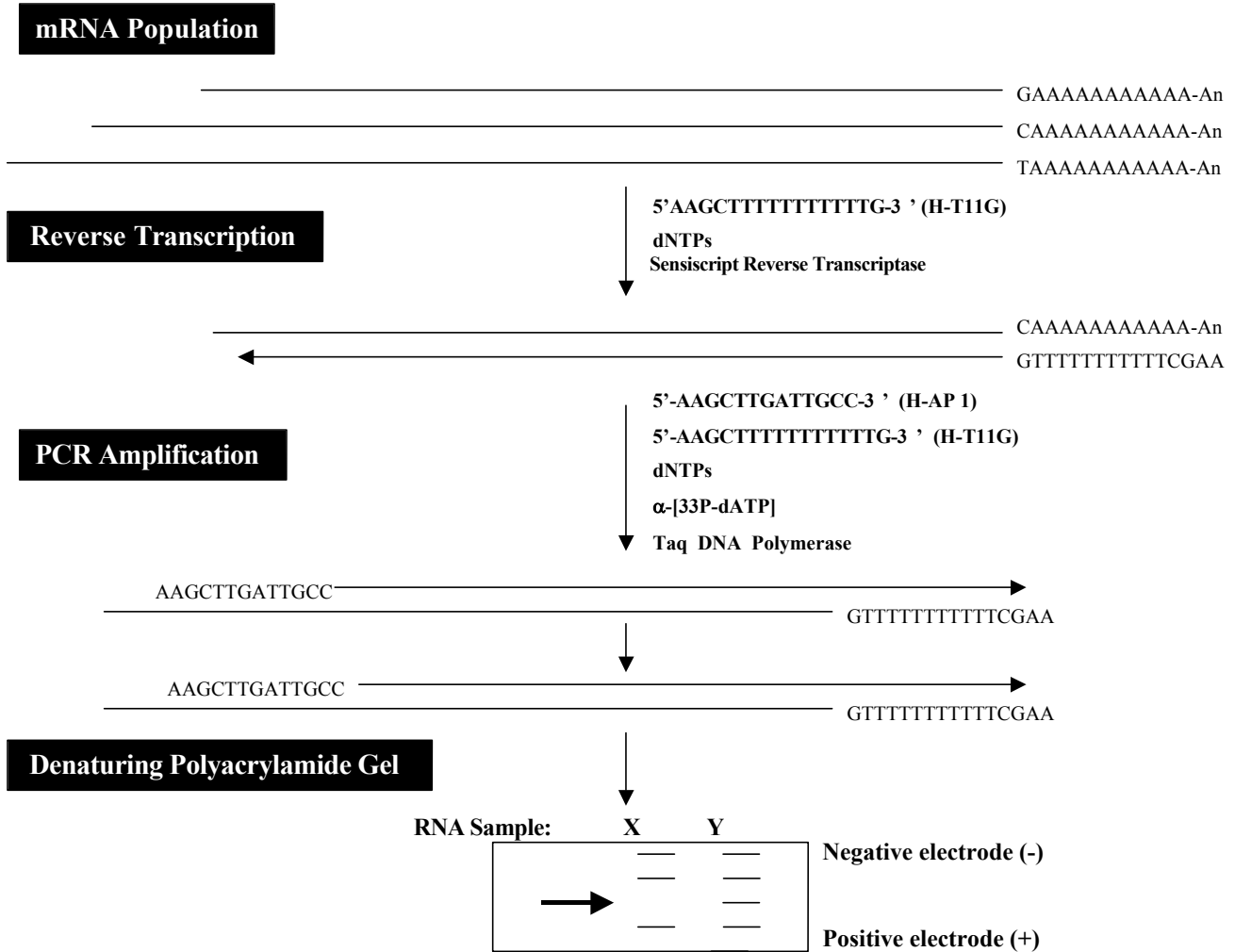
In this study we used a differential display technique to identify candidate genes differentially expressed during D2V infection in HUVEC and ECV 304 cells. The Differential display (DD) technique was developed by Peng Liang and Arthur B. Pardee to identify differentially expressed genes in various cells or under altered conditions (Liang and Pardee, 1992). It is a very powerful method that allows the comparison of similar cells or tissue types and the identification and isolation of differentially expressed genes (Martin and Pardee, 1999). Higher organisms like humans, contain about 100,000 different genes of which only a fraction, perhaps 15%, are expressed in any individual cell the number of aleatory combinations generated from the random primers will in theory, cover the expression repertoire of the cell. ). DD is distinguished from related methods for its low stringency, competitive PCR step that uses primer pairs (arbitrary and anchor primer) to target the 3' ends of messenger RNAs. The anchor 3' primer in DD hybridize with the polyadenylate [poly (A)] tail present in eukaryotic mRNA's to anchor the primer at the 3' end of the mRNA, with one additional 3' bases. Hence, by probability each anchor primer will recognize 1/3 of the total mRNA population because there are 3 (AT, GT, CT) different combinations of the last 3' base, committing T as the penultimate base (Liang and Pardee, 1992). The differentially expressed gene fragments can be excised from the DD gel, identified, and used to prepare specific primers for PCR and to study gene expression levels (Fig. 5). Alternately more complex, Subtractive hybridization and high density DNA microchips are used to study mRNAs expression.

The advantages of DD over subtractive hybridization are that it is much quicker, allows simultaneous detections of both groups (treated and untreated) of differentially

expressed genes, it is a very sensitive technique, and very small quantities of RNA containing the template (200 ng) are sufficient (Bosch et al., 2000) as compared to subtractive hybridization which requires 50 times more RNA. Also, while performing the subtractive hybridization, detection of highly homologous proteins within a gene family or identifying isoforms of the same gene is very unlikely.

DD has advantages over DNA microchips also in some aspects. It is cost-effective and does not require bioinformatics for analysis of the results. Hence, we selected the DD technique, to identify differentially expressed bands in ECV 304 and HUVECs during D2V infection, instead of subtractive hybridization or DNA microchips (Liang and Pardee, 1992; Martin and Pardee, 1999).

# Differential Display Method



**Fig. 5** Various steps involved in differential display technique.



### *Cell Lines*

A transformed human umbilical vein endothelial cell line (ECV 304) and HUVECs were used in the study. The ECV 304 cell line has been shown to be a good model to study dengue infection (Bonner and O'Sullivan, 1998). The advantages of HUVECs over the ECV 304 cell line is that it is a much more biologically relevant endothelial cell system because it is a primary cell line as opposed to ECV 304 which is a transformed cell line.

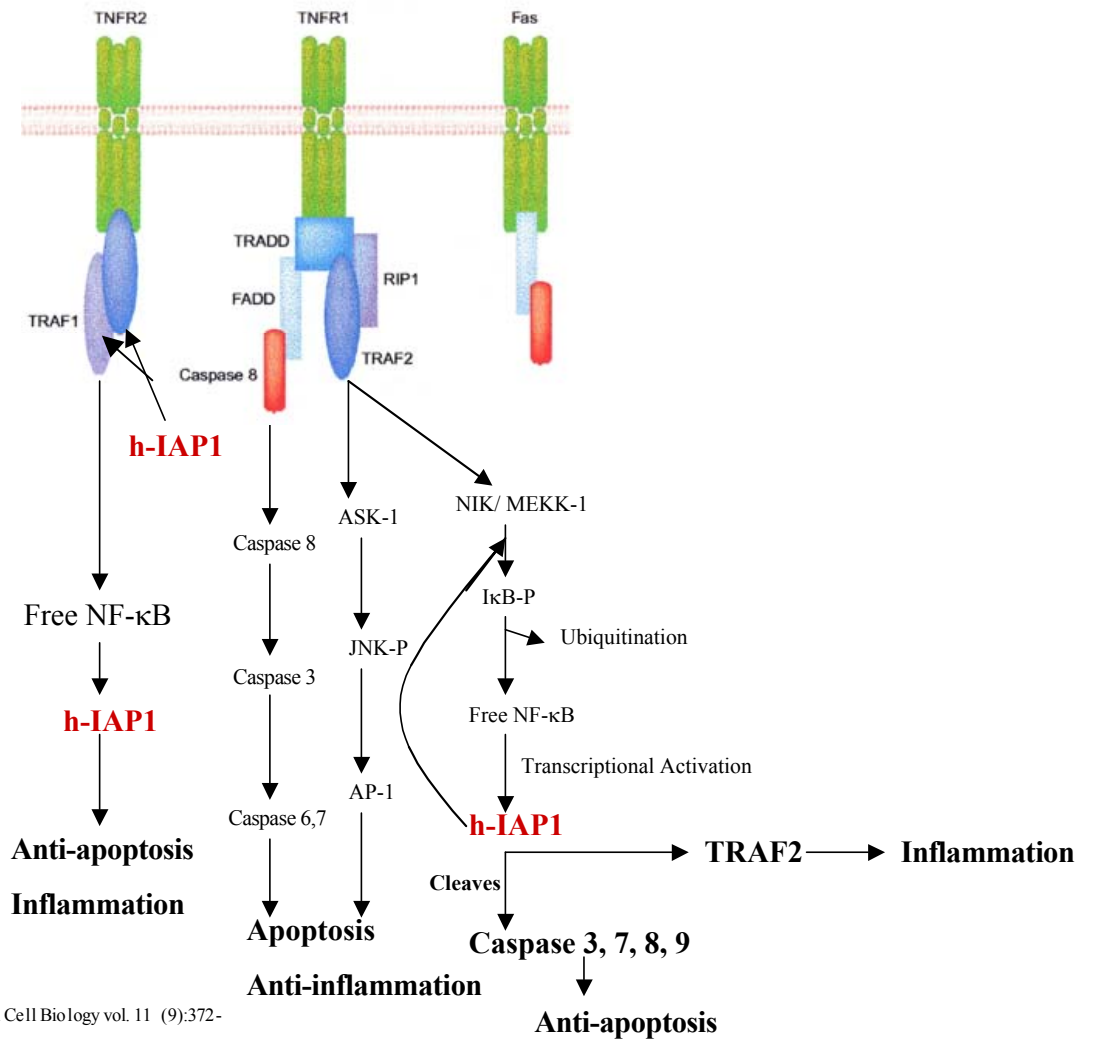
Using the DD technique we found 7 genes to be up-regulated during D2V infection in HUVEC cells, namely h-IAP1, 2'-5' OAS, 2'-5' OAS like, Mx1, MxA, Gal-9 and RGS2, and one gene, (ESDN) was found to be down-regulated in HUVEC cells during D2V infection. The identification of these genes helped us to detect three pathways involved during D2V infection which were previously unknown. The technique and the pathways involved, including the genes they regulate, will be described below.

### *TNF $\alpha$ Signaling Pathway*

Tumor necrosis factor  $\alpha$  (TNF  $\alpha$ ), a potent pro-inflammatory cytokine, has been found to be augmented in DHF patients. It belongs to the ever growing family of trimeric cytokines and cell-surface proteins such as Fas ligand (FasL), lymphotoxin- $\alpha$  (LT- $\alpha$ ), CD40 ligand (CD40L), TNF-related apoptosis-inducing ligand (TRAIL) and receptor-activator of NF- $\kappa$ B (RANKL). TNF  $\alpha$  binds to the two receptors, TNFR1 and TNFR2 and activates 3 different pathways, two transcription factors, NF- $\kappa$ B and AP-1, and the caspases. The NF- $\kappa$ B pathway regulates the expression of cytokines and hence modulates the inflammatory response. It also targets various anti-apoptotic genes thus

partially controlling cell survival. AP-1 leads to activation of caspases which ultimately lead to cell death or apoptosis (Fig. 6). The anti-apoptotic genes that NF- $\kappa$ B is known to regulate are Bcl-2, A20, Bcl-x, IEX-1L and three members of the inhibitor of apoptosis (IAP) family, h-IAP1/ c-IAP2, h-IAP2/ c-IAP1 and XIAP. Of all the known anti-apoptotic genes, it has been shown that NF- $\kappa$ B preferentially up-regulates the expression of h-IAP1. NF- $\kappa$ B transcriptionally regulates the expression of h-IAP1, and h-IAP1 itself can activate NF- $\kappa$ B. Hence, there exists a positive feedback loop between NF- $\kappa$ B and h-IAP1 leading to increased expression of h-IAP1. Also, h-IAP1 is recruited to the TNF receptor by associating with the TNF receptor-associated factors (TRAF1 and TRAF2) (Fig. 6). TRAF2 can interact with TRADD, which interacts, with TNFR2 or directly with TNFR1 but it has higher affinity for TRADD in comparison to TNFR1 or TRAF1 (Baud and Karin, 2001; Heyninck and Beyaert, 2001; Idriss and Naismith, 2000; Locksley et al., 2001; Park et al., 2000; Tracey and Cerami, 1993). Hence, signaling through the TNFR2 receptor is more important since most of the TNF  $\alpha$  signaling occurs through TNFR2. h-IAP1 has been demonstrated to interact with TRAF2 which is essential for the overexpression of h-IAP1. TRAF1 is not essential for up-regulating expression of h-IAP1 but its presence enhances the expression of h-IAP1 indicating that it might stabilize the interaction between TRAF2 and h-IAP1. Overall, this further enhances the expression levels of h-IAP1 gene. Downstream of its expression, h-IAP1 induces the proteolytic degradation of caspase-3, -7 and -9. Previous studies have shown that in Jurkat T cells, HeLa cells and in HUVEC cells TNF  $\alpha$  stimulation leads to increased expression of h-IAP1 (Chu et al., 1997) (Hofer-Warbinek et al., 2000) (Stehlik et al., 1998).

# TNF- $\alpha$ Signaling Pathway



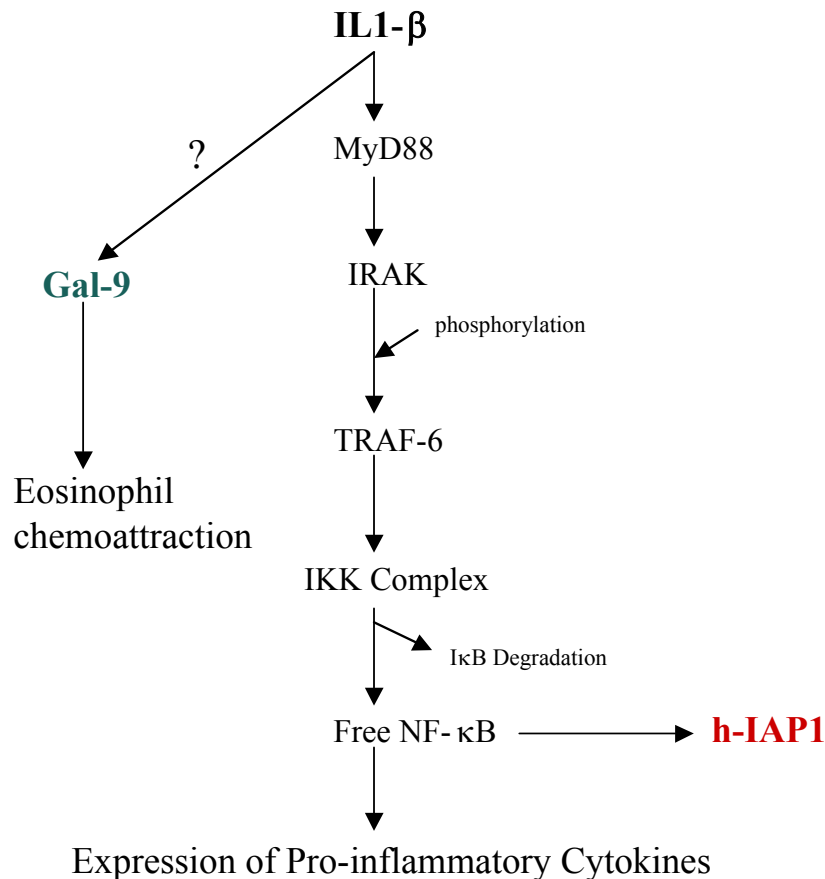
Extracted and modified from Baud and Karin, TRENDS in Cell Biology vol. 11 (9):372-377, 2001

**Fig 6** Signal transduction through the TNF  $\alpha$  signaling pathway

## *IL1-β Signaling Pathway*

Interleukin1- β (IL1-β), also a pro-inflammatory cytokine, which signals through the IL-1 receptor, activates NF-κB. Activation of NF-κB leads to the expression of other pro-inflammatory cytokines (You et al., 2001). It has been demonstrated that IL1-β also can up-regulate the expression of the h-IAP1 gene in HUVEC cells (Bannerman et al., 2002) (Wang et al., 1996) (Wrighton et al., 1996). Also, IL1-β has been shown to enhance the up-regulation of expression of Gal-9, an eosinophil chemoattractant (Fig 7)

## **IL1-β Signaling Pathway**



Toll like receptors (TLR) are a family of innate immune-recognition receptors that induce antimicrobial immune responses on recognizing lipopolysaccharide (LPS) or other microbial products. Toll like receptor-3 (TLR 3) recognizes dsRNA and activates the type-1 IFN pathway in endothelial cells (Miettinen et al., 2001) and NF- $\kappa$ B (Alexopoulou et al., 2001) (Alexopoulou et al., 2001). Hence, TLR3 activation may lead to inhibition of protein synthesis and increased production of inflammatory cytokines, possibly playing a role in the host defense against viruses.

### *Type-1 IFN Pathway*

The type-1 interferons are made by infected cells to directly induce an antiviral effect in neighboring uninfected cells and control viral spread. The IFN pathway is among the first to be activated to induce an antiviral state and fight the viral infection (innate) before an immune response (adaptive) is developed. 2'-5' OAS, PKR and Mx proteins recognize dsRNA and activate the type-1 IFN pathway (IFN- $\alpha$  and - $\beta$ ). IFN- $\alpha$  and - $\beta$  produced in response to viral infection inhibit cells growth, controls apoptosis, and will activate the expression of more genes which will lead to an inhibition of protein synthesis (Paul, 2001; Stark et al., 1998).

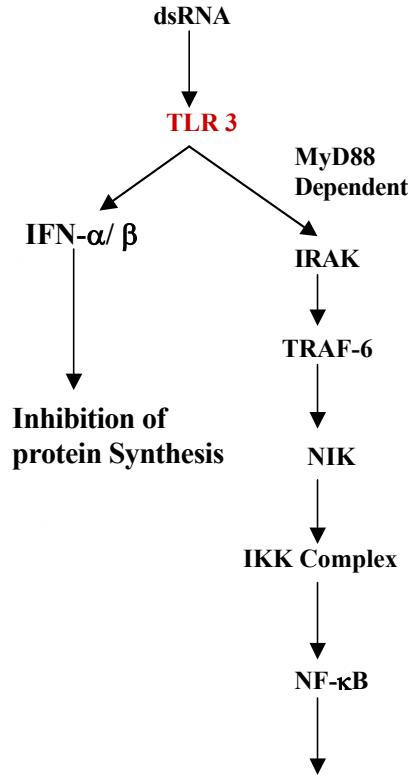
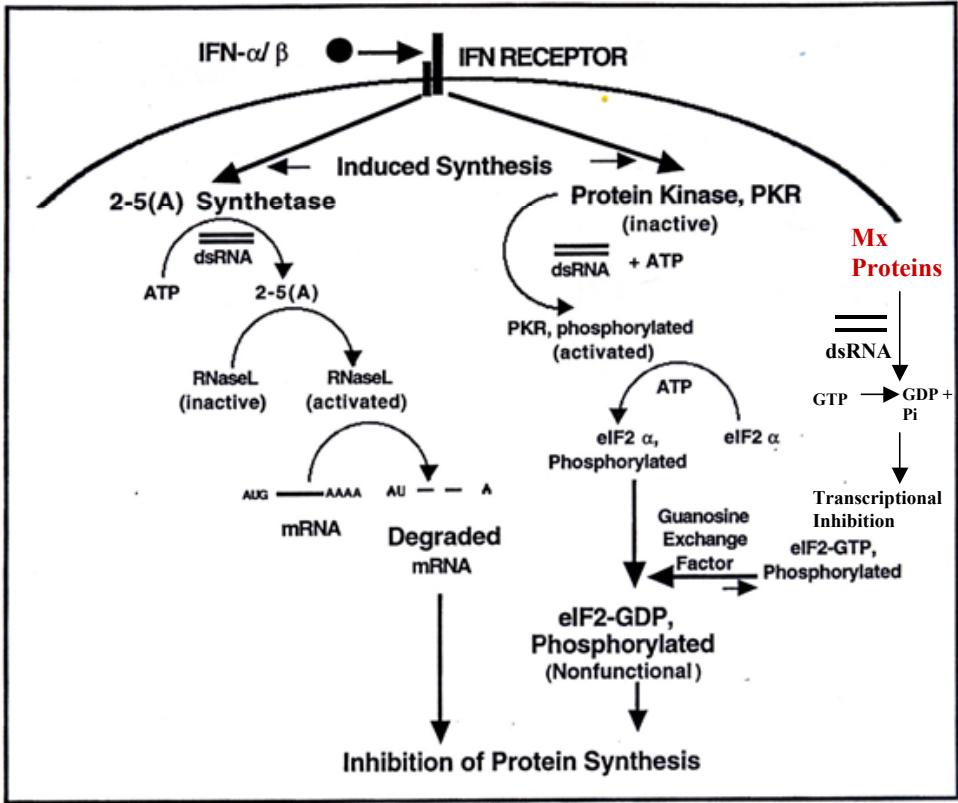
On recognizing dsRNA, PKR gets phosphorylated (activation) and phosphorylates eukaryotic initiation factor 2 (eIF2), which makes it nonfunctional and hence inhibits protein synthesis and viral replication.

IFN inducible 2'-5' OAS is stimulated by dsRNA and produces a series of short 2'-5'-oligoadenylates that converts inactive RNase L to active RNase L, which selectively degrades viral RNA (Bonnevie-Nielsen et al., 1995).

Mx proteins, Mx1 (Tezak et al., 2002) and MxA are also IFN-inducible that belong to the dyx1c1 superfamily of GTPases that are involved in endocytosis and vesicle transport (Fig. 8). The formation of large oligomers is essential for their anti-viral activity (Harcourt et al., 2000; Li et al., 2000; Rebouillat and Hovanessian, 1999; Rebouillat et al., 2000; Stark et al., 1998).

Induction of the IFN pathway also leads to an increase in expression of dsRNA-responsive genes such as E-selectin, ICAM-1, VCAM-1, which regulate the numbers and types of leukocytes that accumulate at the site of infection, and IL-1 $\beta$  and IL-6 that activate the leukocytes and help to contain and eliminate the viral infection (Sen, 2001). Previous studies have shown dengue virus infection induces type I and II- IFN pathway in immune cells and induction of E-selectin and VCAM-1 in endothelial cells (Avirutnan et al., 1998; Diamond et al., 2000; Libraty et al., 2001; Rothman and Ennis, 1999). Also the specific pathways stimulated downstream of the IFN receptors is not known yet.

# dsRNA Dependent Antiviral Activity Pathways



Expression of pro-inflammatory cytokines like **TNF α** and **IL1-β**

Extracted and modified from Fundamental Immunology by Paul, 4th Edition, Chapter 39, page 1302

Fig. 8 dsRNA recognition pathways

## **PROJECT PURPOSE**

The first goal of this project was, to identify potential candidate genes differentially expressed in HUVEC cells during dengue 2 virus infection using differential display technique. The second goal was, to identify prominent cellular pathways that are activated or regulated by these differentially displayed genes during D2V infection to eventually delineate possible interventions to counteract viral persistence and its effect on target cells. We investigated ECV 304 cells and HUVEC cells as model systems for endothelial cells.



## MATERIAL AND METHODS

### *Cell Culture*

HUVEC were purchased (Clonetics Corporation, San Diego, CA) and maintained in endothelial cell culture media (EGM® Bullet Kit; Clonetics Corporation) supplemented with Hydrocortisone (1 mg/ml), 50 mg/ml Gentamicin sulphate Amphotericin-B (GA-1000), 2% Fetal Calf Serum (FCS) 10 µg/ml Human Recombinant Epidermal Growth Factor (hEGF) and 3 mg/ml Bovine Brain Extract (BBE) at 37°C in humidified air containing 5% CO<sub>2</sub> according to the manufacturer's instructions. Only cells from passage two were used for experiments. The human umbilical vein endothelial cell line (ECV 304), obtained from American Type Culture Collection (ATTC) (Manassas, VA) was grown in medium 199 (Life Technologies, Grand Island, N.Y.) supplemented with 10% FCS (Life Technologies), Earle's medium, 2 mM L-glutamine and 2,200 mg/L of sodium bicarbonate at 37°C in humidified air containing 5% CO<sub>2</sub>.

### *Primer Sequences*

The sequences of oligonucleotide primers obtained from Genhunter (Nashville, TN) are shown in Table 1.

**Table 1.**

<b>Gene</b>	<b>Forward primer 5'–3'</b>	<b>Reverse primer 5'–3'</b>
h-IAP1	cagaagacacagacgtcttta	cgaactgtacccttgattgta
2'-5' OAS	actttaaaaaccccattattgaaa	ggagagggggcagggatgaat

2'-5' OAS-like	catcgtgaacccttatgagc	ctgctgggtcttcaatctgc
Gal-9	cctgcccagtgctcagagggtt	gcacatgggtcagctggatga
ESDN	ctacctttaaaccagaagaa	ccccatcccttctctgctc
RGS2	cggaattctgtgaagaaaatat	gcatgaggctctgtggtgattt
MxA	gccaggaccaggtatacag	gcctgcgtcagccgtgc
Mx1	gccaggaccaggtatacag	gctccttcaggagccagc

### ***Infection of Endothelial Cells***

Monolayers of ECV304 cells in 6 well micro titer dishes were resuspended in medium 199 containing 2% FCS, Earle's medium, 2 mM L-glutamine and 2,200 mg/L of sodium bicarbonate. Monolayers of HUVEC cells in 6 well microtiter dishes were resuspended in endothelial cell culture media (EGM® Bullet Kit; Clonetics Corporation) supplemented with half quantity of Hydrocortisone (1 mg/ml), Gentamicin sulphate Amphotericin-B (GA-1000), 2% Fetal Calf Serum (FCS), 10 µg/ml Epidermal Growth Factor Human Recombinant (hEGF) and 3 mg/ml Bovine Brain Extract (BBE). Dengue 2 virus New Guinea C (D2V NGC) was added to confluent monolayers of cells at a multiplicity of infection (M O I) of 1, and incubated at 37°C in humidified air containing 5% CO<sub>2</sub> for 12-14 hours. The culture supernatant was then removed, and fresh growth medium containing 10% FCS was added to each well. After 48 hours of infection, cells were harvested, centrifuged at 500 g for 7 min at 4° C, washed with PBS, pelleted and stored at -70°C for future experiments.

### ***Total Cellular RNA Preparation***

Total cellular RNA was extracted from 20 million ( $20 \times 10^6$ ) uninfected or infected HUVEC or ECV304 cells using an RNA Extraction Kit (Qiagen, Germany). Typically one million cells ( $1 \times 10^6$  cells) produced 10  $\mu\text{g}$  of total RNA. RNA was digested to eliminate residual DNA with DNase I using Message Clean Kit (GenHunter, Nashville, TN). The RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated  $\text{dH}_2\text{O}$  for DD analysis (alternately; Promega RNA Extraction Kits and Stratagene Absolute RNA Kits were used). RNA was quantified by spectrophotometry (Bio Photometer, Eppendorf, Germany) at 260 nm.

### ***Reverse Transcription of RNA for Differential Display***

RT was performed using Sensicript Reverse Transcriptase (Qiagen, Germany). cDNA was synthesized in 20  $\mu\text{l}$  reactions containing 200 ng of HUVEC or ECV304 mRNA, RT buffer (25 mM Tris-Cl, pH 8.3, 37.2 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 5 mM DTT) and 0.5 mM dNTP, 10  $\mu\text{mol}/\mu\text{l}$  of H-T11C anchor primer (5'-AAGCTTTTTTTTTTTC-3') and RNase inhibitor (10 U/ $\mu\text{l}$ ). Reactions were incubated at 37°C for 60 min, then 95°C for 5 min.

### ***Amplification of cDNA for Differential Display***

PCR amplification of cDNA was achieved according to the RNAimage protocol (GenHunter, Nashville, Tx). For the PCR reactions 10% of the RT reaction including Taq DNA Polymerase (Promega, Madison, WI) or Titanium<sup>TM</sup>Taq polymerase that includes an antibody against Taq DNA Polymerase (Clontech, Palo Alto, CA) was used. The PCR

reaction contained 200 nM of each primer (H-T11C anchor primer with either H-AP 1, 12, 15, 21 and 42) (Genhunter, Nashville, Tx), 2  $\mu$ l of 10X PCR buffer (final concentrations of 10  $\mu$ M Tris-Cl, pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 5 mM DTT), 25  $\mu$ M dNTP mix, 3000 Ci/mmol [ $\alpha$ -<sup>33</sup>P] dCTP per reaction and 5 U/ $\mu$ l of Taq Polymerase (Promega) in a total volume of 20  $\mu$ l. Reactions were cycled at 94°C for 30 sec, 40°C for 2 min, and 72°C for 30 sec for 40 cycles, followed by 72°C for 10 min. Reactions which included 0.4  $\mu$ l of Titanium<sup>TM</sup>Taq DNA Polymerase contained 2  $\mu$ l of Titanium Taq PCR buffer (final concentrations of 10  $\mu$ M Tris-Cl, pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 5 mM DTT), 0.4  $\mu$ l of dNTP, and 200 nM primer mix, and 3000 Ci/mmol of [ $\alpha$ -<sup>33</sup>P] dCTP per 50  $\mu$ l reaction in PCR-grade water. Conditions for Titanium Taq PCR were set at 95°C for 1 min, 40 cycles at 93°C for 30 sec, 40°C for 2 min, 68°C for 30 sec, and a final step at 68°C for 10 min.

### ***Gel Electrophoresis of cDNA Fragments for Differential Display***

PCR reactions were electrophoresed on extended-format denaturing 6% polyacrylamide gels using the programmable Genomyx LR DNA sequencer (Beckman Coulter, Columbia, MD).

### ***Re- Amplification of cDNA probes for Differential Display***

The RNAimage protocol was followed to re-amplify candidate bands observed on the DD gels. The bands were extracted from the denaturing polyacrylamide gel by soaking in 100  $\mu$ l of 2X PCR buffer (final concentrations of 10  $\mu$ M Tris-Cl, pH 8.4, 50

mM KCl, 1.5 mM MgCl<sub>2</sub> and 5 mM DTT) for 10 min. This supernatant was treated with 3M Sodium Acetate (10 µl), 5 µl of Glycogen (10 mg/ml) and 450 µl of 100% Ethanol, stored at -70°C for 30 min, and pelleted by centrifugation. The pellet was rinsed with 200 µl Ethanol, air dried and dissolved in 10 µl dH<sub>2</sub>O. This extracted DNA was amplified using the RNAimage protocol (GenHunter). The 40 µl PCR reaction included 4 µl of DNA, 4 µl of 10X PCR buffer (final concentrations of 10 µM Tris-Cl, pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 5 mM DTT), 3.2 µl of dNTP (250 µM), 200 nM of primers, 200 nM of H-T11C and 2.5 U of Taq Polymerase (Promega). Reactions were subjected to 40 rounds of PCR amplification at 94°C for 30 sec, 40°C for 2 min, and 72°C for 30 sec, followed by 72°C for 10 min. L35A DNA served as a control to exclude variations between samples. The amplified PCR products were electrophoresed in 1.5% agarose gels (Life technologies, Grand Island, N.Y.) containing 0.5 µg/ ml ethidium bromide for staining. Results were documented with Quantity One Software version 4.2.3 (Biorad, Italy).

### ***Northern Blot***

Total cellular mRNA was isolated using Trizol reagent (Gibco BRL, Gaithersburg, MD). 10 µg of RNA was electrophoresed on a standard formaldehyde 1% agarose gel. The northern blot protocol from the 2<sup>nd</sup> edition of Molecular Cloning, A Laboratory Manual (Maniatis, 1989) was followed to transfer the RNA to a nitrocellulose membrane. Samples were electroporesed in buffer containing 0.1 M MOPS (pH 7.0), 40 mM sodium acetate and 5 mM EDTA (pH 8.0), and blotted overnight using 20X SSC onto a

nitrocellulose membrane. RNA was cross-linked to the nitrocellulose membrane by exposing to UV light for 3 minutes.

Membranes were hybridized with  $\alpha$ -[ $^{32}\text{P}$ ] dCTP-labeled DNA restriction fragments specific to h-IAP1 using the Random Primed Labeling Kit (Roche, IN, USA). The h-IAP1 fragment (probe) was produced by amplification of h-IAP1 by PCR using 5'cagaagacacagactcttta3' (forward primer) and 5'cgaactgtacccttgattga3' (reverse primer). In this protocol, the h-IAP1 DNA was denatured at 100°C for 10 min, and then cooled on ice. A final volume of 20  $\mu\text{l}$  contained 1  $\mu\text{l}$  of Klenow enzyme (2 U), 3  $\mu\text{l}$  of dATP, dGTP, dTTP mixture (0.5  $\mu\text{M}$ ), 5  $\mu\text{l}$  of [ $\alpha^{32}\text{P}$ ] dCTP (3000 Ci/mmol) and 25 ng denatured DNA in dH<sub>2</sub>O. This reaction was incubated at 37°C for 30 min, followed by 65°C for 10 min to stop the reaction. Unincorporated nucleotides were removed using Quick Spin G-25 Sephadex columns. Initially the membranes were washed with 1X SSC, 0.1% SDS, and later with 0.2X SSC, 0.1% SDS. Membranes were analyzed by autoradiography after exposure to Kodak BioMax films at -70°C for 72 hours using intensifying screens. Radioactivity was quantified using a Phosphoimager.

### ***Gel Extractions and DNA Sequencing***

The QIAquick Gel extraction kit protocol (Qiagen, Germany) was used to extract DNA from samples run on 1.5% Agarose gels. Purified DNA was eluted in dH<sub>2</sub>O instead of Buffer EB (10 mM Tris-Cl, pH 8.5) and quantified by spectrophotometry using a BioPhotometer (Eppendorf, Germany) at 260 nm. These DNA samples were sequenced in the UMASS Medical School sequencing facility using the anchor primer H-T11C.

### ***PCR Extractions***

PCR mixes were subjected to the QIAquick PCR Purification kit protocol (Qiagen, Germany). DNA was eluted in dH<sub>2</sub>O instead of Buffer EB (10 mM Tris-Cl, pH 8.5) and quantified by spectrophotometry using a BioPhotometer (Eppendorf, Germany) at 260 nm. These DNA samples were sequenced using the anchor primer H-T11C.

### ***Semi-Quantitative RT-PCR***

0.2 µg of total cellular RNA obtained from infected and un-infected cells was reverse transcribed using Omniscript/ Sensiscript Reverse Transcriptase (Qiagen, Germany) in the presence of 1 µM of anchor primer, and a dNTP concentration of 500 µM in a volume of 20 µl. Reverse transcription (RT) was performed at 37°C for 60 min, followed by 94°C for 15 min. The PCR reaction was performed using various quantities (0.5, 1 and 2 µl) of the cDNA obtained from infected and un-infected RNA samples in the RT step. The 50 µl PCR reaction included 2.5 U of Taq DNA Polymerase (Clontech, Palo Alto, CA), 100 µM of dNTP, and 100 µM of primer. This reaction mix was subjected to a PCR program: 94°C for 1 min, followed by varying cycles at 58°C for 1 min, 72°C for 45 sec, and 94°C for 30 sec, and then 58°C for 1 min, and 72°C for 10 min. The cDNA obtained from the RT step was also utilized in a real-time PCR reaction.

### ***Real-Time PCR (SYBR Green)***

cDNA samples generated from a semi-quantitative RT step were used as template for a real-time PCR reaction. Each 50  $\mu$ l reaction included 1-4  $\mu$ l of cDNA (containing 200 ng of RNA) and 15.75  $\mu$ l PCR master mix containing 5  $\mu$ l of 10X SYBR PCR Buffer, 6  $\mu$ l of 25 mM MgCl<sub>2</sub>, 4  $\mu$ l of dNTP blend (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 5 mM dUTP), 0.25  $\mu$ l AmpliTaq Gold (1.25 U) and 0.50  $\mu$ l AmpErase UNG (0.5 U) in deionized water. Five dilutions of a plasmid from 100 femtograms (fg) to 1 nanogram (ng) (1/10 dilution series) were used as standard for the experiment, and L35A was used as an internal control for the unknown samples. Reactions were cycled at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, and 60°C for 1 min in a Gene Amp PCR System 5700 (Perkin Elmer).

### ***Fluorescence Detection of Infected HUVEC Cells***

D2V infected primary human umbilical vein endothelial cells were analyzed by FACs using intracellular labeling (Cytotfix/Cytoperm Plus kit, Pharmingen/Becton Dickinson, San Diego, CA) with mouse polyclonal D2V antibody diluted 1:500 in PBS, purified from hyperimmune mouse ascitic fluid (ATCC), and a secondary antibody against mouse IgG coupled with FITC (Sigma).

### ***Multiplex PCR Reactions***

For the multiplex PCR reaction, cDNA template was obtained from the semi-quantitative RT step, and the CytoXpress® Multiplex RT-PCR Kit protocol (BioSource International, CA) was followed. The 50  $\mu$ l reaction contained 4  $\mu$ l of 3.12 mM dNTP, 5



$\mu\text{l}$  of Taq DNA polymerase (2.5 U), 5  $\mu\text{l}$  of 10x MPCR primers, 5  $\mu\text{l}$  of 10x MPCR Buffer, 5  $\mu\text{l}$  of 10x MPCR enhancer, and 5  $\mu\text{l}$  of cDNA (200 ng/ $\mu\text{l}$ ) in dH<sub>2</sub>O. After an initial denaturation step at 96°C for 1 min, the PCR reaction was cycled 2 times at 96°C for 1 min, and 60°C for 4 min, followed by 30 cycles at 94°C for 1 min, and 60°C for 2.5 min and a final step of incubation at 70°C for 10 min. 20  $\mu\text{l}$  of PCR product was electrophoresed on a 1.5% agarose gel (Life Technologies, Grand Island, N.Y.) containing 0.5  $\mu\text{g}$  / ml ethidium bromide for staining DNA.

Primary monocytes were isolated and infection was performed for 48hours. RT-PCR was done as described elsewhere (Bosch et al., 2002).

## RESULTS

### *Differential Display Analysis of Dengue Infected ECV304 and HUVEC Cells*

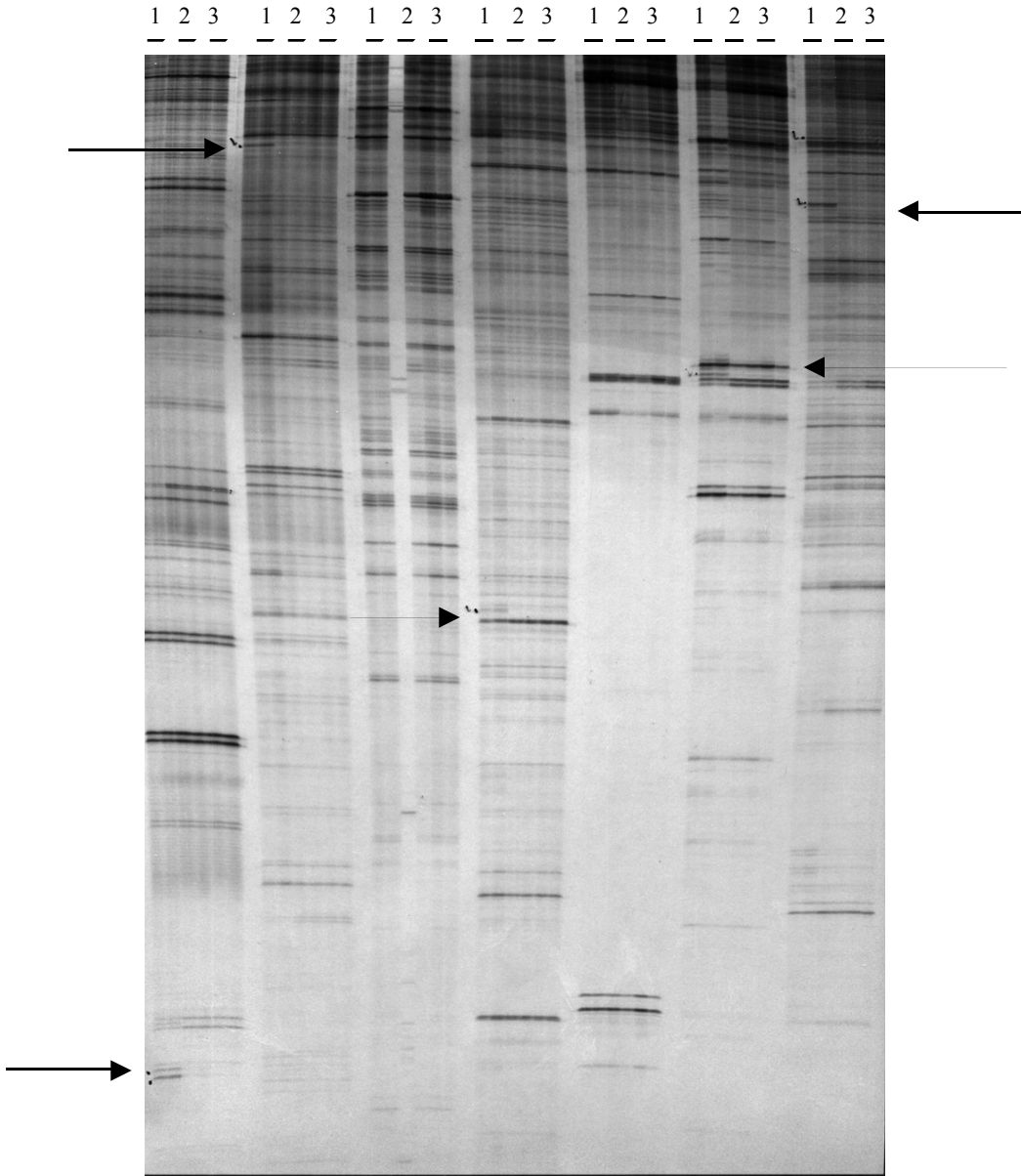
A combination of 41 different primers was used in an effort to identify DD genes in the ECV 304 cell line during D2V infection. The list of the primers used was H-AP 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 30, 31, 32, 41, 42, 43, 44, 45, 46, 47, 48 (Fig 9). One of the bands excised from the D2V infected lanes using the H-AP 21/HT11C primer pair was identified as the human inhibitor of apoptosis-1 (h-IAP1) on sequencing (UMASS Medical School sequencing facility) and BLAST search (Fig 10). This was the only DD-identified band that found an identity with a human expressed sequence tag in Genbank out of 45 bands analyzed by BLAST searches. Most of the Genbank searches found genomic identities. When DNase I treatment was used at the end of the RNA extraction, we observed elimination of the full length genomic DNA Genbank hits. Hence, this might demonstrate that the genomic identities we had earlier encountered may have been due to contamination of the RNA with traces of genomic DNA.

The data from the DD analysis of ECV 304 cells indicated h-IAP1 was increased in dengue infection. But ECV 304 is a transformed endothelial cell line while HUVEC is a primary endothelial cell line. Perhaps the pattern of gene expression would be more accurate in a cell line that the virus usually infects, primary HUVEC cells, as compared to an immortalized cell line that has undergone changes during its transformation. Hence, DD analysis of HUVEC cells (a much more biologically relevant cell, compared to ECV 304) was used to study gene profiling during dengue 2 virus infection. The random primers used were H-AP 1, 3, 4, 6, 8, 12, 15, 16, 19, 21, 22, 23, 41 and 42 in

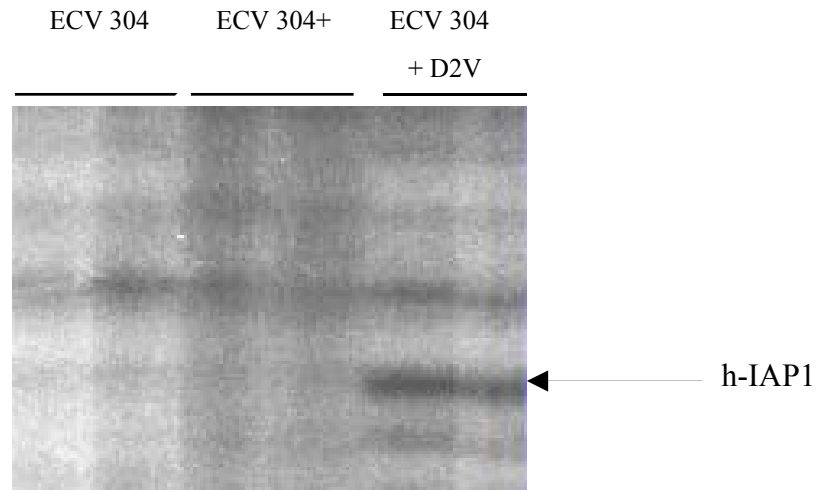
various combinations. Out of the 16 bands extracted from the DD gel, 8 bands found identifications with human expressed tags when the BLAST search against Genbank was used. Six of these bands are shown in Fig 11. The list of these identifications is shown in Table 1.

**Table 2.** Summary of Differentially displayed genes in HUVEC cells

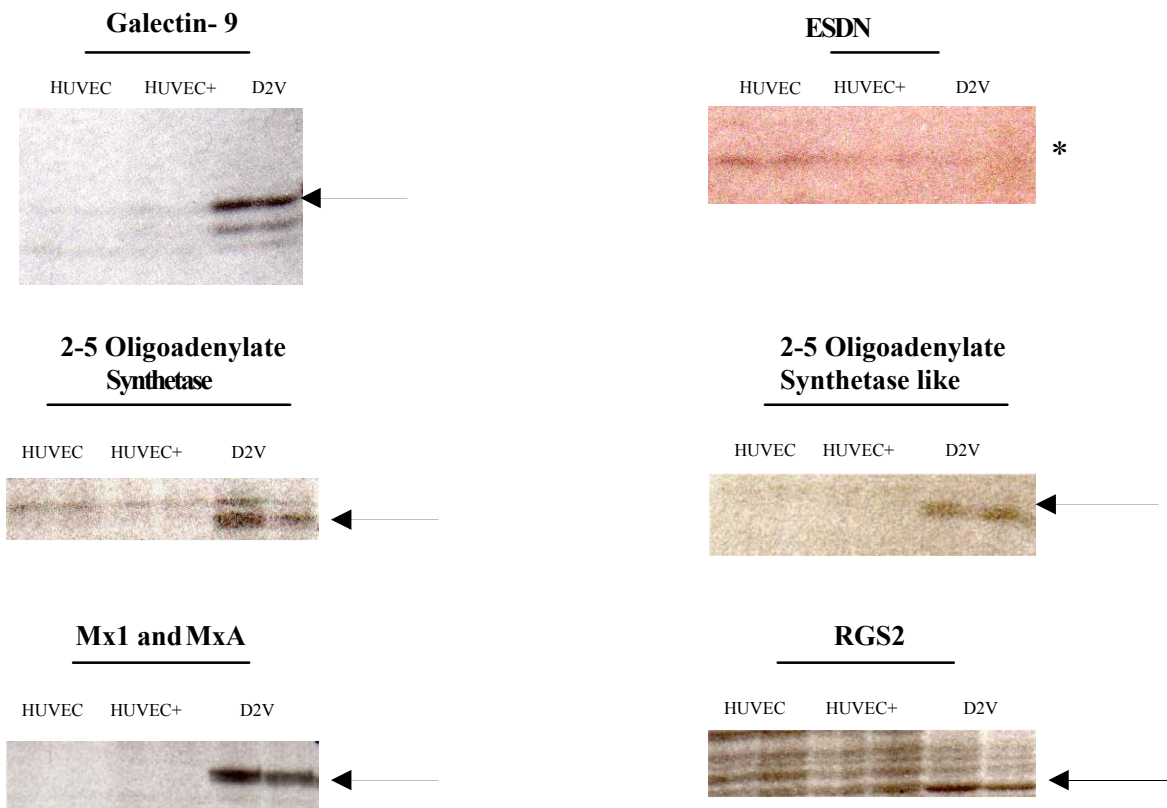
<b>Gene Name</b>	<b>GenBank Acc.</b>	<b>Function</b>
<b>1. h-IAP1</b> Human inhibitor of apoptosis protein-1	AF070674	Anti- apoptotic
<b>2. ESDN</b> Endothelial and smooth muscle cell- derived neuropilin-like protein	AF387547	Regulation of Vascular cell growth
<b>3. 2'-5' Oligoadenylate synthetase</b>	XM_056785	Induction of IFN- $\alpha$ , $\beta$
<b>4. 2'-5' Oligoadenylate synthetase-like</b>		Binds dsRNA but function unknown
<b>5. Mx1</b> Myxovirus (influenza) resistance 1, homolog of murine (interferon-inducible protein p78)	M30817	Type1 IFN response
<b>6. MxA</b> Interferon-induced cellular resistance mediator protein	XM_009773	Type1 IFN response
<b>7. RGS 2</b> Regulator of G-protein signaling 2	XM_029884	Regulates G-protein signaling and T-cell activation
<b>8. Galectin 9</b> Lectin, galactoside-binding, soluble 9	NM_002308	Eosinophil Chemoattractant, cell adhesion, apoptosis and cell proliferation



**Fig. 1** Differential Display using total RNA. Total RNA was reverse transcribed and this cDNA was then radioactively labeled with [ $\alpha$ - $^{33}\text{P}$ ]dCTP during PCR reaction. PCR product was analyzed on a 6% DNA sequencing gel. Lane 1 is infected sample, lane 2 is uninfected + D2V virus and lane 3 is uninfected sample. The arrows indicate a amplified mRNA species found only in D2V infected HUVEC cells.



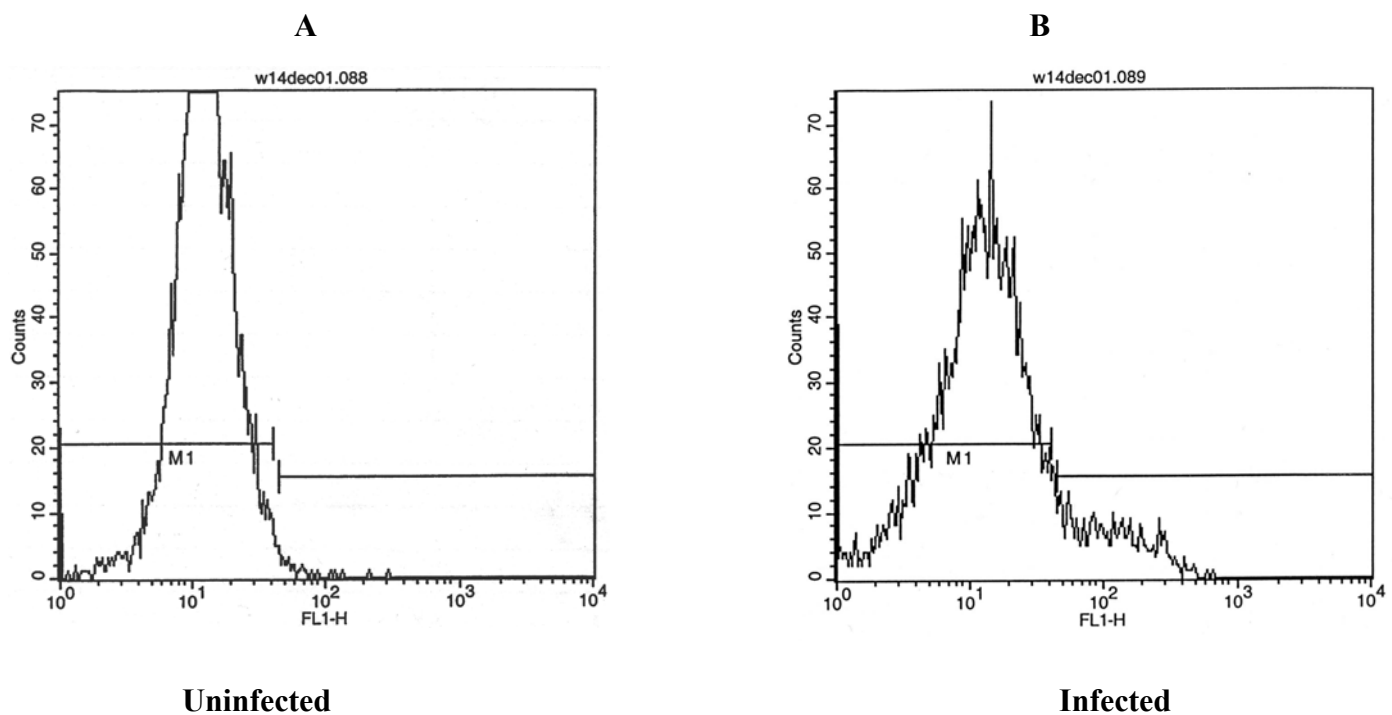
**FIGURE10.** Differentially displayed (DD) h-IAP1 gene in EVC 304 cells during 48 hours of D2V,NGC infection. The arrow indicates a differentially displayed band on a 6% denaturing polyacrylamide gel that was identified using primers HT11C / H-AP21. A sample containing ECV 304 RNA spiked with D2V,NGC virus (ECV 304+) was used to avoid false positives.



**FIGURE 11.** Differentially displayed genes in HUVEC cells during 48 hours of D2V,NGC infection. HUVEC RNA was used to prepare cDNA with Sensiscript reverse transcriptase (limit of detection was 0.2 ng of total RNA in the RT reaction) and HT<sub>11</sub>C primer. The arrows indicate the differentially displayed bands identified when HUVEC cells were infected with D2V,NGC compared to control HUVEC cells (up-regulated in treated cells). The asterisk indicates a down-regulated cDNA when HUVEC cells are infected with D2V,NGC. Its presence can be seen in uninfected cells. H-AP21-1, H-AP12, H-AP42, H-AP15, H-AP21-2, H-AP1 primers correspond to 2-5 OAS, Mx1/MxA, Gal-9, ESDN, 2-5 OAS-like genes respectively.

### *Flow Cytometry Analysis*

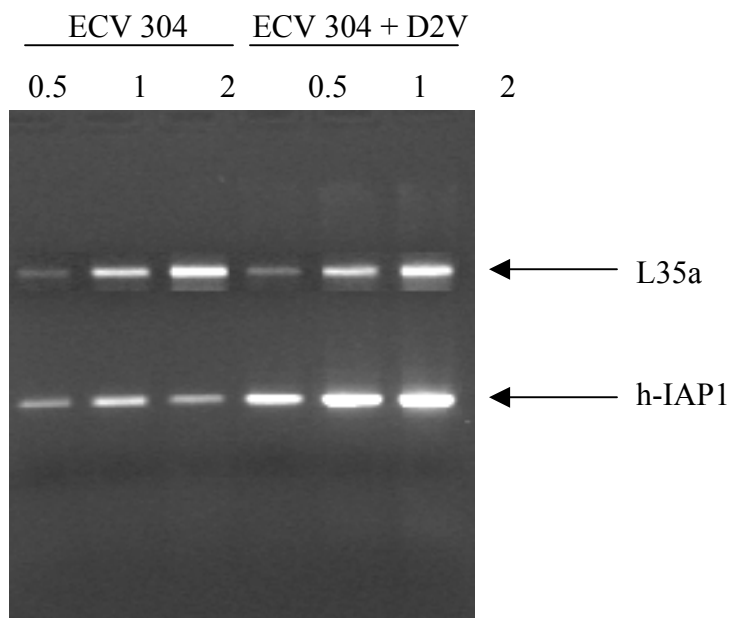
Infected and uninfected HUVEC cells were fixed and permeabilized for detection of viral antigens using immunofluorescence as specified in Materials and Methods. Cells were analyzed by FACs using antibodies against dengue virus to detect percent of infection. 10% infection was detected in one pilot experiment as shown in Fig. 12. The mRNA extracted from these cells was utilized in the time course experiments.



**FIGURE 12.** Flow cytometry analysis to determine the percent infection of HUVEC cells with D2V,NGC. Uninfected (A) and D2V,NGC infected cells (B) were harvested after 50 hours of incubation. The cells were fixed, permeabilized and subjected to intracellular labeling with mouse polyclonal D2V antibody diluted 1:500 in PBS, purified from hyperimmune mouse ascitic fluid (ATCC). The cells were stained with a secondary antibody against mouse IgG coupled with FITC and analyzed by FACscan. The M2 gate corresponds to 10% infection.

### *Semi-Quantitative RT-PCR in ECV 304 Cells*

To confirm the up-regulation of h-IAP1 in ECV 304 cells during D2V infection, a semi-quantitative RT-PCR was performed on the total RNA extracted from uninfected and infected ECV 304 cells. Three different quantities (0.5, 1 and 2  $\mu$ l) of both uninfected and infected cDNA were used in the PCR reaction. h-IAP1 was found to be up-regulated approximately 8 fold during D2V infection of ECV304 cells as shown in Fig. 13. In two independent experiments, the same result was obtained. Ribosomal protein gene L35A was used as a housekeeper gene in both experiments. This data showed that h-IAP1 was indeed up regulated in ECV 304 cells during D2V infection.



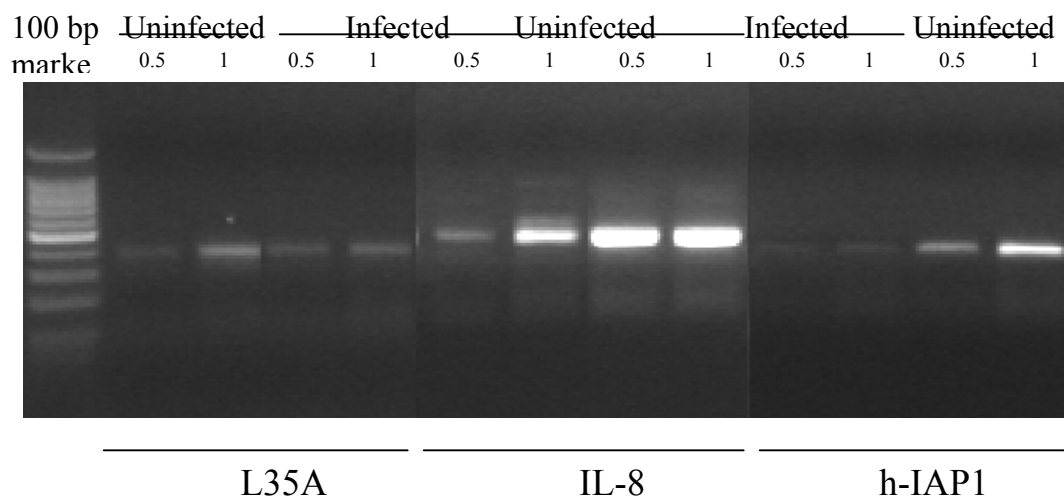
**FIGURE 13.** Up-regulation of h-IAP1 gene (~ 8 fold) in D2V,NGC infected ECV 304 cells. ECV 304 cells were incubated with D2V,NGC for 48 hours and total RNA was obtained from uninfected and infected cells. RT-PCR was performed to check for up-regulation of the h-IAP1 gene. Three different quantities of cDNA (0.5, 1, 2  $\mu$ l) were used in the PCR step. L35a, a ribosomal protein gene was used as a control.



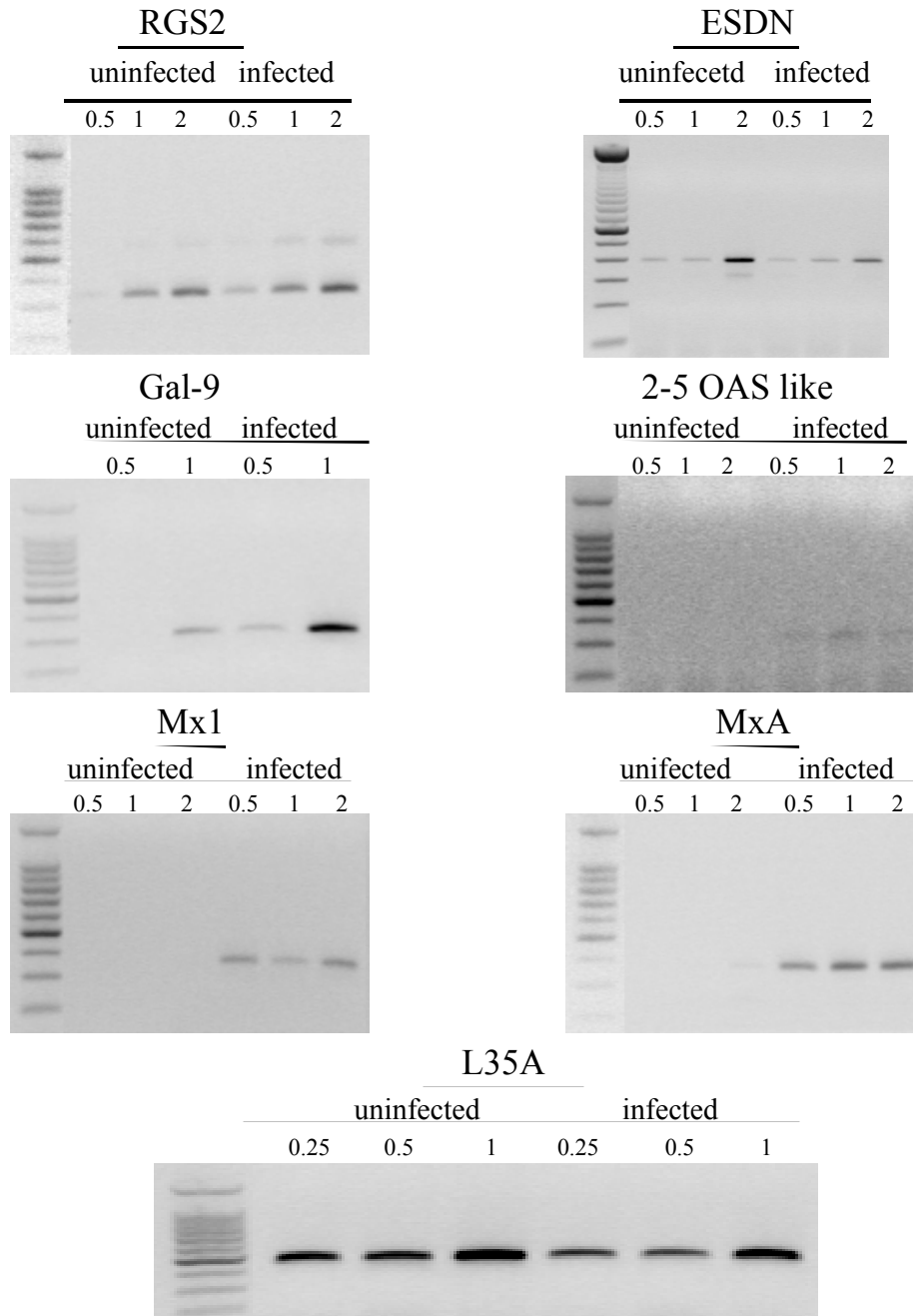
### *Semi-Quantitative RT-PCR in HUVEC Cells*

Semi-quantitative RT-PCR analysis was performed for all the 8 genes identified by DD as potential candidates for differential expression during D2V infection of HUVEC (e.g., Fig 14). The L35A gene was used as a housekeeper gene to standardize the results obtained in the experiments (Fig 15). The fold induction (up-regulation or down-regulation) of the 8 genes is shown in Table 2 (right column).

We performed RT-PCR on the 7 genes in ECV 304 mRNA confirmed to be differentially expressed in primary HUVEC cells. 3 out of the 7 genes differentially expressed in HUVEC cells were not found to be differentially expressed in ECV 304 cells (data not shown). This strongly indicates that the ECV 304 cell line model system appears not to be ideal for studying gene profiling in endothelial cells during D2V infection.



**FIGURE 14.** Induction of h-IAP1 mRNA in D2V,NGC infected HUVEC. Total RNA was extracted from uninfected and infected cells and RT-PCR was performed after 48 hours of D2V,NGC infection. L35a a ribosomal protein gene was used as a control and IL-8 was used as a positive marker of infection. In the PCR step two different quantities of cDNA were used (0.5, 1 µl).



**FIGURE 15.** Semi-quantitative RT-PCR analysis showing up-regulation of 6 different genes in D2V ,NGC infected HUVEC. Total RNA was extracted from uninfected HUVEC 48 hours post-infection. L35a was used as housekeeper gene. 0.5, 1 and 2 μl of cDNA of uninfected and infected samples was used in the PCR step for all reactions except for L35a in which 0.25, 0.5 and 1 μl of cDNA was used.

### ***Real-Time PCR with SYBR® Green***

A recent study indicates that real-time RT-PCR approach is well suited for validation of differential expression since it is quantitative and rapid and requires 1000-fold less RNA than conventional assays (Rajeevan et al., 2001). Hence, to confirm the results obtained by semi-quantitative analysis and to more precisely quantify the fold induction of these differentially expressed genes, the SYBR®Green Signal Detection System 5700 (Applied Biosystems) was used. For normalization of the data, ribosomal protein L35a was used. This analysis (see Table 2, middle column) shows that h-IAP1 is over expressed 8.1 fold in HUVEC cells after 48 hours of exposure to dengue virus (D2V NGC). This value is in close agreement with the 7.79 fold up-regulation identified by semi-quantitative RT-PCR.

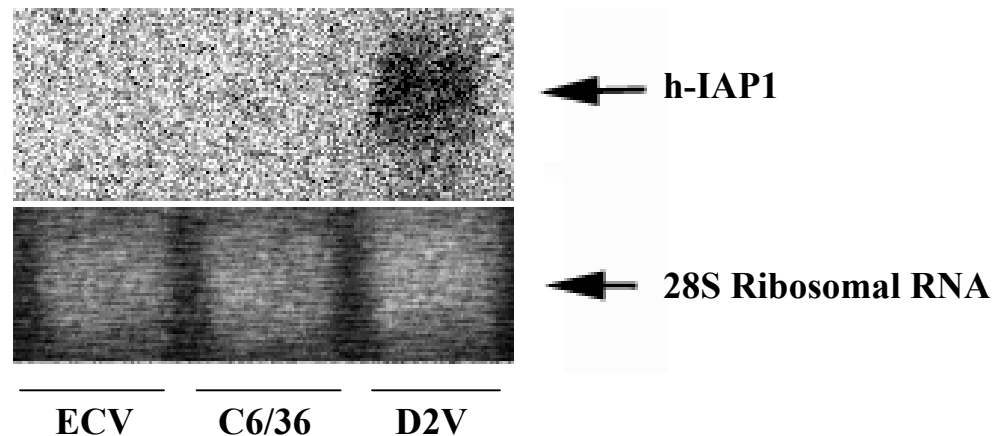
**Table 3.** Comparison of fold induction of genes obtained from SYBR® Green Real Time PCR and RT-PCR

<b>Genes</b>	<b>Fold Induction</b>	
	<b>SYBR® Green</b>	<b>RT-PCR</b>
h-IAP1	8.1	7.79
2'-5' OAS	72.85	> 2.65
2'-5' OAS-like	>100	> 3.55
RGS2	2.8	3.9
Gal-9	4.59	4.7
Mx1	N.D	>2.9
MxA	>100	>11
ESDN	N.D	0.45

TNF $\alpha$	3.76	3.82
IL1- $\beta$	40.77	10.23

***Northern Blot Analysis to Confirm Up-Regulation of h-IAP1***

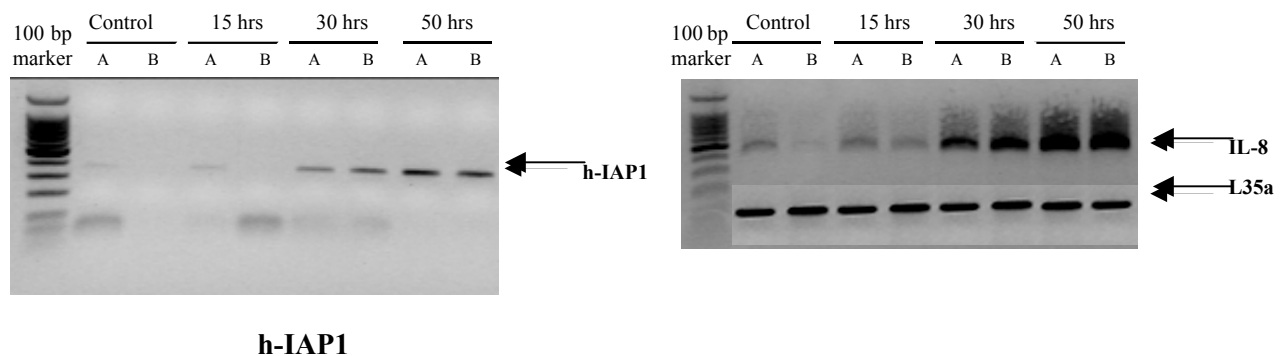
To further confirm the up-regulation of h-IAP1 expression during D2V infection Northern Blot analysis was performed on ECV 304 RNA. 10  $\mu$ g of total cellular RNA was electrophoresed on a denaturing agarose gel and blotted overnight to a nitrocellulose membrane. This membrane was probed with  $\alpha$ -[<sup>32</sup>P] dATP-labeled oligonucleotide specific for h-IAP1 (Fig 16). Ethidium bromide staining (lower panel) was used to confirm equal loading of mRNA samples in each lane. A band was observed only in the D2V infected ECV 304 lane (right side) and was completely absent in the uninfected and C6/36 infected lane. This result confirmed our previous conclusion that h-IAP1 is up-regulated in HUVEC cells during D2V infection.



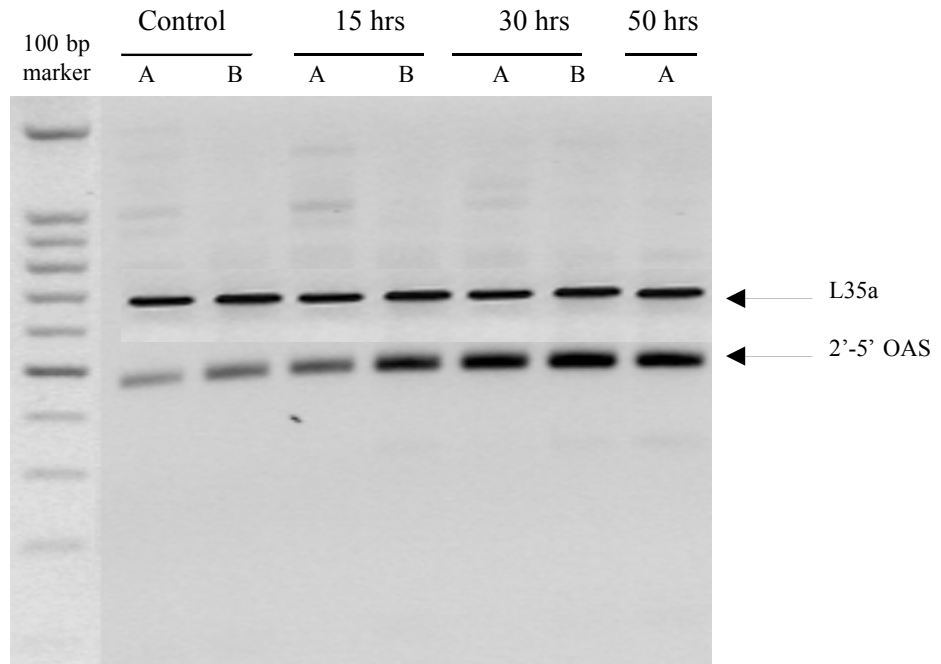
**FIGURE 16.** Northern blot analysis was used to confirm the expression of h-IAP1 in D2V,NGC infected HUVEC. 10  $\mu$ g of total RNA was probed with  $\alpha$ -[<sup>32</sup>P] dCTP labeled h-IAP1 fragment as described in Materials and Methods section. Ethidium bromide staining was used as control to confirm equal loading of RNA in each lane. The position of 28S ribosomal RNA (5200 bp) was used to determine the approximate length of the h-IAP1 transcript (5256 bp).

### ***Time-Course Study of the Expression of h-IAP1 and 2'-5' OAS***

To study in more detail the level of expression of h-IAP1 and 2'-5' OAS genes in D2V infected HUVEC cells, a time-course RT-PCR study was performed over an interval of 15, 30 and 50 hours of D2V infection. In both the experiments, the expression pattern of IL-8 gene (previously shown to be up-regulated in D2V infection) (Bosch et al., 2002) was used as positive control for D2V infection of HUVEC, while L35A was used as a housekeeper gene. The data from the h-IAP1 experiment (Fig 17, Left panel) indicated that its expression was up-regulated by 15 hours of infection, and that the expression of the gene increased with increasing time of D2V infection. Un-detectable levels of h-IAP1 were found in control samples. The expression of 2'-5' OAS was also increased with increasing time of D2V infection in HUVEC cells (Fig 18). As early as 15 hours post-infection, an increase in the expression of 2'-5' OAS gene was observed. In two independent experiments the results were almost identical.



**FIGURE 17.** Time course RT-PCR study for expression of h-IAP1 in HUVEC. D2V,NGC infected and control cells were incubated with D2V,NGC for 15, 30 and 50 hours and total RNA was isolated. IL-8 was used as a positive marker for infection, and the ribosomal RNA, L35a as a housekeeper gene. A,B represents two independent experiments.



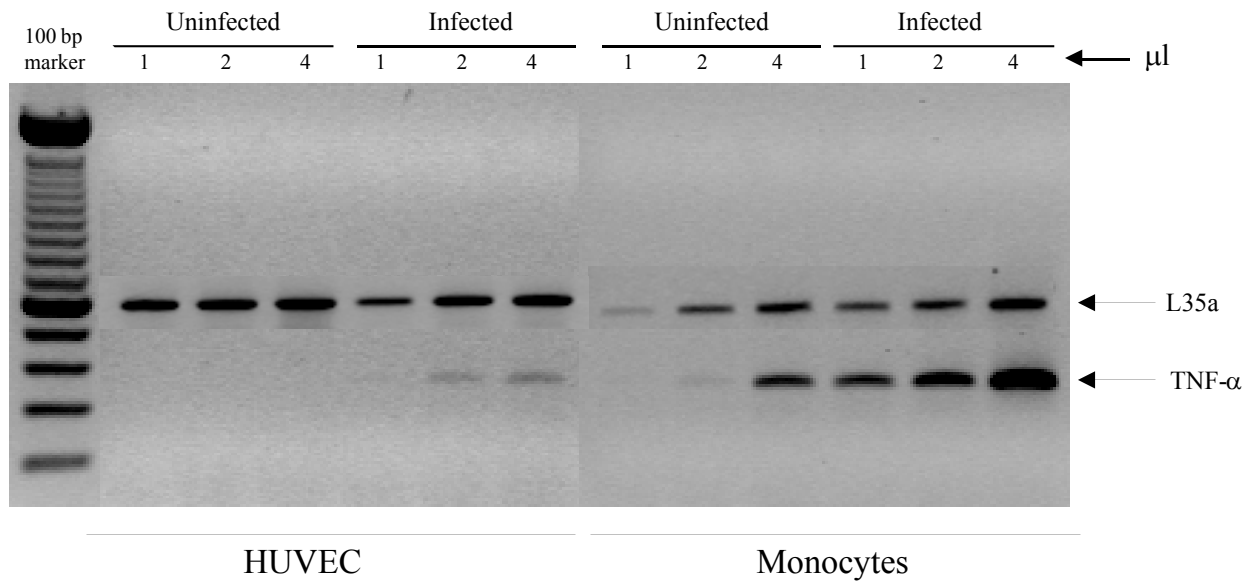
### 2-5 Oligoadenylate Synthetase

**FIGURE 18.** Semi-quantitative analysis to study expression of 2-5 OAS at different time points of D2V,NGC infection. HUVEC cells were infected with D2V,NGC for 15, 30 or 50 hours. RT-PCR was performed on total RNA extracted from infected and uninfected cells. The constitutively expressed ribosomal gene L35a was used as control. Two independent experiments were done (A,B)

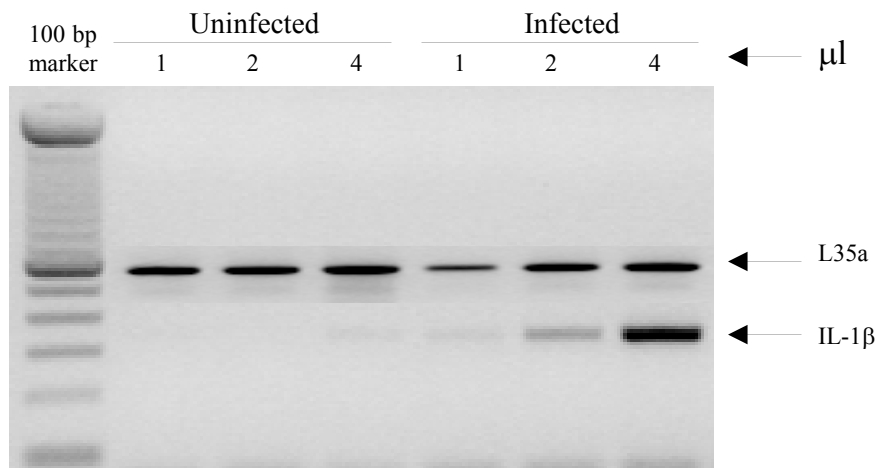
### ***TNF- $\alpha$ Expression in HUVEC Cells and Monocytes***

In previous experiments (Ballard et al, 1997) where HUVEC cells were stimulated with TNF  $\alpha$ , an up-regulation in the expression of h-IAP1 was observed. So we were interested to know if the up-regulation of h-IAP1 expression in HUVEC during D2V infection involved higher TNF  $\alpha$  expression. To find out whether TNF  $\alpha$  expression increased during D2V infection, a semi-quantitative RT-PCR was performed and L35A was used as a housekeeper gene. The data from the experiment (Fig 19) confirmed that TNF  $\alpha$  expression was increased 3.82 fold when HUVEC cells were infected with D2V (Left panel) and 10.94 fold when monocytes were infected with D2V (Right panel).

It has also been shown previously that stimulation with IL1- $\beta$  also increased the expression of h-IAP1 gene (Bannerman et al., 2002) (Wang et al., 1996) (Wrighton et al., 1996). Hence, we performed a semi-quantitative RT-PCR using different quantities of uninfected and D2V infected HUVEC cDNAs (0.5, 1 and 2  $\mu$ l) in the PCR step to compare expression of IL1- $\beta$ . The data from the experiment (Fig 20) showed a 10.23 fold induction in the expression of IL1- $\beta$  (lower band in the Fig 20) in D2V infected HUVEC samples.



**FIGURE 19.** D2V,NGC infection(48 hours) induces TNF  $\alpha$  expression in HUVEC and monocytes. The different amounts of cDNA 1 ,2 and 4  $\mu$ l were used in each group. TNF  $\alpha$  over-expression in lane 4 was 3.82 fold in HUVEC and 10.94 fold for monocytes.

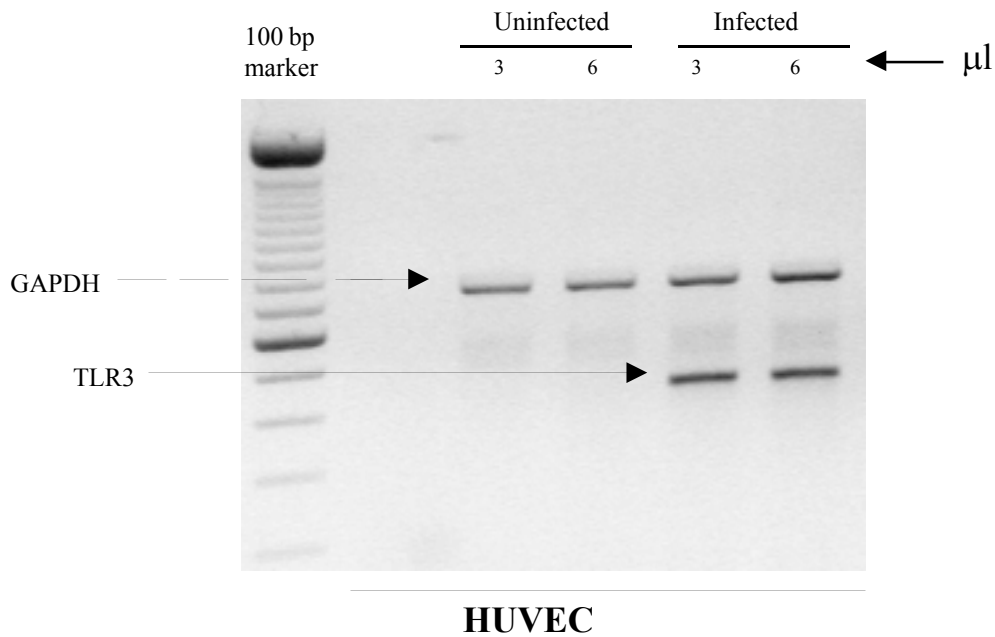


**FIGURE 20.** Quantification of IL-1 $\beta$  up-regulation in D2V,NGC infected HUVEC cells (48 hours). The RT-PCR product was subjected to agarose gel electrophoresis. L35a was used as loading control. 1 ,2 and 4  $\mu$ l of cDNA were used in the PCR reaction.



### ***TLR (1-5) Expression***

We found in the present work that D2V infection of HUVEC cells increases the expression of 2-5 OAS which is one of the three pathways through which dsRNA recognition leads to IFN- $\alpha/\beta$  signaling pathway activation, the other two pathways being Protein kinase-R (PKR) and Mx. Toll-like receptor 3 (TLR 3) has been shown to recognize dsRNA and activate NF- $\kappa$ B. Hence we wanted to find out if TLR 3 (or any other TLR from 1-5) was regulated during D2V infection in HUVEC and Monocytes. A multiplex RT-PCR reaction was performed (Fig 21) to find out which of the TLR from 1-5 were involved in D2V infection. We found that only TLR3 expression was up-regulated 4.07 times during D2V infection in HUVEC but not in monocytes (data not shown) while in monocytes only the expression of TLR2 was increased 1.85 times (data not shown) and this was not seen in HUVEC cells (Fig. 21). Hence in HUVEC cells dsRNA recognition by TLR 3 that activates NF- $\kappa$ B via a Myd88 dependent pathway might lead to production of pro-inflammatory cytokines during D2V infection.



**FIGURE 21.** Multiplex PCR for expression of Toll-like receptors (TLR)1-5 in uninfected and D2V infected HUVEC and Monocytes. cDNA template was obtained from a RT step performed on total RNA isolated from control and D2V,NGC infected cells 48 hours post infection. GAPDH is given as a control for equal loading. In the PCR step two different quantities of cDNA were used (3, 6 μl). The solid arrows indicate the TLR genes that were up-regulated during D2V,NGC infection in HUVEC and monocytes. The dotted arrow indicates constitutive expression of GAPDH.

## DISCUSSION

A Differential Display (DD) technique was used for gene profiling in a ECV 304 and in HUVEC. Gene expression was compared between un-infected, samples to which dengue 2 viral RNA was added during cDNA synthesis (viral RNA spiked) and dengue 2 virus infected ECV 304 cells. We have incorporated the novel approach of spiking the uninfected RNA samples to avoid false positive results. h-IAP1 sequence identity was confirmed by BLAST searches on the combined Genbank/EMBL nonredundant (nr) and expressed sequence tag libraries (dbEST), accessed through the National Center for Biotechnology Information homepage (<http://www.ncbi.nlm.nih.gov/BLAST>). In the initial experiments, the majority of differentially displayed bands turned out to be amplified pieces of genomic DNA. This problem was solved once we started treating the extracted RNA samples with DNaseI (Martin and Pardee, 1999). This indicates that it might be essential to avoid minor amounts of genomic DNA. After using 41 different arbitrary primers in ECV 304 cells human inhibitor of apoptosis-1 (h-IAP1) was the only gene found to be differentially expressed during D2V infection. For HUVEC cells in contrast, with 16 different arbitrary primers we found 7 genes to be differentially expressed during D2V infection. In terms of levels of infection, we detected 10% infection with D2V NGC for HUVEC (Fig 12), and similarly 8% infection for ECV 304 cells. HUVEC cells were a better model system than ECV 304 to study gene profiling during D2V infection. Also, we had decreased background and less non-specific amplification when Titanium® Taq DNA polymerase was used instead of Taq DNA polymerase (Bosch et al., 2000). Hence, we suggest the use of Titanium® Taq DNA

polymerase for future experiments while studying gene profiling in endothelial cells. The list of the differentially expressed genes that found identifications when queried by BLAST search were h-IAP1, 2'-5' OAS, 2-5 OAS-like, ESDN, RGS2, Mx1, MxA and Gal-9.

In a previous study differential display technique was performed to identify mRNAs with enhanced expression levels in cytomegalovirus-infected cells as compared to mock-infected cells. They found that cytomegalovirus induced expression of interferon-responsive mRNAs (Zhu et al., 1997).

h-IAP1/ cIAP2 belongs to the family of human inhibitors of apoptosis (IAP). The family of human IAP genes (six genes including xIAP, h-IAP1, h-IAP2/ cIAP1, survivin and nIAP) was first described in baculovirus, and they share the baculovirus IAP repeat (BIR) which seems to be essential and sufficient to cause the anti-apoptotic effect (Deveraux and Reed, 1999; Vucic et al., 1998). IL1- $\beta$ , TNF  $\alpha$  and lipopolysaccharide stimuli, which activate NF- $\kappa$ B, have been shown to up-regulate the expression of h-IAP1 in HUVEC (Stehlik et al., 1998). Hence, h-IAP1 might not only have an anti-apoptotic but also a pro-inflammatory effect. It has been demonstrated that flaviviruses, including dengue, elicit a response in which NF- $\kappa$ B dependent expression of cytokines occurs, and that NF- $\kappa$ B regulates the expression of anti-apoptotic genes (including h-IAP1) through the TNF  $\alpha$  pathway to protect the cells from apoptosis (Hong et al., 2000). It is also known that h-IAP1 modulates the TNF  $\alpha$  induced NF- $\kappa$ B activity to inhibit apoptosis (Bosch et al., 2002; Chu et al., 1997; Stehlik et al., 1998). Hence, there is a positive feedback mechanism operating between NF- $\kappa$ B and h-IAP1 that contributes to cell survival following TNF stimulus, initially in which NF- $\kappa$ B activates the expression of h-

IAP1, and in turn h-IAP1 interacts with I $\kappa$ B $\alpha$  (a cytoplasmic inhibitor of NF- $\kappa$ B) and stimulates its proteolytic breakdown to release free NF- $\kappa$ B. This free NF- $\kappa$ B is subsequently translocated to the nucleus and further activates h-IAP1 expression (Chu et al., 1997). h-IAP1 has also been shown to interact with TNF-receptor associating factor 2 (TRAF2) and increase its expression in the absence of TNF  $\alpha$  stimulation (Chu et al., 1997; Rothe et al., 1995; Uren et al., 1996). Overall, in the TNF signaling pathway, h-IAP1 seems to potentiate its own expression, by interacting with TRAF2, a cytoplasmic protein and, by phosphorylation of I $\kappa$ B using the c-terminal domain of h-IAP1, which in turns leads to the activation and translocation of NF- $\kappa$ B. Once expressed h-IAP1 exerts its anti-apoptotic effect downstream in the TNF  $\alpha$  signaling pathway by stimulating proteolytic cleavage of terminal caspase-3, -7 and cytochrome-c induced caspase-9 thereby inhibiting protein degradation and caspase-mediated apoptosis (Deveraux et al., 1998; Lee and Collins, 2001; Roy et al., 1997). We found the h-IAP1 gene to be up-regulated 8.1 fold in HUVEC during dengue 2 virus infection. h-IAP1 along with the other members of the IAP family of proteins has been shown to suppress the cell death response to viral infection. Inhibition of apoptosis involving h-IAP1 may be a possible mechanism by which apoptosis is controlled in HUVEC cells during infection, and consequently viral persistence and inflammation prevail. Hence from the DD results obtained we can conclude that D2V over expresses h-IAP1 in endothelial cells, which may help to overrule the apoptotic signals and increase cell survival.

It is known that NF- $\kappa$ B up-regulates the expression of h-IAP1 by TNF  $\alpha$  stimulation. IL1-  $\beta$  has also been shown to up-regulate expression of h-IAP1 in HUVEC

cells (Wang et al., 1996; Wrighton et al., 1996). This up-regulation is very likely to be NF- $\kappa$ B dependent because IL1- $\beta$  activates NF- $\kappa$ B through the kinase cascade. Since h-IAP1 was up regulated during D2V infection we investigated whether D2V infection leads to higher levels of TNF  $\alpha$  and IL1- $\beta$  mRNA. There was an increase in the expression of TNF  $\alpha$  in HUVEC as well as in primary human monocytes and of IL1- $\beta$  in HUVEC after D2V infection. Previous studies have shown that dengue infection elicits a response, which leads to NF- $\kappa$ B dependent over expression of cytokines (Gagnon et al., 1999). Both IL-1 $\beta$  and TNF  $\alpha$  are up regulated during D2V infection, which would in theory indicate that the expression of hIAP-1 could be caused by the upstream effect of IL-1- $\beta$  and TNF  $\alpha$ . We found that TNF  $\alpha$  and IL1- $\beta$  expression increased 3.8 fold and 10-40 fold respectively. TNF  $\alpha$  and IL1- $\beta$ , pro-inflammatory cytokines could contribute to vascular leakage at the site of inflammation in dengue infection and regulate the expression of proteins that modulate inflammatory response (Reynolds et al., 2002). Hence, the up-regulation of TNF  $\alpha$  and IL1- $\beta$  may be partly responsible for the plasma leakage during D2V infection.

### ***Antiviral Response***

The expression levels of 2'-5' OAS, Mx1 and MxA were elevated during D2V infection in HUVEC. All 3 of these genes are involved in recognition of dsRNA and belong to the IFN-induced antiviral pathway that provides an early line of defense against viral infections. Mx1 and MxA proteins, which belong to the Mx pathway, and the 2'-5' OAS gene, which belongs to the 2'-5' OAS pathway, are induced preferentially by the

IFN-type1 pathway (IFN  $\alpha/\beta$ ). In the presence of dsRNA 2'-5' OAS binds to inactive 2'-5' OAS dependent RNase (RNase L) and converts it to activated RNase L, which is a reversible process. RNase L leads to extensive degradation of viral RNA and selectively blocks viral replication (Hovanessian, 1991). Mx proteins (MxA, Mx1), which belong to the family of GTPases, have been shown to impair viral growth at the transcriptional level and might also block the intracellular trafficking of nucleocapsids (Hefti et al., 1999; Horisberger, 1992; Horisberger, 1995; Landis et al., 1998; Muller et al., 1992). Our findings indicate that 2'-5' OAS, Mx1, and MxA are up-regulated 72.85, more than 2.9 and more than 100 fold respectively.

The finding that 2'-5' OAS system is activated during D2V infection is of particular importance because genetically deficient PKR-RNase L mouse cells retained sensitivity to inhibition of IFN suggesting that IFN response during dengue virus infection is 2'-5' OAS and PKR independent (Diamond and Harris, 2001). NS5A from hepatitis C virus (HCV) (Flavivirus) has been shown to inhibit IFN-induced PKR to maintain global mRNA translation rate during early infection (He et al., 2001). This indicates that instead of a PKR independent IFN response, NS5 protein from dengue virus might be blocking IFN-induced PKR to favor higher levels of viral replication.

The up-regulation of genes involved in the type-1 IFN pathway is important because the IFN system (innate primary response) plays a role in limiting viral spread before the activation of the immune system involving T-cell and B-cell response in dengue virus infection.

2'-5' Oligoadenylate synthetase-like gene (OASL) is highly homologous to 2'-5' OAS (OAS). It is a 56-kDa protein, which shares the functional domain with the OAS

proteins, binds DNA and dsRNA but it cannot activate RNase L to cause viral RNA degradation like the 2'-5' OAS genes (Hartmann et al., 1998; Hovnanian et al., 1999; Rebouillat et al., 1998). The catalytic activity OASL is still not known. We found 2'-5' OAS to be up-regulated more than 100 fold in HUVEC cells during D2V infection.

TLR3 has been shown to recognize dsRNA, activate NF- $\kappa$ B and induce cytokine production through the MyD88 dependent pathway (Alexopoulou et al., 2001). It is also known that NF- $\kappa$ B regulates the expression of h-IAP1, hence TLR3 could also be involved in the increased expression of h-IAP1 found here. dsRNA recognition by TLR3 also stimulates IFN type1 pathway in endothelial cells (Miettinen et al., 2001). Hence, TLR3 might be involved in induction of cytokines and the anti-viral pathway in endothelial cells. In our experiments, TLR3 was up regulated approximately 4 fold in HUVEC cells during D2V infection.

MxA, Mx1, 2'-5' OAS and TLR3 recognize dsRNA and induce IFN type1 pathway. So theoretically TLR3, 2-5 OAS, MX1 and MXA could block the translation of D2V viral genes.

The Regulator of G-protein signaling- 2 (RGS2) is a member of the Regulators of G-protein signaling proteins that regulate G-protein linked signaling pathways by enhancing the hydrolyses of GTP to GDP thus limiting the time of activation. RGS2 also induces T-cell proliferation. Cell stress has been shown to induce higher RGS2 mRNA levels (Kehrl and Sinnarajah, 2002; Song et al., 2001). It was found to be up regulated



3.9 fold in infected HUVEC cells. The Mx genes, which belong to the type-1 IFN pathway, have GTPase activity (GTP → GDP) and protein-protein interaction interactions, which indicates their importance in regulating signaling through the G protein-coupled receptor (GPCR) and protein trafficking (Horisberger, 1992). It has been demonstrated that Kaposi's sarcoma herpesvirus (KSHV) encodes a G protein-coupled receptor (GPCR) that induces endothelial cells to constitutively express NF-κB and increased secretion of the chemokine IL-8 (Shepard et al., 2001). It is important to note that all chemokines like IL-8 act via seven-transmembrane domain receptors that are coupled to heterotrimeric GTP-binding proteins (GPCR) (Loetscher and Clark-Lewis, 2001). Regulators of G-protein signaling (RGS) negatively regulate signaling through G-protein coupled receptors. We found that RGS2 expression was up-regulated 3.9 fold during D2V infection in endothelial cells. Classical symptoms of dengue virus infection are inflammation and plasma leakage. Hence, we postulate that RGS2 expression might be up-regulated in HUVECs in response to dengue virus infection to regulate chemokine secretion which are mediators of inflammation and also to potentiate signaling through Mx pathway similar to T-cells.

Among other genes that were differentially expressed during HUVEC D2V NGC infection was ESDN. ESDN, which was recently discovered, is a novel neuropilin-like type-1 transmembrane protein that is considered to regulate vascular cell growth. Neuropilins mediate chemorepulsive as well as chemoattractive signals. In endothelial cells neuropilin-1 acts as a co-receptor for VEGF, enhancing VEGF's biological function. Also, ESDN expression is up-regulated in injured arteries and its up-regulation causes decreased cell growth (Kobuke et al., 2001). ESDN expression was found to be down

regulated 2.2 fold in infected HUVEC cells. The function of ESDN is not clearly known. It might involve chemoattractive or chemorepulsive responses during D2V infection of HUVEC cells. But, the significance of its down-regulation is not quite clear. This fluctuation in expression deserves further study.

Lastly, Gal-9 belongs to the family of Ca<sup>2+</sup> dependent galactoside-binding lectins that modulate a variety of cellular activities such as cell proliferation/cell death, cell adhesion and chemoattraction (Barondes et al., 1994; Lahm et al., 2000; Matsumoto et al., 1998; Oda et al., 1991; Paroutaud et al., 1987; Tureci et al., 1997; Wada and Kanwar, 1997; Wada et al., 1997). Gal-9 is an eosinophil chemoattractant expressed in HUVEC cells and in immune cells like macrophages, B and T-cells and mast cells (Chabot et al., 2002). Hence, Gal-9 is one of the chemoattractments that accumulates eosinophils to the site of inflammation. The pro-inflammatory cytokine IL1- $\beta$  (itself up-regulated during dengue virus infection) has been shown to enhance the expression of Gal-9 (Yoshida et al., 2001). Dengue virus infection leads to inflammation and plasma leakage in vascular endothelia. This might be a result of action of cytokines and cationic chemokines that act on the vascular endothelium and cause transient leakage. Gal-9 was found to be up regulated 4.7 fold in D2V infected HUVEC cells. Hence, there is a possibility that Gal-9 is responsible for the accumulation of eosinophils at the surface of vascular endothelium leading to inflammation and plasma leakage during dengue virus infection. In fact, it is possible that IL1- $\beta$  leads to over expression of Gal-9 in HUVEC cells during D2V infection. Gal-9 has also been described as a urate transporter / channel (Spitzenberger et al., 2001).

Most of the genes found by DD to be regulated at the level of mRNA can potentially explain in part the pathogenesis of dengue virus infection in HUVEC cells. The three main pathways found to be activated during D2V infection in this thesis are, first the TNF  $\alpha$  signaling pathway, second one, the IL1- $\beta$  signaling pathway, and third, the type-1 IFN pathway (IFN- $\alpha$  and IFN- $\beta$ ). The genes involved in the TNF  $\alpha$  signaling pathway that we found to be up-regulated are TNF  $\alpha$  itself and h-IAP1. h-IAP1 expression is transcriptionally controlled by NF- $\kappa$ B, which regulates expression of pro-inflammatory cytokines like TNF  $\alpha$  and IL1- $\beta$  (Bannerman et al., 2002; Chu et al., 1997; Hong et al., 2000; Stehlik et al., 1998). Hence, dengue virus might be activating these two genes to induce a pro-inflammatory and an anti-apoptotic response. IL1- $\beta$  can also activate h-IAP1 expression (Wrighton et al., 1996). IL1- $\beta$  expression leads to activation of NF- $\kappa$ B and hence could potentially regulate h-IAP1 expression. Hence, to identify the function of h-IAP1 expression during D2V infection further study is required. IL1- $\beta$  also has been shown to enhance expression of Gal-9, which is an eosinophil chemoattractant (Yoshida et al., 2001). Hence, the possibility that IL1- $\beta$ , a pro-inflammatory cytokine, might up-regulate expression of Gal-9 and h-IAP1 makes its regulation during dengue virus infection very important. Moreover, TNF  $\alpha$  and IL1- $\beta$  has been shown to induce significant microvascular leakage in a recent study (Reynolds et al., 2002). Plasma leakage is clearly involved during dengue virus infection. Overall from previous data about the TNF  $\alpha$  and IL1- $\beta$  pathway, and the findings in our experiments, indicate that these two pro-inflammatory cytokines might play a very important role during dengue virus infection. The type-1 IFN pathway might be activated as an anti-viral mechanism to fight against D2V infection before the immune response involving T-cell and B-cell

activation occurs which will help to control the spread of viral infection. Contrary to earlier findings, we found the 2'-5' OAS pathway gene to be up-regulated during D2V infection along with Mx pathways genes, but we still do not know whether PKR, the 3<sup>rd</sup> pathway that belongs to the type-1 IFN pathway is activated due to D2V infection in HUVEC cells. The study identifies three pathways activated in HUVEC during D2V infection not known previously (Fig. 22).

The rationale for the activation of antiapoptotic pathway during the viral infection is to allow for the virus to replicate before pathogenic responses destroy the host cell. The time in which apoptosis is inhibited would serve the virus to complete its cycle of infection and form viable particles that are passed to neighboring cells.

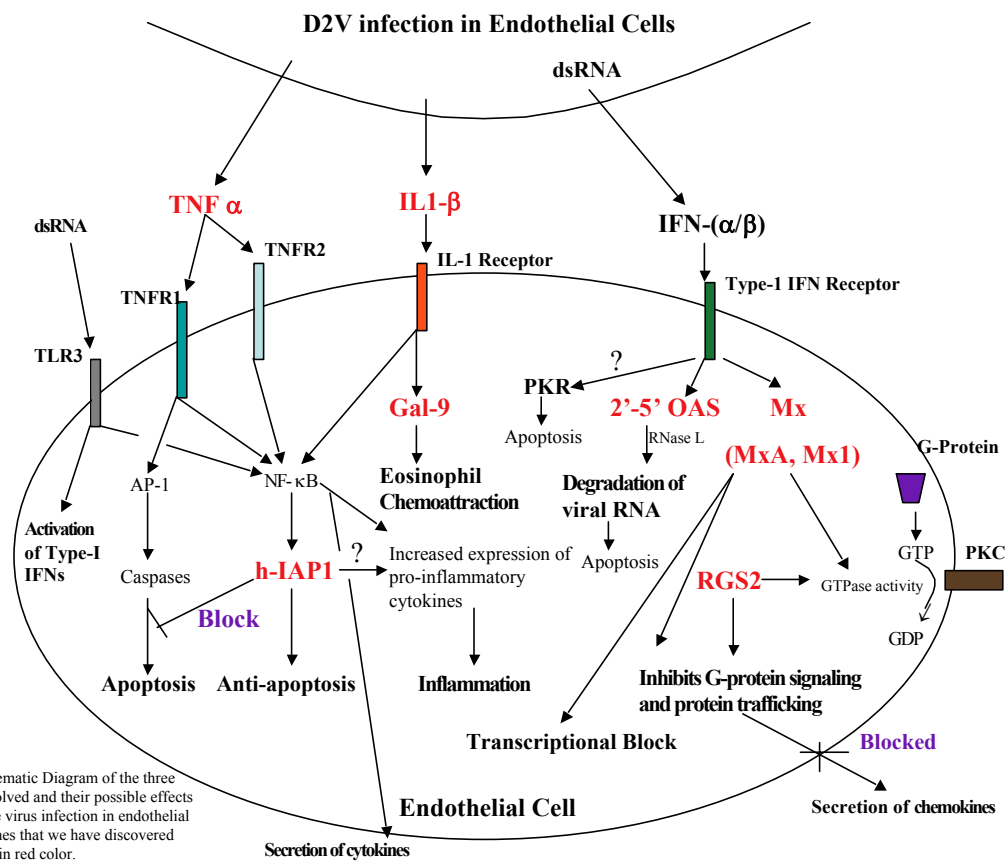
On the other hand, if there is a limit to the infectivity of the virus, eventually, the viral load would be high enough to cause cellular death. Therefore, there are antiviral mechanisms in place, like the type-I IFN response, the 2'-5'OAS the Mx (MxA and Mx1) genes whose expression can target viral destruction by uncapping the viral RNA and interfere with translation. There must be a fine tuning of those mechanisms that the virus has evolved to allow its own replication as well as those mechanisms that are evolved to control the progression of the infection. As a product of the first type of viral interaction, inflammation occurs, which in turn activates the antiviral response through IFN  $\alpha/\beta$ . The second type of response, the one directed toward downplaying the cellular response, would be signaling through G protein coupled receptors. G protein coupled receptors, necessary for chemokine secretion are regulated by RGS family of proteins. RGS2, a member of the RGS family of proteins was found to be upregulated by dengue virus

NGC infection. This may be a way in which the cell down regulates the inflammatory response once it has activated during dengue virus NGC infection.

The infected host has two roles:

To activate the innate response, by increasing the IFN Type-1 IFN ( $\alpha/\beta$ ) response, 2'-5' OAS, MxA and Mx1 expression to generate antiviral response to block infection. Also, to stimulate the immune system to generate an immune response and to cause inflammation and stimulation of the immunological effectors cells. Therefore, the infected cell is responsible of controlling the infection, directly and indirectly through antibody and cell mediated response.

Hence, for future experiments it is important to study the activation of PKR pathway and its phosphorylation to study if PKR is involved during dengue virus infection. Also, perform co-immunoprecipitation studies for NS1 and NS5 proteins from D2V NGC to see if it regulates Type-I IFN pathway by interacting and blocking PKR pathway. It is interesting to study if the genes that were differentially expressed in HUVECs during D2V infection are also differentially expressed in other Flavivirus infection (e.g. West Nile, Yellow Fever).



**Fig.22** Schematic Diagram of the three pathways involved and their possible effects during dengue virus infection in endothelial cells. The genes that we have discovered using DD are in red color.

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