2008-04-14

Molecular Dissection of the Cellular Reponse to Dengue Virus Infection

Rajas V. Warke
University of Massachusetts Medical School

Molecular Dissection of the Cellular Response to Dengue Virus Infection

A Dissertation Presented

By

Rajas V. Warke

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

In

IMMUNOLOGY AND VIROLOGY

APRIL 14, 2008

Molecular Dissection of the Cellular Response to Dengue Virus Infection

A Dissertation Presented

By

Rajas V. Warke

Approved as to style and content by:

Paul Clapham, Ph.D., Chair of Committee

Neal Silverman, Ph.D., Member of Committee

Christopher Sassetti, Ph.D., Member of Committee

Ana-Fernandez-Sesma, Ph.D., Member of Committee

Irene Bosch, Ph.D., Thesis Advisor

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Program in Immunology and Virology

April 14, 2008

COPYRIGHT NOTICE

Parts of this dissertation have appeared in separate publications:

Warke RV, Xhaja K, Martin KJ, Fournier MF, Shaw SK, Brizuela N, de Bosch N, Lapointe D, Ennis FA, Rothman AL, Bosch I. Dengue virus induces novel changes in gene expression on human umbilical vein endothelial cells. J Virol. 2003 Nov;77(21):11822032.

Ramirez-Ortiz ZG, Warke RV, Pacheco L, Xhaja K, Sarkar D, Fisher PB, Shaw SK, Martin KJ, Bosch I. Discovering innate immunity genes using differential display: a story of RNA helicases. J Cell Physiol. 2006 Dec;209(3):636-44.

Warke RV, Martin KJ, Giaya K, Shaw SK, Rothman AL, Bosch I. TRAIL is a novel antiviral protein against dengue virus. J Virol. 2008 Jan;82(1):555-64.

Becerra A, Warke RV, de Bosch N, Rothman AL, Bosch I. Elevated levels of soluble ST2 protein in dengue virus infected patients. Cytokine. 2008 Feb;41(2):114-20

Warke RV, Becerra A, Zawadzka A, Schmidt D, Martin KJ, Giaya K, Dinsmore JH, Woda M, Hendricks G, Levine T, Rothman AL, Bosch I. Efficient Dengue Virus (DENV) Infection of Human Muscle Satellite Cells Up-Regulates Type I IFN Response Genes And Differentially Modulates MHC I Expression on Bystander AND DENV-infected Cells. J Gen Virol. In Press

Becerra A, Warke RV, Martin KJ, Xhaja K, de Bosch N, Rothman AL, Bosch I. Role of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in the inhibition of proinflammation induced by DENV. Submitted to Infection and Immunity for review.

ACKNOWLEDGEMENTS

After I finished my thesis defense talk, many professional colleagues told me that I should be given the award for the best and the longest acknowledgements in the history of UMASS Medical School. I was happy to hear that since I consider myself lucky enough to have family and friends who have helped me along every step of the way.

As I contemplate on the number of people who have made this possible for me, I realize how important these people have been in my success.

First and foremost, I will like to thank Dr. Francis Ennis for reminding me always to look at the "BIG PICTURE" while designing experiments and interpreting the implications of experimental data.

I would like to thank Dr. Irene Bosch, my thesis advisor, for giving me the freedom to think outside the box. I was always allowed to question any scientific hypothesis as long as the alternative was logical. I am quite amazed at her fighting spirit and her capacity to absorb and learn from failure and continue her scientific endeavor to understand neglected diseases and hopefully help find treatment to diseases. Hopefully, I have learned these virtues from her.

Dr. Alan Rothman has also been instrumental is my success during my graduate research. He always made sure that my feet were grounded and I was not over-interpreting the experimental data. Alan has instilled qualities like asking questions, hard work and ethics in me.

I am grateful to my TRAC committee members for guiding me to stay focused and answer questions using scientifically sound experiments. They always told me that I was underselling my data and the amount of work I had done. These encouraging remarks always kept me motivated.

I would also like to acknowledge all the past and present members of CIDVR who have been my family for the last six years supporting me personally and scientifically through my journey at UMASS Medical School. I cannot thank them enough.

I will also like to thank Kris Giaya who practically runs the lab since my advisor traveling quite often. I would like to thank Kris for guiding through my personal issues. Also, I am grateful to Kathy J. Martin without whom the project success would not have been possible.

I am quite lucky to have had great friends around me during all stages of my education supporting me and helping me stay steady during times of failure. I will miss Diego a lot for all the FUN, agreements, disagreements, discussions and sometimes

arguments we have had over very diverse tips over the last eight years. Also, I will miss Alex, Hema and Serena for their support and criticism for the time they have known me.

I am grateful to Dan and Erin for accepting me as I am. I will miss them a lot when I go back to India. I thank them for unconditionally supported me through my troubled times.

To Sheetal Chaudhari, I salute for agreeing to take the journey of life with me as her partner and believing in me more than I believe in myself. She is my strongest supporter and my steadfast partner.

Finally but not the least, I would like to thank my parents and family for unconditionally loving me and for never giving up on me. My sister always told me what Thomas Edison had quoted “Genius is 99 percent perspiration and one percent inspiration”. I would like to thank my uncle Dr. Ghanashyam Patil for reminding me the famous words “The harder I work, the luckier I get” by Henry Ford. I would be nothing without my parents support.

Today when I look back at my graduate school, I realize that in any journey, the journey itself is more important and than the result.

ABSTRACT

The immune response to viral infection involves a complexity of both innate and adaptive pathways at the cellular and the molecular level. There are many approaches to begin to define the pathways at work to control viral pathogenesis. The approach favored in this thesis was to conduct a broad screen of the innate immune response at the gene expression level of infected cells.

The innate immune response is critical to the control of viral infections. Type I interferons (IFN), IFNα and IFNβ, are antiviral proteins that are an integral part of the innate immune response. Furthermore, by virtue of their effects on maturation and activation of antigen-presenting cells, IFNs are a pivotal link between the innate and adaptive immune systems. Most cell types produce type-I IFN when exposed to viruses. However, viruses have evolved multiple strategies to suppress IFN production or signaling. It is imperative to understand the virus-host interaction at the molecular level in order to identify as yet unknown mechanisms of the host antiviral response; these additional pathways may be useful in counteracting the viral suppression of IFN. Type-I IFNs regulate expression of at least five hundred genes, suggesting a complex network of signaling pathways. Depending on the cell type different proteins regulate the induction of IFN or the expression of IFN-inducible genes. Identification of proteins that induce selected IFN-inducible genes may provide synergistic activity with or may have an advantage over type-I IFN for anti-viral therapy in the future.

Many diseases are untreatable if identified late in their progression. In resource-limited countries, many diseases are diagnosed clinically, which can lead to incorrect or delayed diagnosis and treatment. The identification of biomarkers of disease has the

potential to guide the correct therapy in a timely fashion. The objective of this thesis was to identify novel anti-viral therapies and disease biomarkers for dengue virus (DENV) infection.

DENV is a mosquito-borne positive-sense single-stranded RNA virus, which causes an estimated 50 million infections annually. Most DENV infections result in a febrile illness called Dengue fever (DF). Less frequently, infections cause Dengue hemorrhagic fever (DHF), a potentially fatal vascular leakage syndrome associated with the production of pro-inflammatory cytokines. At present patients infected with DENV can only be treated by intravenous fluid support to prevent hypovolemia and hypotensive shock. This treatment is less effective in severe cases if the diagnosis is delayed. Identification of therapeutics with both antiviral and immune-modulatory activity may lower patient mortality and reduce the burden of DENV on society.

DENV infection is cleared in most individuals after a short period of viremia {Libraty, 2002 #2225}. Based on in vitro and mouse models, type-I and type-II IFN signaling pathways are thought to be critical in the regulation of DENV infection. Higher serum levels of type I and type II IFNs during acute DENV infection in patients lend support to the above hypothesis {Kurane, 1993 #2152; Libraty, 2002 #2225}.

To understand the DENV-human host cell interaction at the molecular level, we performed global gene expression analysis on DENV-infected primary human cells using Affymetrix GeneChips (HG-U133A). We studied dendritic cells (DC), monocytes, B cells and human umbilical vein endothelial cells (HUVECs), all of which are known to be permissive to DENV infection. We first identified genes commonly regulated in multiple cell types in response to DENV infection; we hypothesized that understanding this

common gene expression profile would identify signaling pathways involved in regulation of viral spread, activation of immune cells or induction of inflammation. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), one of the 23 common response genes, was identified as a key link between type I and type II interferon response genes. Pretreatment of cells with recombinant TRAIL (rTRAIL) inhibited DENV replication in monocytes, B cells, HUVECs and DCs. Using the DC infection model, we showed that this inhibition of viral replication was apoptosis-independent. Type-I IFN receptor (IFNR) blocking experiments showed that signaling through the type-I IFN receptor played an important role in the antiviral activity of exogenous rTRAIL. Furthermore, TRAIL also significantly reduced the expression of mRNA and protein of pro-inflammatory cytokines (TNFα, MIP-1β and IFNα) and chemokines (MCP-2, IP-10 and IL-6) in response to DENV infection. The data that TRAIL inhibits both viral replication and pro-inflammatory cytokine production suggest that TRAIL has therapeutic value in dengue.

The endothelial cell is the site of pathology in DENV infection in vivo (vascular permeability and plasma leakage). To understand the direct effect of DENV infection on endothelial cells and its role in the induction of genes regulating vascular permeability, we compared gene expression in DENV-infected HUVECs to that of uninfected cells and cells infected with other RNA and DNA viruses, including flaviviruses (West Nile, yellow fever, and Japanese encephalitis viruses), bunyaviruses (Sin Nombre and Hantaan viruses), Epstein-Barr virus and vaccinia virus. Among the genes confirmed for their differential expression, ST2 (Interkeukin-1 receptor-like-1 protein-IL1RL1) and indoleamine 2,3-dioxygenase (IDO) were identified to be upregulated specifically in

response to DENV infection. Higher serum soluble ST2 (sST2) levels were detected in DENV-infected patients than in patients with other febrile illnesses (OFI) at the end of the febrile stage and at defervescence ($p=0.0088$ and $p=0.0004$, respectively). In addition, patients with secondary DENV infections had higher serum sST2 levels compared with patients with primary DENV infections ($p=0.047$ at the last day of fever and $p=0.030$ at defervescence). Higher levels of IDO activity ($p<0.02$) were also detected in dengue patient serum during the febrile stage of the disease as compared to OFI patient serum. Treatment of cells with 1-methyl-tryptophan, an antagonist of IDO, reversed the inhibitory effect of IFNγ on DENV replication, suggesting that IDO activity is important for IFN-γ mediated antiviral function against DENV. These data suggest that sST2 and IDO may have value as biomarkers for acute DENV infection.

In conclusion, global gene expression analysis identified novel proteins with promising characteristics for the treatment and/or diagnosis of DENV infection. Although further studies will be needed to validate the clinical utility of TRAIL, sST2, and IDO, these studies demonstrate the utility of this unbiased genomics approach to identify therapies to currently incurable diseases.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

APC antigen presenting cell

DC dendritic cell

DC monocyte derived dendritic cell

Mo Monocyte

HUVEC human umbilical vein endothelial cell

DC-SIGN dendritic cell-specific ICAM3-grabbing non-integrin

DF Dengue fever

DHF Dengue hemorrhagic fever

DSS Dengue shock syndrome

DENV Dengue virus

WNV West Nile Virus

SNV Sin nombre virus

EBV Epstein barr virus

JEV Japanese encephalitis virus

YFV Yellow Fever Virus

ICS Intracellular cytokine staining

Type-I IFN Type-I Interferon

IFNα Interferon alpha

IFNβ Interferon beta

IFNγ Interferon gamma

TRAIL Tumor necrosis factor related apoptosis inducing ligand

TNFSF10 Tumor necrosis factor superfamily 10

IL1RL1 Interleukin 1 receptor like 1

IDO Indoleamine 2,3-dioxygenase

MHC major histocompatibility antigen

PBMC Peripheral blood mononuclear cells

NS Non-structural

IL Interleukin

MIP Macrophage inflammatory protein

MCP macrophage chemoattractant protein

TNF Tumor necrosis factor

IP Interferon gamma inducible protein

MOI multiplicity of infection

NO Nitric oxide

L-Trp L-Tryptophan

Th T helper cells

HAECs Human aortic endothelial cells

OFI Other febrile illness

EMCV Encephalomyocarditis virus

HCMV Human cytomegalovirus

SD Standard Deviation

ADE Antibody dependent enhancement

Chapter I

Introduction

Dengue - A Disease of Global Importance

Dengue virus (DENV) is an arthropod-borne flavivirus, which has re-emerged as a major global health problem, particularly among children. DENV infection is either an epidemic or endemic health threat in many tropical and sub-tropical areas like South-East Asia, South America and Africa. Each year about 50-100 million people are infected with DENV, 500,000 cases of DHF are hospitalized and 20,000 to 50,000 people die of DENV infection, mostly children (68, 71). Moreover, the lack of proper diagnostics and inability to control mosquito populations makes it a major public health issue in the developing world where this disease is prevalent. No preventative therapy such as vaccines or anti-viral treatments is currently available for DENV infections currently, despite its major impact on the world population. Timely treatment, through intravenous hydration along with close medical monitoring, reduces the mortality due to dengue infections. DENV infection is clinically defined either as dengue fever (DF), a milder form of the disease or dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), more severe and potentially fatal forms of the disease. All four antigenically distinct serotypes of DENV [DENV1, DENV2, DENV3 and DENV4] are able to infect and produce all grades of the disease. Infection with one serotype of DENV induces life-long immunity only to the infecting serotype. Secondary infections with any other serotype (heterologous serotype) of DENV inducing ADE and a heterologous T-cell response has been hypothesized to cause DHF/DSS, the severe forms of the disease. A large

number of people in the tropical countries have already been exposed to DENV infection. This presents complications in the design of an effective vaccine. Currently, the concept of a tetravalent vaccine is being implemented with yet, no implications of protection in humans as of yet.

The mechanisms that underlay the DENV infection induced immune pathology have not yet been deciphered completely. Moreover, the type of cells and tissues infected are being investigated currently. Severe vascular permeability and elevated pro-inflammatory cytokines have been demonstrated during severe dengue infections (97, 229). Since DENV infection-mediated pathology (plasma leakage) is observed when the virus already has disappeared from the systemic circulation, theoretically a strong antiviral agent against DENV administered early in the disease would prevent the occurrence of pathology associated with severe cases of DENV infection. Limiting the levels of circulating virus should also help reduce the transmission of DENV by mosquitoes biting dengue-infected individuals. Hence, identification of novel antiviral agents to treat the disease is a priority for research studies on DENV. In addition, identification of markers of severity, early during infection would help begin the right line of therapy to reduce disease severity.

Role of Innate Immune Response in DENV Infection

Animal models are extensively used to find vaccines and new treatments to various diseases. Due to the lack of a true animal model for DHF it has been challenging to propose effective therapies although vaccine research has taken place in non-human primates. However, the non-human primate model of dengue infection does not provoke the immune activation mediated pathology seen in humans. As a

consequence, research studies on DENV are largely focused on clinical samples (cells, serum or plasma) to find correlates of disease, or *in vitro* infection models. Various research groups are developing a DENV mouse model but DENV-infected mice weakly support virus replication and do not present with clinical symptoms observed in human DENV-infected patients (116). Findings from *in vitro* and mouse studies have suggested that the innate immune response plays an important role in the control of spread of DENV. Moreover, innate immune response cytokines are expressed at higher levels in DENV-infected patient serum and or plasma during the acute stage of the disease. For example, higher IFNα and IFNγ protein levels are detected in DENV patient serum (111, 112). *In vitro* and mouse models of DENV infection have shown that IFNα and IFNγ are important for control of DENV replication (51, 52, 193, 194).

The main objective of the present work was to define the molecular determinants of the cellular response to dengue virus. We hypothesized that gene expression analysis of DENV-infected primary human cells known to be permissive to DENV infection would provide information about host response genes involved in inhibition of viral replication, activation of the immune cells or in disease pathogenesis, by identifying genes that are differentially regulated in response to DENV infection. We selected primary human monocytes, B cells, dendritic cells and endothelial cells (HUVECs), which are known to be targets for DENV infection *in vivo* (92, 104, 107, 117, 230). DENV infection has been previously shown to activate these cells inducing expression and/or release of adhesion molecules, pro-inflammatory chemokines and cytokines (30, 154). However at the individual cell

level, DENV infection induces inefficient maturation and activation of infected monocyte-derived DCs (DCs) and muscle satellite cells, respectively as compared to neighboring uninfected bystander DCs or muscle satellite cells (125, 166)(Warke RV et al. J Gen, Virol,In Press).

In vitro studies in primary human cells and studies in mice have shown that the innate immune response is critical to limit levels of and spread of DENV. Based on these findings, our group postulates that a common innate immune response in the cells susceptible to DENV infection will limit DENV replication and spread. To identify common signaling pathways activated or repressed in monocytes, B cells and HUVECs we isolated RNA from DENV-infected and uninfected cells and then hybridized the labeled RNA to GeneChips and analyzed the gene expression data using gene analysis software. We identified a set of 23 genes commonly upregulated in all cell types studied in response to DENV infection. Most of the genes in the common gene expression profile are regulated upstream either by type-I or type-II IFN signaling pathways. Tumor necrosis factor receptor apoptosis inducing ligand (TRAIL), or Tumor necrosis factor, superfamily member 10 (TNFSF10), was one of the 23 common response genes identified as a potential common linker for most of the common profile genes. TRAIL expression was detected only in DENV antigen-negative DCs (bystander cells) suggesting that TRAIL expression was inhibited in DENV-infected DCs. We further found that TRAIL functions as an antiviral protein during DENV infection in an apoptosis-independent manner (224). In addition, the experiments on mechanism of action of TRAIL antiviral activity suggest that TRAIL inhibits DENV by an IFNα dependent (majority) and independent mechanism.

Furthermore, exogenous recombinant TRAIL inhibited the production of pro-inflammatory chemokines (MCP-2, IP-10 and IL-6) and cytokines (TNFα, MIP-1β), suggesting an anti-inflammatory function for TRAIL as well.

The endothelium is the target site for DENV infection-mediated pathology such as vascular permeability, capillary fragility (evidenced by positive tourniquet test done in patients), bleeding, coagulopathy and hypovolemic shock during the acute phase of DHF/DSS. *In vivo*, the pathophysiology clinically observed is considered to be a result of both direct (viral infection) and indirect (pro-inflammatory cytokines, chemokines released by activated leukocytes) effects on endothelial cells. We were interested in understanding the endothelial cell responses to DENV infection.

With the goal of identifying unique molecular markers of DENV infection in endothelial cells, we performed global gene expression analysis in HUVECs infected with DENV or with other RNA or DNA viruses. We identified a set of 30 genes upregulated mostly in DENV-infected HUVECs. However, some of the genes were also differentially regulated in HUVECs infected with related flaviviruses, Yellow Fever virus (YFV) and hantavirus, a bunyavirus. Among these 33 potential DENV infection response genes, we selected two genes, sST2 and IDO (indoleamine 2,3-dioxygenase), for further study. The dengue patient PBMCs used to confirm the relative specificity of these genes were obtained with NIH approved clinical protocols and consents in the dengue clinics of Venezuela and Thailand, from established collaborations with Dr. Alan Rothman and Dr. Norma de Bosch. We confirmed the specificity of sST2 and IDO upregulation following DENV infection at the mRNA by

quantitative reverse transcriptase PCR (qRT-PCR). Serum sST2 levels (ELISA) and IDO activity (mass spectrometry) were both able to distinguish patients with dengue from patients with other febrile illnesses (OFI) or healthy individuals. In addition, the data suggest that sST2 can differentiate between primary and secondary DENV infections. Blocking IDO enzyme activity by addition of 1-methyl tryptophan, a competitive inhibitor of IDO inhibited a significant level of the IFNγ-mediated antiviral activity against DENV. These results indicate that IDO might play an important role in the antiviral function of IFNγ against DENV.

In summary, we identified novel antiviral molecules, TRAIL and IDO, with possible *in vivo* application as therapeutic agents against dengue. The ability of TRAIL to suppress the production of pro-inflammatory cytokines, which have been implicated in dengue disease pathogenesis, provides further support for its potential therapeutic value. We also identified two potential novel serum markers (sST2 and IDO) for DENV infection. Serum sST2 is the first biomarker described that might allow discrimination of secondary DENV infections from primary DENV infections. These data may improve the prospects of identification and treatment of future DENV infections.

Identification of Novel Cellular Targets for DENV Infection

Symptomatically, patients with dengue often present with general muscle affection as well as severe, persisting myalgia, head-ache and rash (35, 70). Higher serum levels of creatine and elevated creatine-phosphokinase (CPK), which is specifically produced by muscle cells may be elevated in DENV patients (98, 134, 177). Similar to DENV infection, individuals affected with Chikungunya virus

present with persisting myalgia (35, 70, 226). A recent study involving muscle biopsies of acutely infected individuals demonstrated that Chikungunya virus infects muscle satellite cells in patients during acute phase of the disease (165). These findings suggest that myalgia, muscle weakness and elevated serum CPK levels in dengue might be a result of direct viral infection of muscle satellite cells. Hence, we performed flow cytometry, immunoflourescence and plaque assay to determine susceptibility of muscle satellite cells (CD56 and desmin positive) (6) to DENV. Results from these experiments demonstrate that primary human muscle satellite cells are susceptible to *in vitro* DENV infection and also support replication of DENV.

This Introduction gives an overview of the dengue virus, viral genome, life cycle and cells susceptible to DENV infection. A section that explains the aspects of innate and adaptive immune response activated during DENV infection follows. A very brief explanation of the disease, its clinical stages (DF, DHF/DSS) of DENV infection and the host factors involved in protection or pathogenesis during DENV infection are discussed in the next section. An overview of various global gene expression tools and how these tools have been applied to understand dengue virus infection follows. The last section of the Introduction is dedicated to providing previously published information on genes (TRAIL, ST2 and IDO) to put our findings in a global perspective of these protein functions.

Dengue Virus (DENV) Genome and Life cycle

Dengue is the most rapidly expanding arboviral disease in the tropics and subtropics. Almost half of the world's population is estimated to be at risk, with an

estimated 50 million infections a year. One million severe dengue cases were reported for the Americas in 2005. There are currently close to 70 countries reporting dengue cases to the World Health Organization from Asia, Africa and the Americas (157).

Proteins such as GRP78, DC-SIGN, glycosaminoglycans like heparan sulphate, heat shock proteins, CD14-associated molecules and mannose receptor (MR) have been proposed as human cellular receptors for DENV (38, 93, 141, 178, 211). Several studies have shown that the C-type lectin dendritic cell specific adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN, CD209) mediates the high rate of dengue virus infection in DCs, which naturally express DC-SIGN (158, 211).

DENV is an enveloped, single-stranded positive-sense RNA virus. The ~11 kb genome, which is capped at the 5' end, encodes 10 (seven non-structural and 3 structural) proteins. The viral genome is initially translated as a single polyprotein, which is then cleaved into structural (C, PrM/M and E) and non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins by viral and host proteases. DENV is thought to enter the cell via receptor-mediated endocytosis. Following endocytosis, acidification of the endosome initiates the conformational change of E to form trimers that exposes the active site for fusion with the membrane. The inner side of the vesicle membrane and the viral membrane fuse, allowing the formation of a pore which as it opens releases the RNA attached to the viral nucleocapsid into the cytoplasm. Once in the cytosol the viral RNA disassociates from the viral nucleocapsid, which allows for initiation of viral translation. The structural protein E and non-structural (NS) NS1 protein contain signal sequences for insertion into the

ER membrane where host and viral proteases cleave them (20, 202). The PrM and E proteins translocate into the ER lumen while the capsid protein remains associated with the ER on the cytoplasmic side. The remainder of the NS proteins localize in the cytoplasm, also possibly nuclear, in the case of NS5 (173) due to its DNA binding domains. Virion assembly occurs at the ER membrane; the E and M proteins undergo modifications in the Golgi and the infectious viral progeny are exocytosed via secretory vesicles.

Also, stable transfection of DENV non-permissive cells with DC-SIGN or MR makes them susceptible to DENV infection (141). Among the seven NS proteins, which are part of viral genome, NS3 (viral protease, RNA replication and capping of viral RNA) and NS5 (RNA-dependent RNA polymerase, viral RNA capping) are the two largest proteins. The NS1 protein has complement fixing activity and serum levels of NS1 protein correlate with the severity of the disease (9). NS2A protein functions to cleave NS1 and NS2B proteins (83). Also, NS2A, NS4A and NS4B proteins have been shown to interfere with type-I IFN signaling pathway so that the virus can evade the innate immune system (152, 153).

Cellular Targets for DENV Infection

In vivo cellular targets for DENV infection have not been extensively studied. DENV is often not detected systemically when the hemorrhagic symptoms, vascular permeability and plasma leakage occur. Since bleeding and vascular fragility are a part of infection-mediated pathology, collection of biopsy samples of internal organs is not considered safe for the patient. Some autopsy studies have been performed to

identify the cell types susceptible to DENV infection *in vivo*. The limitation of autopsy studies is that not all cell types that support DENV survival during the acute stage of the infection may stain positive for DENV. Monocytes have for a long period of time been considered the primary sites for DENV infection (107). Recently, circulating B cells were shown to be targets for DENV infection in DENV patients (104) and hepatocytes, sinusoidal endothelial cells and lung vascular endothelium also have stained positive for DENV antigen (92) in autopsy studies. Wu et al. made a major contribution to the field by demonstrating that DENV infects skin-resident Langerhans cells and monocytes-derived dendritic cells (230). Immature DCs (Langerhans cells) that reside in the dermis (the site of the mosquito bite), interstitial dendritic cells and precursor interstitial dendritic cells ($CD14^+$ interstitial cells) have been shown to efficiently uptake DENV (117, 230).

The high susceptibility of dendritic cells to DENV infection has been attributed to the expression of DC-SIGN. Mannose receptor (MR) was recently identified as another putative receptor for DENV in monocytes (141). Furthermore, in U937 and K562 cell lines, FcγRI and FcγRII receptors can increase virus uptake by the phenomenon of ADE (130). Hence, it is not clear whether there is a single specific receptor for entry of DENV into susceptible cells.

Due to the lack of a good animal model for DENV infection, primary cells and cell lines have been exposed to DENV *in vitro* to identify susceptible cell types. Mast cells, fibroblasts and activated T cells have been shown to be susceptible to DENV infection *in vitro* (113, 139). Moreover, we have found that primary muscle satellite cells are highly susceptible to DENV infection *in vitro* (Warke et al. J Gen

Virol. In press). Cell lines derived from different lineages, including 293 (epithelial), 293T (type), HepG2 (hepatoma), U937 (monocytic), K562 (kidney embryonic), HPMEC1.1 (endothelial), and ECV304 (epithelial) can be infected with DENV *in vitro*. DENV infection of 293T cells, ECV304 cells and monocytes induced expression of IL-8 transcriptionally through the trans-activation of its promoter by NFκB (24).

DENV mediates endothelial cell activation via both direct infection and indirectly as a result of cytokine induction (10, 50, 92, 201). The endothelium forms the primary barrier of the circulatory system and functional dysregulation of endothelial cells during acute dengue disease is thought to contribute to dengue pathology. Monocytes are important targets for DENV infection and are thought to play a critical role in the ADE model of dengue pathogenesis. *In vivo*, DENV RNA and antigen have been detected in multiple cell types. (85, 92, 104, 230).

Among the accepted sites for DENV infection and replication *in vivo*, DCs are most highly susceptible to DENV infection *in vitro,* which can be determined by various techniques (125, 166, 230). In addition, DCs are important in initiation and modulation of the innate and adaptive immune response. Hence, DC is a good *in vitro* cell model to study the effects of DENV infection on DENV-infected and bystander cells.

Muscle Satellite Cells as Targets for DENV Infection

No clinical report published to date has looked at presence of DENV in muscle cells. However, a clinical study found that seven (44%) out of sixteen patients

positive for acute DENV infection clinically and serologically (IgM ELISA), presented with acute flaccid weakness (98). These seven DENV-positive patients had an incidence of acute motor quadriplegia along with elevated serum levels of CPK and SGPT, muscle weakness, pain and tenderness, which are findings consistent with myositis (98). In addition, Rajajee et al. found that 50% of the children presenting with clinical and laboratory features of benign acute childhood myositis were positive for DENV by serological tests (PAN BIO, DUO IgM and IgG capture ELISA) (177). Furthermore, Malheiros SM et al. detected moderate perivascular mononuclear infiltrate in twelve out of fifteen muscle biopsies performed in serologically positive DENV-infected individuals (134). These studies indicate that human skeletal muscle cells are indirectly or directly damaged during the acute stage of DENV infection. Previous studies of DENV infection in mice have also reported biochemical and ultrastructural changes in skeletal muscle (156, 177) as well as detected infectious DENV progeny in the muscles (5). Also, fatal myocarditis has been observed during acute DENV infection (Dr. Iris Villalobos, Dr. Jairo Rodriguez, personal communication).

Immune Response to DENV Infection

(i) Innate Immune Response

The innate immune response plays an important role in the recognition of pathogens, for example, in initiating a potent antiviral response to virus infection. Uptake of the pathogen by antigen-presenting cells (dendritic cells, macrophages) activates the pro-inflammatory response as well as the type-I IFN signaling pathway

that is the upstream regulator of antiviral response genes. Type-I IFNs (α/β), which are produced by most cell types, play a central role in the innate antiviral response to a wide variety of viruses including DENV. However, since type-I IFNs can modulate maturation and activation of dendritic cells (46, 189), they perform a vital function to bridge the innate and adaptive parts of the host immune response. Type-II IFN (IFNγ) also can induce expression of antiviral genes. However, most of the type-II IFN is produced by activated NK and T cells and not by virus infected cells.

NK cells are an important piece of the innate immune response partly by producing massive amounts of IFNγ. NK cells upregulate their expression of activation markers (CD69, HLA-DR and CD38), adhesion molecules (CD44, CD11a) and cytotoxic granules (TIA-1) early during the acute phase of dengue disease (11). However, the role of NK cells in the regulation of DENV infection has not been studied in depth. Higher levels of type-I and type-II IFNs have been detected in dengue patient serum during the acute stage of the disease (111, 112). Intravenous DENV (D2S10) infection in AG129 mice, which lack both type-I IFN and type-II IFN receptors, resulted in productive infection and TNFα-mediated lethal disease (195). Also, in the *in vitro* model of dendritic cell infection with DENV, IFNα and IFNγ inhibited viral replication even though it has been shown that DENV infection inhibits type-I IFN signaling within infected cells (78, 152, 153).

(ii) Adaptive Immune Response

The adaptive immune response involves antibodies and T lymphocytes. Depending on the serotype of DENV both aspects of adaptive immunity have been

associated with secondary DENV infection-mediated pathogenesis (159, 179). Passive transfer of antibodies to E, NS1 and prM proteins has been shown to protect mice against lethal DENV challenge (60, 118). Cross-reactive antibodies from previous DENV infections at sub-neutralizing concentrations can increase uptake of non-neutralized virus by binding to FcRII on cell surface (120).A study by Kliks et al. found high DEN-2 antibody-dependent enhancing activity as a risk factor for DHF (106). However, studies by Endy et al. (58) and laoprasopwattana K et al. (120) did not find a correlation between titers of preexisting neutralizing DENV-antibodies and the severity of the secondary infections.

During secondary DENV infections, cross-reactive T cells presumably generated from the previous DENV infection proliferate more than the T cells specific to the newly infecting serotype (145). This phenomenon is called "Original antigenic sin". DENV serotype cross-reactive cytotoxic T cells show higher avidity for antigen and, as compared to DENV serotype-specific cytotoxic T cells, produce higher pro-inflammatory cytokines like IFNγ and TNFα (55). Furthermore, other cytokines like IL-2, IL-10 and IL-8, which can be produced by T cells, are elevated before the onset of plasma leakage (112). The altered immune response and higher production of pro-inflammatory cytokines during a heterologous secondary infection might play a role in vascular leakage and activation of the coagulation cascade.

Protection

The longevity of the protection afforded by primary DENV infection against the homologous serotype is not completely understood. The major target for antibody

response is to the E protein of DENV virion. *In vitro* antibodies against E protein both inhibit viral binding to cells and neutralize infectious virus (181). Furthermore, passive transfer of antibodies to E protein can protect mice against challenge with DENV (100). IFNα and IFNγ were found to be critical for early and late immune response and resistance to DENV infection in mice, respectively (194). Further, a mouse-adapted strain of DENV injected peritoneally killed IFNα/β- and IFNγ-deficient mice (94). IFNα can inhibit DENV replication in multiple cell types *in vitro* (51, 52, 78). However, IFNα cannot block DENV replication if added after DENV infection suggesting that DENV has evolved to counter IFNα's antiviral effects. IFNγ also strongly inhibits DENV replication in DCs, but unlike IFNα, IFNγ treatment after DENV infection can still inhibit virus replication (78). Overall, immune responses and their effects to limit DENV replication are hypothesized to be protective against the development of the severe form of the disease.

Clinical Stages of DENV Infection

DENV infection, which has an incubation time of 3-7 days, is asymptomatic in a majority of individuals. Dengue fever (DF), Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are three major classifications for grades of severity of dengue disease. DHF is further sub-classified into grades I, II, III and IV by the World Health Organization (WHO). Grades III and IV constitute DSS. However, positive tourniquet test, one of the minimal criterion to diagnose a case as DHF has been unsuccessful in discriminating between DF and DHF effectively in multiple studies. In two studies, only 18% and 61% of the children with shock met

the criteria classified by WHO for DHF (14, 170). Hence, prominent researchers in the field are in favor of modifying the current WHO standards of dengue classification (48).

Dengue Fever

DF is a mild, self-limited febrile illness typically associated with the following symptoms: retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, leukopenia and headache. Most of the individuals recover after the acute febrile period, which may last from 2 to 10 days, without any specific treatment (68). There is lower risk of death in DENV patients presenting clinical symptoms for DF.

Dengue Hemorrhagic Fever

DHF is a more severe manifestation of DENV infection and can be caused by all four serotypes of DENV. A minority of people (~3%) infected with DENV develop this more severe form of disease according to the WHO classification criteria (68). During the febrile phase of the disease, patients present with similar clinical symptoms as DF. However, at the time of defervescence or shortly afterward, patients with DHF abruptly develop a vascular permeability/plasma leakage syndrome associated with thrombocytopenia, a bleeding diathesis. Vascular leakage and increased endothelial cell permeability resolve following the acute phase of the disease, suggesting dysfunction rather than structural damage to endothelial cells during DHF. A majority of cases of DHF are associated with secondary DENV infections (114) (DHF.CAB Int.Oxford,1997). Studies have found that children of

ages 4 years and above are at higher risk of developing DHF following DENV infection while children under 3 years of age who were seropositive for DENV did not present clinical symptom of infection (73). However, the cause of this DENV infection-mediated pathogenesis is not well understood. “Original Antigenic Sin” and “Antibody Dependent Enhancement” models have been postulated to be the immunopathogenesis mechanisms that lead to increased severity of disease (145, 149). The cytokine profile resulting from heterologous immunity might add to the inflammation-induced immunopathogenesis (55, 145). Recent clinical studies have provided data suggesting that certain DENV genotypes are associated with DHF (55). The two models of pathogenesis are further explained in detail in the “Immunopathogenesis” section of the introduction.

Dengue Immunopathogenesis

Activation of the immune response associated with the ADE and original antigenic sin models during heterologous DENV infection might be responsible for the immunopathogenesis observed during DHF/DSS. Antibodies produced during primary DENV infection bind to virions of heterologous DENV serotypes during subsequent (secondary) infections. *In vitro* studies have shown that macrophages but not DCs (125, 230) efficiently uptake these virus-antibody complexes, which leads to an increase in viral replication by a phenomenon called antibody-dependent enhancement (ADE). Moreover, antibodies from a previous infection can also cause immune activation via innate immune reactions and complement activation (233). Levels of T cell expansion are also enhanced during secondary DENV infections (55,

145). However, expansion of lower-avidity memory T cells over high-avidity naïve T cells specific for the infecting serotype generates a more inflammatory and less cytotoxic T cell cytokine and chemokine response called original antigenic sin (145). Sub-optimal viral clearance and disturbance of homeostasis of the blood clotting system might occur. In addition, ADE might lead to increased DENV antigen presentation to T cells that might further contribute to the activation of inefficient T cells and the immunopathology of heterologous secondary DENV infections. Circulating levels of pro-inflammatory cytokines and chemokines like TNFα, IL6, IL-2, IL8 and MCP1 and regulators of inflammation like IL-10 and sST2 are further correlated with a severe outcome during acute stage of the disease (17, 37, 63, 65, 96, 121, 176).

This overview on DENV and factors associated with the disease presented in the introduction shows that large bodies of experiments have been performed to understand the dengue disease. These studies have found that viral and many host factors correlate either with protection or pathogenesis during DENV infection. However, due to the absence of a reliable animal model, the significance of these correlations associated with the pathogenesis is not well understood. Hence, current focus in the field of dengue research is to understand the molecular changes effected by DENV infection to identify aspects of host immune response that can be induced or inhibited to develop a prophylactic or therapeutic vaccine against dengue.

Gene Expression Analysis as a Tool for Discovering Genes Involved in the Cellular Response to Infection

Global gene expression analysis using reverse transcription differential display (RT-DD), subtractive hybridization or DNA microarays followed by quantitative RT-PCR is an approach that has been successfully used to identify candidate differentially expressed genes to understand host response to pathogens or disease (191, 199, 225). This approach allows for the identification of markers of disease and / or severity of a disease. RT-DD has advantages over subtractive hybridization because it requires small amounts of sample, is relatively fast, simpler and allows simultaneous detection of genes from the control and sample group (23). However, many RT-DD experiments are required to cover the entire transcriptome. In contrast, microarray analysis provides expression patterns of thousands of genes at once (20,000-40,000). Hence, the major advantage of microarray over RT-DD and subtractive hybridization is that it can identify multiple genes and reveal multiple functional signaling pathways in a single experiment. Significant conclusions can be obtained from a minimum of 2 microarrays using the right statistical analysis.

Our lab used DD and Affymetrix oligonuceotide microarray analyses followed by qRT-PCR (225) to study gene expression changes in HUVECs during infection with DENV. Using these 2 techniques, we identified several genes, some of which were involved in the innate antiviral response. Researchers have also identified upregulation of cellular support for pathogen replication and downregulation of MHC expression using microarrays, which can allow the pathogen to escape the immune system (47, 190). A microarray study on Salmonella enterica typhimurium showed

induction of genes involved in cell cycle arrest and cell death (49). Genomic analyses have also been used to understand innate and adaptive immunity, to diagnose pathogens, tumors, metabolic pathways and to understand the efficacy of a drug and vaccine research (29). Recently, Fink et al. and Popper et al. have reported gene expression profiles of *in vitro* infected cells and PBMCs of acute infected dengue patients, respectively (61, 198). Very encouraging findings from these two other groups were found as some of the genes reported in their study coincided with our analysis. Simmons et al. also observed lower abundance of type I interferon inducible genes in gene transcripts associated with DSS as compared to non-DSS patients. In addition to human cell analysis, Sariol et al. have looked at PMBCs from rhesus monkeys infected with dengue virus and found that interferon response genes are highly expressed in infected rhesus monkey PBMCs (182).

New Antiviral, Immunomodulatory and Biomarker Proteins Identified in this Thesis

This thesis identified TRAIL as a promising candidate for further study using genomic analysis in primary human cells susceptible to DENV infection (224). Genomic and proteomic analysis approach also discovered sST2 and IDO as potential biomarkers for DENV infection (17).

Here we present published information on TRAIL, ST2 and IDO genes, which are identified as antiviral, immunomodulatory or biomarker proteins against DENV.

(i) Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

TRAIL (APO2L, TNFSF10) is a member of the TNF family that specifically promotes apoptosis in cancerous (tumor) cells by binding to and activating the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) (44, 84). Two decoy receptors DcR1/DR6 and DcR2/DR7 and intracellular proteins c-FLIP, XIAP, G1P3 and p38αγ are regulators of the signaling downstream of the two TRAIL death receptors (3, 39, 129, 221). TRAIL is a 32.5 kDa protein composed of 281 amino acids, and is a type II membrane glycoprotein, with a signal peptide at the N-terminus (147). The first 38 amino acids are intracellular and transmembrane, while the C terminus of the protein (39-281) is extracellular. The extracellular domain of TRAIL binds the TRAIL receptors DR5 and DR4 producing a trimeric structure that activates signaling downstream of the two death receptors (34). TRAIL protein is not only secreted into the extracellular space but also can localize on the surface of the cell or be stored in the cytoplasmic compartment of the cell (56, 147, 219, 224). Monleon I et al. observed that APO2L/TRAIL is stored inside cytoplasmic compartments approximately 500 nm in diameter, with characteristics of multivesicular bodies (microvesicles) (147). However, the origin of the soluble form is not fully understood. In their study anti-CD59 Ab stimulation induced release of TRAIL containing microvesicles into the supernatant. However, TRAIL was localized in plasma-membrane-enriched/secretory-granule fraction in neutrophils (103). Overall, the origin of soluble TRAIL in other systems is not understood. The expression of both TRAIL and TRAIL-Rs is differentially regulated in PBMCs (56, 74). Activation of either of the two death receptors results in recruitment of adaptor protein FADD

(Fas-associated death domain). TRAIL receptors are widely expressed (74, 167); thus most tissues and cell types are potential targets for TRAIL. FADD recruits procaspase-8 into the death receptor complex, thereby causing autoproteolytic cleavage of procaspase-8, which in turn activates the apoptosis signaling pathway (213).

In vitro and *in vivo* studies have demonstrated tumoricidal activity of TRAIL without significant toxicity toward normal cells or tissues (171, 184). TRAIL-mediated killing of EMCV and HCMV infected but not uninfected cells has been demonstrated (183, 188). In addition, blocking TRAIL protein delayed the clearance of influenza virus in mice (89). TRAIL-mediated killing of tumor cells by NDV, reovirus and HCV has also been shown (40) (57) (43).

TRAIL has also been shown to negatively regulate innate immune responses and inflammation independent of apoptosis (54, 82, 185, 200, 218). TRAIL induction of ERK/Akt activation is mainly associated with proliferation and survival of endothelial cells (186) and can induce proliferation of T cells and synovial fibroblasts (148, 197).

IFNs enhance the expression of TRAIL, while, on the other hand, TRAIL treatment can enhance expression of IFN-inducible genes like IFITM1, IFIT1, STAT1, LGal3BP, and PRKR as well as IFN-β itself (108). Hence, TRAIL binding to its receptors activates downstream signaling pathways that can affect any one of the above-mentioned cellular responses, depending on the activation and inhibition of the regulators of the TRAIL signaling pathway.

(ii) Indoleamine 2,3-dioxygenase (IDO)

IDO is an enzyme ubiquitously distributed in mammalian tissues and cells. It catalyzes the initial and rate-limiting step in the catabolism of L-tryptophan along the kynurenine pathway (210). IFNγ (12) and LPS (62) can induce expression of IDO while IL-4 represses its expression (155). Other cytokines such as IFNα/β, TNF-α and IL-1 cannot induce IDO expression individually. However, TNF-α and IL-1 enhance IDO expression induced by IFNγ (150). Overall, IFNγ is the most potent known inducer of IDO expression (180). IDO activity is also induced under pathological conditions such as viral and bacterial infections (45, 237), allograft rejection (140) and tumor development (217). IDO-expressing cells inhibit T cell proliferation and function by depleting L-tryptophan in the surrounding microenvironment (150). This process is termed "immunosuppression by starvation". T cells stop proliferating in a tryptophan-reduced microenvironment since this tryptophan-sensitive checkpoint is at the G1 phase of the cell cycle (150). Studies in the Th1-mediated trinitrobenzene sulphonic acid-induced colitis model (69) demonstrated that 1-MT treatment to block IDO activity abolished tolerance. In addition, tumor and allogenic fetal rejection models (151, 217) have directly shown that 1-MT treatment abolishes tolerance *in vivo*. Uyttenhove et al. (217) found that tumors partly resist attack by tumor-specific CTLs through the expression of IDO, which leads to the degradation of tryptophan in the tumor microenvironment. The same study also demonstrated that, in addition to tumor cells, tumor-infiltrating dendritic cells but not tumor-specific CTLs stained for IDO.

IFNγ-induced activation of IDO has been shown to restrict *Toxoplasma gondii* growth in human microvascular endothelial cells (45). In addition, a recent study by Adams et al. (4) showed that IFNγ-induced IDO expression restricts herpes simplex virus (HSV) replication in human astrocytes (4).

(iii) Interleukin-1 receptor like-1 (IL1RL1 or ST2)

The Interleukin-1 receptor like-1 (IL-1RL-1, ST2) protein is a member of the interleukin-1 receptor (IL-1R) family of proteins. It was originally detected as one of the primary response genes in growth-stimulated murine fibroblasts (BALB/c-3T3) (214, 236) and as a HA-ras oncogene-responsive gene (228). Alternative splicing of the gene generates three mRNAs, corresponding to a longer membrane-anchored form (ST2L), a shorter released form (sST2) and a membrane bound variant form (ST2V) (21, 215, 235). The expression of the three forms has been detected in various human tissues and cells, including hematopoietic and endothelial cells (109). ST2L has been proposed as a marker for Th2 CD4+ T cells since it is selectively expressed on Th2 but not on Th1 CD4+ T cells (231, 234) and might be involved in the effector phase of Th2 immune responses (216). The sST2 protein can be induced *in vitro* by pro-inflammatory stimuli like lipo-polysaccaride (LPS), IL-1β, TNFα and IL-6 in human and murine inflammatory models(109, 208). In mice, the production of pro-inflammatory cytokines precedes sST2 expression (164). Elevated levels of sST2 are found in patients with inflammatory disorders associated with abnormal Th2 mediated responses, including some autoimmune diseases (115), asthma (161, 163), idiopathic pulmonary fibrosis(208), and sepsis(28); elevated sST2 levels are also

found in patients with other inflammatory conditions, like LPS-induced inflammation (162) and myocardial infarction (192). Additionally, sST2 has been proposed as a biomarker for heart failure (227). These findings suggest that increase in sST2 might reflect the development of inflammation and might play a role in its regulation.

General Thesis Objectives

The general objective of this thesis is to dissect the cellular response to dengue virus infection by studying its differential gene expression. We specifically studied the global gene expression response to DENV infection *in vitro* to identify and test gene function involved in major cellular events that might help to understand protective and/or immunopathological responses observed in DENV patients *in vivo*.

We postulated that the global gene expression analysis approach to understand commonly regulated gene expression analysis in DENV-permissive cells would result in identification of genes / signaling pathways involved in inhibition of DENV spread. Endothelial cells are the primary target site for DENV infection-mediated pathogenesis. Hence, we reasoned that comparing endothelial cell response to DENV infection with infection by other RNA viruses that also affect endothelial cells might help us to identify factors that might represent, and allow future understanding of DENV infection mediated vascular dysfunction.

Hence, we set out to identify whether (a) infection of DENV permissive cells (monocytes, B cells, HUVECs and dendritic cells) would activate type-I and type-II IFN activated antiviral molecule/s that inhibit DENV infection and replication (b)

infection of endothelial cells would activate DENV-specific genes when compared to other RNA viruses tested that also infect endothelial cells.

Chapter II

Materials And Methods

Isolation of primary human cells

Blood samples were obtained from healthy U.S. volunteers at The University of Massachusetts Medical School. Monocytes and B cells were negatively selected from heparin-anticoagulated blood using a rosetting antibody precipitation kit (StemCell, Seattle, WA) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. Sample purity was determined by cell surface staining of freshly isolated monocytes ($CD14^+$, $CD4^-$, $CD8^-$, $CD19^-$) and B cells ($CD14^-$, $CD4^-$, $CD8^-$, $CD19^+$).

Primary HUVECs were obtained from pooled umbilical cords (two to five donor pools per culture). Human subject protocols were approved by Harvard University Medical School. HUVECs were maintained by the Core Facility of the Center for Excellence in Vascular Research at Harvard Medical School in M199 medium supplemented with 10% FCS, 1 mM glutamine, endothelial cell growth stimulant, porcine intestinal heparin, and antibiotics. HUVEC cultures were split at a ratio of 1:3 or 1:4 for up to two passages. The protocol for preparation and passage of HUVECs is posted at http://vrd.bwh.harvard.edu/core_facilities/cx_huvec.html. For isolation of human monocyte-derived dendritic cells, CD14 microbeads (catalog number 120-000-305; Miltenyi Biotec) were used to positively select CD14-positive cells (monocytes) from Ficoll-isolated peripheral blood mononuclear cells from healthy donors. Monocytes ($1.5 – 1.75 \times 10^6$ cells/ml) were cultured for 7 days in RPMI 1640 medium supplemented with 800 U/ml of granulocyte-macrophage

colony-stimulating factor, 500 U/ml of interleukin-4, and 10% FCS. Cells were stained for CD1a, CD14, HLA-DR, and CD83 on day 6 to determine the monocyte to immature DC conversion.

Cryopreserved muscle satellite cells were derived from muscle biopsies taken from the thigh muscle of healthy human donors and were donated by Mytogen, Inc (Charlestown, MA). The donor biopsies were obtained under an approved clinical study protocol (53). The donor identification protocol number was erased after cell preparation and culture of the satellite cells. Connective tissue was removed after the biopsy samples were placed in Ringer solution with heparin followed by 3 cycles of enzymatic digestion (0.5mg/ml Trypsin, 0.5mg/ml collagenase). The first cycle of enzymatic digestion was carried out for 60 min followed by two 40 min cycles. The temperature was maintained at 37^{o}C and a total volume of 5ml of fresh digestion mixture was used for each cycle. The cells after each cycle of digestion were trypsinized and centrifuged in complete growth medium at 1000 r.p.m. for 5 min at 4^{0}C. Cells were seeded in one T175 flask per digestion cycle. All cells were expanded for up to 10 – 12 doublings for purposes of optimizing yields and viability and then cryopreserved. The muscle satellite cells were thawed and formulated in DMEM + 15% FCS (fetal calf serum), recombinant human epidermal growth factor (rhEGF: 10 ng/mL), and dexamethasone (0.39 μg/mL) for growth. Muscle satellite cells at approximately 15 doublings were used for experiments in this study. No myotubes were evident by microscopic inspection of the culture at the time of infection. The conditions described here are considered optimal for muscle satellite cell phenotype and survival for transplantation purposes (53).

Virus Propagation

Dengue virus was propagated as previously published (113). Briefly, C6/36 cells were infected with low passage (passage 2-4) DENV at a multiplicity of infection (M.O.I.) of 0.1 and maintained in RPMI 1640 media containing xx% non-essential amino acids, (concentration) sodium pyruvate, 10% FCS and (concentration) penicillin/streptomycin for approximately 7 days at 5% CO_2 and 26^0C. Culture supernatants were collected after centrifugation and stored at -70^0C. DENV-infected C6/36 cell supernatants were titrated on Vero cells using the plaque assay technique. Uninfected C6/36 cell supernatants were collected for use as a negative control.

Dengue Virus Infection:

D2V New Guinea C (NGC), DENV1 Hawaii strain, D3V CH53489 and DENV4 strain 814669 of DENV were used for infection of primary human cells. D2V New Guinea C (NGC) is a laboratory-adapted prototype Southeast Asian D2V strain. All four dengue serotypes were obtained from Dr. Dubois (Yale University).

(i) HUVECs

HUVECs were obtained through collaborations with Dr. Sunil Shaw at Harvard University. Monolayers of HUVECs were resuspended in fresh cell culture medium. DENV strain New Guinea C (NGC) grown as described above was added to confluent monolayers of cells at a multiplicity of infection (MOI) of 0.5 to 1.5. Virus was allowed to adhere for 2 hours. The culture supernatant was then removed, and

fresh growth medium containing 10% FBS was added to each well. After 48 h of infection, cells were harvested, centrifuged at 700 x *g* for 7 min at 4°C, washed with phosphate-buffered saline (PBS), pelleted again at 700 x *g* for 7 min at 4 C, and stored at -70°C for analysis.

(ii) Monocytes, B cells, PBMCs

Monocytes/macrophages, B cells and PBMCs were obtained as described above. Cells were infected for 2-2.5 hours at 0.1 to 1 PFU/cell or cultured with uninfected C6/36 cell supernatant in RPMI medium containing 2% FCS at 37°C and 5% CO_2. After 2-2.5 hours, the medium was supplemented with FCS to bring the final concentration of FCS to 10% in the media. At the 48 hour timepoint cells were collected, washed and either stained for flow cytometry or cell pellets were stored at – 70^0C until use. Aliquots of the supernatant were kept at 70°C until use.

(iii) DCs

DCs were infected with DENV at a M.O.I of 0.1 for 2 hours at 37°C and then washed thoroughly, resuspended in RPMI 1640 supplemented with 10% FCS, 500 U/ml of IL-4 and 800 U/ml of GM-CSF and incubated for 12, 24 or 48 hours. For TRAIL pre-treatment experiments, DCs were treated with 20 μg/ml of recombinant TRAIL (rTRAIL, Biomol International, PA) for 24 hours prior to infection.

(iv) Muscle satellite cells

Monolayers of muscle satellite cells maintained in fully supplemented medium were washed with fresh cell culture media with no FCS. DENV, previously grown in C6/36 cell monolayers and titrated in Vero cells, was added to confluent monolayers of muscle satellite cells at a multiplicity of infection (M.O.I) of 1-2. The cell culture supernatant was removed after two hours, the monolayers were carefully washed and fresh growth medium containing 10% FCS was added to each well. After 48 hours of infection, the culture supernatant was collected and stored at -70^{o}C for plaque assay and ELISA. The cells were trypsinized and centrifuged at 700 x g for 5 min (twice) and kept on ice for antibody staining or stored at –70^{o}C for RNA extractions.

Plaque Assay

Cell culture supernatants from uninfected and DENV-infected cells were incubated with a monolayer of LLCMK2 cells for 2 hours with intermittent gentle mixing. Cell monolayers were washed with PBS and overlaid with medium viscosity carboxy methylcellulose (Sigma-Aldrich, St. Louis, MO). Carboxy methylcellulose was removed after 6 days by gently shaking the wells with 2ml PBS added to each well. The plate was washed four times with PBS. The washed cells were fixed and stained with 0.2% crystal violet in ethanol. Plaques were counted visually. Each sample was assayed in duplicate wells.

Recombinant Protein and Antibody Treatments

(i) Antibody Treatment:

Monocytes, B cells, and HUVECs were pretreated for 24 hours with TRAIL blocking monoclonal antibody (50 µg/ml; R&D Systems) or purified immunoglobulin G1 isotype control antibody (BD Biosciences) followed by infection with DV, as mentioned above, for 48 hours at an MOI of 1. The antibodies were left in the culture during the infection. The cells were collected by centrifugation at 500 x g, washed twice in phosphate-buffered saline (PBS), and cell pellets were stored at –70°C until analysis.

(ii) Recombinant Protein Treatment:

Monocytes were pre-treated for 24 hours with rTRAIL at concentrations from 5 to 20 µg/ml (Merck, Germany) and infected with DENV at an MOI of 0.1. At 24 and 48 hours post-infection, cells were washed twice with PBS and the cell pellets and supernatants were stored at –70°C.

DCs were only pre-treated with rTRAIL (20 ng/ul, Biomol, PA) and infected with DENV at an MOI of 0.1. Two hours post-infection DCs were washed to remove residual virus used for infection and maintained in fresh RPMI 1640 medium containing 10% FCS, interleukin-4 (500 U/ml), and granulocyte-macrophage colony-stimulating factor (800 U/ml). The media added post-infection was not supplemented with 20 µg/ml rTRAIL.

DCs were pre-treated for 24 hours with rTRAIL (20 ng/ul, Biomol, PA) and / or IFNR blocking Ab (0.5-20 ng/ul) and infected with DENV at an MOI of 0.1 for 48 hours. For the time-course of rTRAIL pre-treatment, DCs were treated for 24hour,

12-15hour, 30min or 0hour with rTRAIL (20 ng/ul, Biomol, PA) and infected with DENV at an MOI of 0.1 for 48 hours. DCs were washed at 2 hours post-infection to remove residual virus used for infection. Cells were maintained in fresh RPMI 1640 medium containing 10% FCS, interleukin-4 (500 U/ml), and granulocyte-macrophage colony-stimulating factor (800 U/ml) till the end time-point of the experiment. At each time point, cells were stained for DENV antigen (DENV-fluorescein isothiocyanate), CD1a-allophycocyanin, HLA-DR-peridinin chlorophyll protein-Cy5.5, and CD83-PE. Supernatants were stored at –70°C for future use.

1-Methyl Tryptophan (1-MT) and IFNγ treatment

Dendritic cells were pre-treated with 500 U/ml of IFNγ or 500 U/ml of IFNγ and 0.1 or 1 mM 1-MT followed by infection with DV for 48hour at a M.O.I. of 1 as described above.

The cells were collected by centrifugation for 5 min at 1,500 r.p.m., washed twice in PBS, and stored at -70^0C until analysis. RNA was extracted from the cell pellets using RNAeasy (Qiagen) and DENV RNA levels were quantified by TaqMan qRT-PCR.

Affymetrix GeneChip hybridization and Analysis

RNA isolated from DENV-infected, C6/36 treated and uninfected cells using the RNeasy kit (Qiagen) was biotin-labeled and hybridized to human oligonucleotide microarrays (Affymetrix HG-U133A) using standard methods as follows. First-strand cDNA was prepared by using a T7-(dT)24 primer and SuperScript II reverse

transcriptase (Invitrogen/Life Technologies) from 8 μg of total cellular RNA. Second strand synthesis was performed by using the Superscript Choice system (Invitrogen/Life Technologies), and the product was purified using a Qiagen PCR purification kit. Synthesis of biotin-labeled cDNA was carried out using a BioArray high-yield transcript labeling kit (Enzo), and cDNA product was purified by using RNAeasy kit (Qiagen). cDNA was fragmented and spiked with bacterial control genes (*bioB*, *bioC*, *bioD*, and *cre*) according to the Affymetrix protocol (ref manual).

Hybridization to Affymetrix HG-U133A oligonucleotide microarrays (22,283 genes) was carried out overnight with 10 μg of biotin-labeled cDNA product. Microarrays were washed using the GeneChip Fluidics Station (Affymetrix) according to the manufacturer's protocol. Staining with R-phycoerythrin-streptavidin (Molecular Probes) was followed by antibody amplification with a biotinylated streptavidin antibody (Vector Laboratories). Scanning was carried out using the GeneArray scanner (Hewlett-Packard). The data were collected with MAS5 software (Affymetrix) and analyzed using the Statistical Expression Algorithm to give signal values. Experiments displaying Affymetrix present call rates of >30% were included in the analysis.

Signal values from each of the 22,283 probe sets were calculated using robust multiarray analysis (Irizarry RA,2003,4:249-264,Biostatistics). Signal values were transformed using the inverse natural log. For repeated experiments, inverse natural log-transformed robust multiarray analysis results were normalized based on the median and geometric means and were calculated prior to importing data into GeneSpring software (Agilent). Each GeneChip was independently normalized to the

median expression level of all genes on the chip. Each gene was then normalized to the median expression levels of that gene. Signals with low expression levels were excluded, and expression of statistical filters was applied as indicated. We analyzed our data by hierarchical cluster analysis using a Pearson correlation. Reproducibility was assessed as previously described (225).

For the determination of the common response, the following analysis was performed: expression signals for 22,283 genes were filtered to exclude those with very low signals (2,000 genes), and the remaining genes were analyzed by one-way analysis of variance (ANOVA) to identify genes with statistically significant differences between DENV-infected samples (five samples) and uninfected samples (five samples). HUVECs (n = 2), monocytes (n = 2), and B cells (n = 1) represent cells exposed *in vitro* to DENV for 48 hours. The gene expression levels in DENV-infected samples were normalized to C6/36 insect cell supernatant-treated samples for each cell type independently. For the determination of the DENV-specific response: expression profile for 22,283 genes were filtered to exclude those with low signals and remaining genes were analyzed by ANOVA to identify genes with statistically significant differences between DENV-infected HUVECs (n=2) and untreated HUVECs (n=4), HUVECs treated with C6/36 (n=1), poly I/C (n=1), HUVECs infected with other RNA [WNV (n=1), YFV (n=1), Hantaan (n=2), SNV (n=1)] and DNA [EBV (n=1), vaccinia (n=2)] viruses. Variances were calculated using the cross-gene error model, parametric test, a P value cutoff 0.05, and the Benjamin and Hochberg false discovery rate multiple testing correction. For hierarchical cluster

analysis a Pearson correlation was used. n = number of independent experiments performed.

Total RNA Extraction

Total cellular RNA was extracted from as much as 2 x 10^7 uninfected or DENV-infected cells by using an RNA extraction RNAase column (Qiagen, Hilden, Germany). Typical yields were 10 µg of total RNA per million cells. Total RNA was digested with DNase I to eliminate residual DNA using the Message Clean Kit (GenHunter, Nashville, Tenn) when specified. The RNA was resuspended in diethyl pyrocarbonate-treated 0.01% distilled H_2O (Ambion, Austin, Tex.) quantified by spectrophotometry (Bio Photometer, Eppendorf, Germany) at 260 nm and stored at -80C until use.

Quantitative RT-PCR

(i) **Semi-Quantitative RT-PCR**:

A total of 0.2 µg of total cellular RNA obtained from infected and uninfected cells cultures was reverse transcribed using Omniscript/SensiScript reverse transcriptase (Qiagen) in the presence of 1 µM anchor primer and a 500 µM dNTP concentration in a volume of 20 µl. RT was performed at 37°C for 60 min, followed by 94°C for 15 min. PCR was performed with 0.5, 1, or 2 µl of cDNA. The 50-µl PCR included 2.5 U of *Taq* DNA polymerase (Clontech), 100 µM dNTP, and 100 µM of primers. PCR was run at 94°C for 1 min, followed by 25 cycles of 58°C for 1 min, 72°C for 45 s, and 94°C for 30 s, followed by 58°C for 1 min, and finally by 72°C for 10 min. L35a

(human ribosomal gene) was used as an endogenous control for all the experiments.

Semi-quantitative RT-PCR Primer sequences specific to sST2 and ST2L isoforms of ST2 gene.

sST2 - Forward Primer 5'AGGCTTTTCTCTGTTTCCAGTA 3'

sST2 - Reverse Primer 5' CAGTGACACAGAGGGAGTTCA 3'

ST2L - Forward Primer 5'AGGCTTTTCTCTGTTTCCAGT 3'

ST2L - Reverse Primer - 5' GGCCTCAATCCAGAACATTTTT 3'

(ii) **TaqMan Quantitation**:

RNA was extracted from the cell pellet using RNeasy (Qiagen) and subjected to TaqMan qRT-PCR for detection of TRAIL and DENV RNA. qRT-PCR of DENV RNA was performed as previously described (225). TRAIL mRNA was quantified using the same reaction conditions with TaqMan primer and probes obtained from Applied Biosystems. cDNA was obtained as described for semiquantitative analysis using 50-100 ng of total RNA, Taqman Reverse Transcription kit (Applied Biosystems) and oligo-dT primer. For PCR, we used a 25 or 50-µl reaction and Applied Biosystems (ABI) Universal 2X PCR Master Mix. A calibration curve containing five points ranging from 100 fg to 1 ng was used as a 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 and then 94.5°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in the TaqMan PCR signal detection system 7300 (ABI). β-actin was used as an endogenous control for all the experiments. A standard curve was run using a precalibrated DENV sample for

absolute quantification of gene expression. Results were calculated using the standard curve method or relative quantification method using quantitative PCR software (Applied Biosystems).

(iii) **Microfluidic Cards**:

For microfluidic card analysis and qRT-PCR, total RNA was extracted from cells using a Qiagen RNeasy kit. RNA was subjected to 384-well microfluidic card (relative quantitation) analysis as described by the manufacturer (Applied Biosystems). One hundred nanograms of total cellular RNA was reverse transcribed using TaqMan reverse transcription reagents in the presence of random hexamers as primers. Reverse transcription was performed at 25°C for 10 min and 48°C for 30 min, followed by 95°C for 5 min. For PCR, a 100-µl reaction mixture through a single port provided 2 µl of total reaction mixture per sample. The 100-µl PCR reaction mixture included 5 µl of cDNA, 45 µl of RNase/DNase-free water, and 50 µl of TaqMan Universal PCR Master Mix (2x). PCRs were cycled at 50°C for 2 min and 94.5°C for 10 min, followed by 40 cycles of 97°C for 30 s and 59.7°C for 1 min in the PCR signal detection system 7900 (Applied Biosystems). β-actin and GAPDH were used as endogenous controls to equalize loading of total RNA between samples. Each data point was measured in quadruplicate, and the standard error was determined.

Flow Cytometry

Live cells were collected at the end of an experiment. 200,000 to 500,000 cells were aliquot to each tube and washed twice with 1ml of PBS. The cells were then

washed with FACS Buffer (2% FBS and 0.1% sodium azide in PBS) and stained with cell surface antibodies for 20 min at 4^0C in the dark. The surface stained cells were washed with FACS buffer and fixed and permeabilized with Cytofix / Cytoperm reagent (BD Pharmingen) for 20 min at 4^0C in the dark. The cells were then washed with Perm Wash solution (BD Pharmingen) and intracellular staining was performed for DENV (DENV 2H2 Ab conjugated to FITC, Chemicon, MA) or TRAIL (TRAIL-PE) for 20 min at 4^0C in the dark. Stained cells were washed twice with FACS buffer and fixed in 0.5% paraformaldehyde in PBS and stored in the dark at 4^0C till the samples were run on FACsAria. Single color fluorescence compensation controls were performed for multi-color stained samples. Data were acquired on a FACsAria machine (BD Biosciences) and analyzed using FlowJo software version 6.0 or 8.6 (TreeStar). Monocytes and DCs were stained for cell surface TRAIL using monoclonal anti-TRAIL-phycoerythrin (PE) antibody (BD Biosciences) and cell surface markers following the same surface staining protocol. The 2H2 Ab is a n antibody to PrM which recognizes the mouse anti-dengue virus complex monoclonal antibody (99).

Detection of apoptosis in DCs

(i) Poly(ADP-ribose) polymerase 1 (PARP-1) protein cleavage detection

Cells undergoing apoptosis cleave PARP-1 protein (116-kDa) into two smaller fragments (85 and 25 kDa) (110). PARP-1 protein cleavage is the last known stage in cell death to differentiate between apoptosis and necrosis. DCs infected with DENV for 48 hours were washed twice with PBS and stained with PARP-1-PE antibody (BD

Biosciences) followed by flow cytometry analysis.

(ii) Caspase-3 and Live/Dead Aqua stain

Live/Dead Aqua stains dying cells while active caspase-3 is an early marker for cells undergoing apoptosis (66). DENV-infected DCs either untreated or treated with TRAIL were stained for apoptosis using caspase-3 and Live/Dead Aqua dye at 12, 24, and 48 hours postinfection. DCs incubated in a 65°C water bath for 20 min and camptothecin B (2 mM)-treated THP-1 cells were used as positive controls for Live/Dead Aqua staining. DCs treated with camptothecin B (2 mM/ml) for 4 hours were used as a positive control for caspase-3 staining. Cells were stained using the protocol described above.

Intracellular Cytokine Staining (ICS)

DCs were infected with DENV as described above at a multiplicity of infection (M.O.I.) of 0.1 for 12, 24, and 48 hours. Cells were treated with brefeldin A for the last 8 hours of each time point to inhibit secretion of proteins. At the end of each timepoint cells were collected, washed and cells were surface stained for DC markers ($CD1a^+$ $CD14^-$) and total (surface and intracellular) TRAIL protein using monoclonal anti-TRAIL-phycoerythrin (PE) antibody (BD Biosciences).

Quantification of Proteins in Cell lysates, Serum or Culture Supernatants

Commercial ELISA assays were used to measure levels of TRAIL (R&D Systems), sST2, MCP-2, IP-10, IL-6, ITAC and IFNα (Ray Biotech Inc., R&D Systems) in

monocytes cell lysates, DC culture supernatants, or sera, following the manufacturers' instructions.

Mass-Spectrometry

Standard and Sample Preparation:

Prism RP 100x1.5um was used for delivery of the mobile phases A (2% Acetonitrile, 2% Formic acid), B (40% Acetonitrile, 2% Formic acid).

Serum concentrations of Tryptophan and kynurenine were measured using reverse-phase HPLC. Frozen sera were thawed at room temperature. Sera were deproteinized by adding 1/10 (v/v) of 2.4 M perchloric acid. The sera were vortexed and centrifuged for 5 min at 3500g and 4^0C. The supernatant was pipetted into another tube and 6% trichloroacetic acid (TCA) was added and the tube was mixed well (vortex) to obtain a final concentration of 1% (w/v) TCA. The tube was centrifuged at 14,000 r.p.m. for 5 min at 4^0C. The sample was directly injected into the separation column. The samples were stored at -20^0C in the dark for later use.

Statistical Analysis: The SPSS 15.0 software was used for statistical analysis. Due to the small sample size, and a non-Gaussian pattern of sample values, a non-parametric method (Mann-Whitney test) was used to compare groups.

Clinical Samples from collaborators.

Dengue clinical samples were obtained from clinical cases by collaborations with other researchers. The patients were defined following the World Health

Organization (WHO) guidelines (1). Diagnostics of dengue patients were done virologically, by detection of DENV serotype-specific reverse transcription and polymerase chain reaction (RT-PCR) was performed using the One-step PCR kit (QIAGEN) and primers described in Lanciotti et al. (119) adapted to a one-step RT-PCR, using reverse primer and serotype specific forward primers. Dengue patients were also described by clinical signs and symptoms, hematological and liver functionality tests and coagulation tests. Becerra et al. 2008 has more complete description of the cohort used in Chapter IV. Serologic studies were also conducted in the patient samples. Dengue antibodies were measured in paired enrollment and convalescence sample, using IgM-ELISA and hemagglutination inhibition assay (HI), at the Instituto Nacional de Higiene Rafael Rangel, Caracas, Venezuela. Patients were classified as Dengue or as an Other Febrile Illness (OFI) based on the detection of dengue RNA, presence of IgM antibodies and / or at least a four-fold increase in HI levels in S2 compared to S1. The HI levels were used to further classify dengue patients as a primary infection (HI titer $\leq$ 1:1280) or secondary infection (HI titer > 1:1280).

Chapter III

Identification of the cellular response of primary human cells to dengue virus (DENV) infection

The first objective of this section of the thesis was to utilize global gene expression analysis to identify genes involved in the cellular response to DENV infection and also to propose proteins markers that can help identify DENV infection from other viruses. We utilized an *in vitro* DENV infection model to identify genes with potential antiviral or biomarker function. DENV-susceptible primary human cells, including monocytes, B cells and HUVECs, were exposed to DENV, *in vitro*. Finally, we investigated a novel primary human cell type, muscle satellite cells, to evaluate their susceptibility and responses to DENV infection.

Source and Purity of primary human cells

We first assessed the purity of monocytes and B cells isolated from blood of healthy US donors using commercially available negative selection kits (RosetteSep, StemCell). Monocytes (Mo) and B cells isolated from blood were ~79% $CD14^+$ and ~87% $CD19^+$, respectively (Table 1). These data indicate that the majority of the cells isolated using the negative selection protocol were either $CD14^+$ or $CD19^+$ for the monocytes and B cell isolation kit, respectively. We detected very low percentage of $CD4^+$, $CD8^+$ and $CD19^+$ cells or $CD4^+$, $CD8^+$ and $CD14^+$ cells using the B cell and monocyte negative isolation kit, respectively (Table 1).

Monocytes were converted into dendritic cells (monocyte-derived DCs, DCs) using the protocol mentioned in detail in Chapter II. On average, by the 6^{th} day of

Table 1: Purity of primary monocytes, B cells isolated from healthy donor blood and percent conversion of monocytes to dendritic cells (DCs) as analyzed by FACS.

	Cell Type	Mean Percent +/- SE
Surface Markers	**B cells (N=5)** CD19+ CD14+ CD4+ CD8+	 86.4 +/- 2.078 1.8 +/-0.41 0.8 +/- 0.051 0.3 +/- 0.19
Surface Markers	**Monocytes (N=5)** CD19+ CD14+ CD4+ CD8+	 1.0 +/- 0.447 78.3 +/- 0.87 1.0 +/- 0.179 0.3 +/- 0.224
Surface Markers	**Dendritic Cells (N=10)** CD83+ CD1a+	 0.45 +/- 0.12 80.34 +/- 3.98

culture, 60-91% of monocytes were converted to DCs ($CD1a^+$ cells) (Table 1). The $CD1a^+$ cells were stained for CD83 on the 6^{th} day of culture to confirm that the DCs were immature. For all donors, very few (<1%) $CD1a^+$ cells stained positive for CD83 surface antigen (Table 1). A good generation of immature DCs ($CD1a^+$, $CD14^-$ and $CD83^-$) from monocytes was also observed using our cell culture protocol (Chapter II). DCs also expressed high level of HLA-DR protein.

HUVECs were obtained from the Core Facility of the Center for Excellence in Vascular Research at Harvard Medical School. The detailed purification procedure is described at (http://vrd.bwh.harvard.edu/core_facilities/mlec.html).

III.1. Identification of common gene expression response following DENV infection of HUVECs, B cells and Mo *in vitro*

Innate immune response [type-I IFN and type-II IFN] is considered important in limiting DENV spread based on studies in mice and human cells, *in vitro* (51, 52, 194, 196). Since, innate immune response is activated in a wide variety of cells *in vivo* on exposure to pathogens, we hypothesized that cells susceptible to DENV infection might limit DENV replication and spread by inducing a common innate immune response. To identify common gene expression changes in primary human cells in response to DENV infection, we analyzed the transcriptome of the infected cells by global gene expression profile in HUVECs, monocytes and B cells using Affymetrix GeneChip arrays. These cell types are known to be susceptible to DENV infection *in vitro* and have been reported to show evidence of infection during DENV infection *in vivo*. All the cells tested were infected with DENV

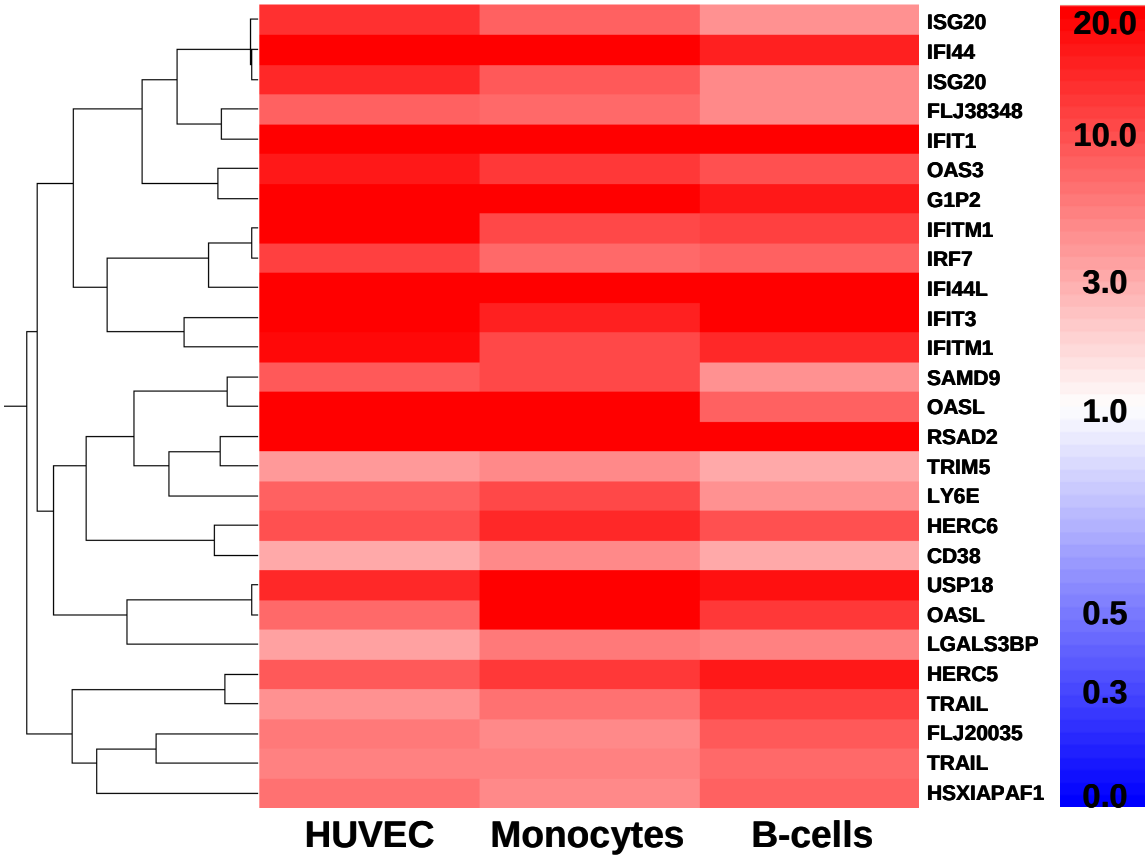


Figure 1A. Gene expression analysis using HG-U133A Affymetrix GeneChips. Expression levels for the 23 DENV common response genes in cells exposed to DENV. HUVECs (n = 2), monocytes (n = 2), and B cells (n = 1) represent cells exposed *in vitro* to DENV for 48 hours. *In vitro* infection profiles were normalized to C6/36 insect cell supernatant-treated samples for each cell type independently. Hierarchical cluster analysis used a Pearson correlation. Color indicates relative changes in induction (n-fold). On the color scale, dark red represents 20-fold up-regulation, white indicates no change and blue indicates down-regulation. Affymetrix microarray analysis was performed using GeneSpring software (Agilent).

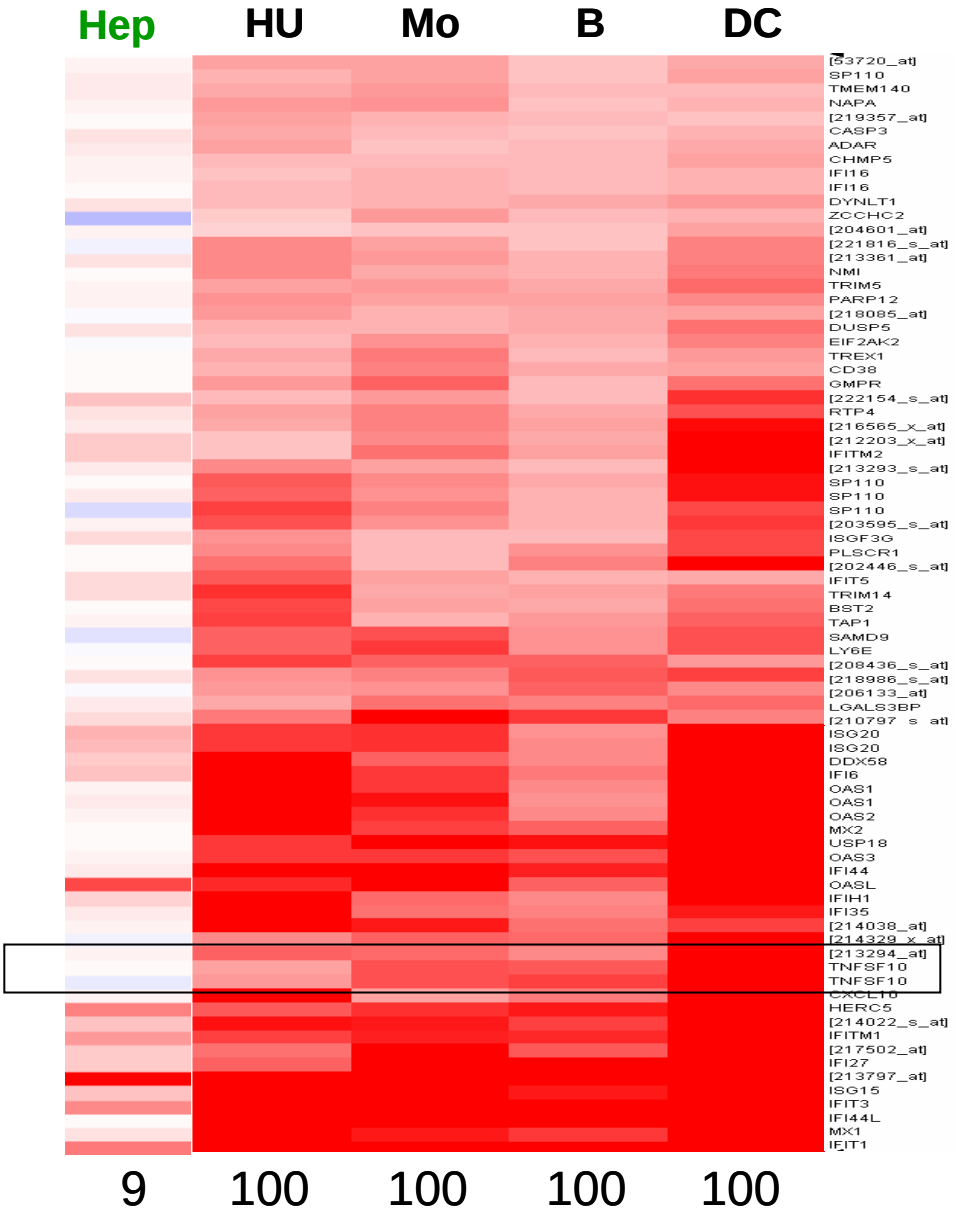


Figure 1B. Comparison of gene expression analysis in DENV-infected HepG2 cells to expression in primary human cells. Monocytes (n=2), B cells (n=1), HUVECs (n=2) and DCs (n=2). This figure shows the expression levels of the 79 common response genes (monocytes, B cells, HUVECs and DCs) to expression in HepG2 (Hep) cells exposed to DENV, *in vitro*. Hep stands for HepG2 cells. Numbers at bottom indicate the percentage of genes that were induced at least 2.5-fold. n represents number of independent experiments. Hierarchical cluster analysis used a Pearson correlation. Color indicates relative changes in induction (*n*-fold). On the color scale, dark red represents 20-fold up-regulation, white indicates no change and blue indicates down-regulation. Affymetrix microarray analysis was performed using GeneSpring software (Agilent). Box – Expression of TNFSF10 (TRAIL) in DENV-infected primary cells and HepG2 cell line.

propagated in C6/36 mosquito cells. Control samples were mock infected with uninfected C6/36 supernatants.

Of the 23 genes that comprised the common dengue response profile, functions and/or descriptions of 19 genes have been found in the literature (Table 2). Figure 1A shows 27 genes in the list of 23 different genes due to repetition of OASL, IFITM1, ISG20 and TRAIL in the analysis output. These include classical anti-viral response genes (OAS3 and IRF7), more recently identified anti-viral genes (ISG15, HERC5, RSAD2, TRIM5, TRAIL, OASL, ISG20), genes regulating ubiquitination (USP18), cell adhesion and cyclic ADP-ribose (cADPR) metabolism (CD38), apoptosis (XAF1), immune suppression (IFITM1), immune activation (LGALs3BP), anti-proliferative (SAMD9) and eight other genes (FLJ20035, FLJ38348, HERC6, IFI44, IFI44L, IFIT1, IFIT3, LY6E) currently with unknown function.

Gene expression analysis of DENV-infected HepG2 cells showed a very different pattern of expression for genes as compared to DENV-infected primary (monocytes, B cells, HUVECs and DCs) cells (Figure 1B). TRAIL (TNFSF10), the gene identified as the potential common linker from DENV-infection of primary cells (Figure 2) was not induced in HepG2 cells (Figure 1B box). This data indicates that primary cells might be better model to understand changes in expression of cellular genes following DENV infection.

Validation of the differential expression of the common response genes

ANOVA identified a set of 23 transcripts that demonstrated a significant change in expression (P of <0.05) in all cell types studied (Fig. 1A and Table 2). To

Table 2. Biological functions of the 23 DENV response genes

Name	Functional Category	Description	GenBank	Affy ID
G1P2	Anti-viral	Interferon, alpha-inducible protein (clone IFI-15K)	NM_005101	205483_s_at
IRF7	Anti-viral	Interferon regulatory factor 7	NM_004030	208436_s_at
ISG20	Anti-viral	Interferon stimulated gene 20kDa	NM_002201	204698_at
OAS3	Anti-viral	2'-5'-oligoadenylate synthetase 3, 100kDa	NM_006187	218400_at
OASL	Anti-viral	2'-5'-oligoadenylate synthetase-like	NM_003733	205660_at
RSAD2	Anti-viral	Radical S-adenosyl methionine domain containing 2	AI337069	213797_at
TRIM5	Anti-viral	Tripartite motif-containing 5	AF220028	210705_s_at
HSXIAPAF1	Apoptosis	XIAP associated factor-1	NM_017523	206133_at
TNFSF10	Apoptosis	Tumor necrosis factor (ligand) superfamily, member 10	U57059	202687_s_at
CD38	Cyclic ADP-ribose metabolism	CD38 antigen (p45)	NM_001775	205692_s_at
HERC5	HECT E3 Ubiquitin ligase	Hect domain and RLD 5	NM_016323	219863_at
IFI44	Interferon-Inducible	Interferon-induced protein 44	NM_006417	214453_s_at
IFI44L	Interferon-Inducible	Interferon-induced protein 44	NM_006820	204439_at
IFITM1	Immune suppression	Interferon induced transmembrane protein 1 (9-27)	AA749101	214022_s_at
LGALS3BP	Immune Activation	Lectin, galactoside-binding, soluble, 3 binding protein	NM_005567	200923_at
USP18	Ubiquitination	Ubiquitin specific protease 18	NM_017414	219211_at
SAMD9	Anti-proliferative	Sterile alpha motif domain containing 9	NM_017654	219691_at
FLJ20035	unknown	Hypothetical protein FLJ20035	NM_017631	218986_s_at
FLJ38348	unknown	Hypothetical protein FLJ38348	AV755522	213294_at
HERC6	unknown	Hect domain and RLD 6	NM_017912	219352_at
IFIT1	unknown	Interferon-induced protein with tetratricopeptide repeats 1	NM_001548	203153_at
IFIT3	unknown	Interferon-induced protein with tetratricopeptide repeats 3	NM_001549	204747_at
LY6E	unknown	Lymphocyte antigen 6 complex, locus E	NM_002346	202145_at

confirm the gene expression changes detected by Affymetrix GeneChip, 11 genes for which primers and probes were available were selected for validation by qRT-PCR.

qRT-PCR confirmed up-regulation (greater than threefold) of all the 11 genes in DENV-infected HUVECs and monocytes (Table 3). Also, the qRT-PCR and GeneChip results for gene expression were consistent for HUVECs (Table 4).

Identification of TRAIL as a common linker of the common response genes

To identify signaling pathways associated with the host cell response to DENV infection, we analyzed our common response gene list using Pathway Architect software (Stratagene).

This analysis found the commonly regulated genes to be predominantly regulated by type-I IFN (IFNα/β) and type-II IFN (IFNγ) signaling pathways (Figure 2). In addition, TRAIL/TNFSF10, one of the 23 common response genes, was identified as a potential linker of these two signaling pathways.

III.2: Identification of the gene expression response specific for DENV infection of HUVECs *in vitro*

Endothelial cells are targets for DENV infection based on *in vitro* and *in vivo* studies. In addition, vascular dysfunction is considered the reason for plasma leakage, vascular permeability and bleeding; clinically symptoms detected during DENV infection-mediated pathogenesis (DHF/DSS). Hence, we used the *in vitro* endothelial cell model for infection with DENV and other RNA viruses in an effort to detect DENV infection-specific responses.

Genes	qRT-PCR Fold Induction	
	Monocytes	HUVECs
C1orf29	12	31
CEB1	9	6
G1P2	8	15
HSXIAPAF1	2	8
IF144	23	10
IF1T1	6	32
IRF7	6	24342
ISG20	13	3
LGALS3BP	11	15
LY6E	17	4
USP18	37	5

Table 3. qRT-PCR validation of the 11 DENV response gene. *In vitro* DENV infections in HUVECs were analyzed by qRT-PCR using microfluidic cards. Relative induction was calculated with qRT-PCR software (Applied Biosystems). The qRT-PCR and GeneChip results showed similar fold induction for HUVECs and monocytes.

Gene Name	GeneChip	PCR
	Fold Induction	Fold Induction
ISG20	15	3
LYE6E	8	4
USP18	14	5
HERC5	9	6
HSXIAPAF1	7	8
IFI44	27	10
G1P2	28	15
LGALS3BP	3	15
IFI44L	58	31
IFIT1	126	32
IRF7	12	>100

TABLE 4.GeneChip and PCR expression comparison of the common response genes. *In vitro* DENV infections in HUVECs were analyzed by GeneChip and qRT-PCR analysis. Relative induction was calculated with qRT-PCR software (Applied Biosystems).

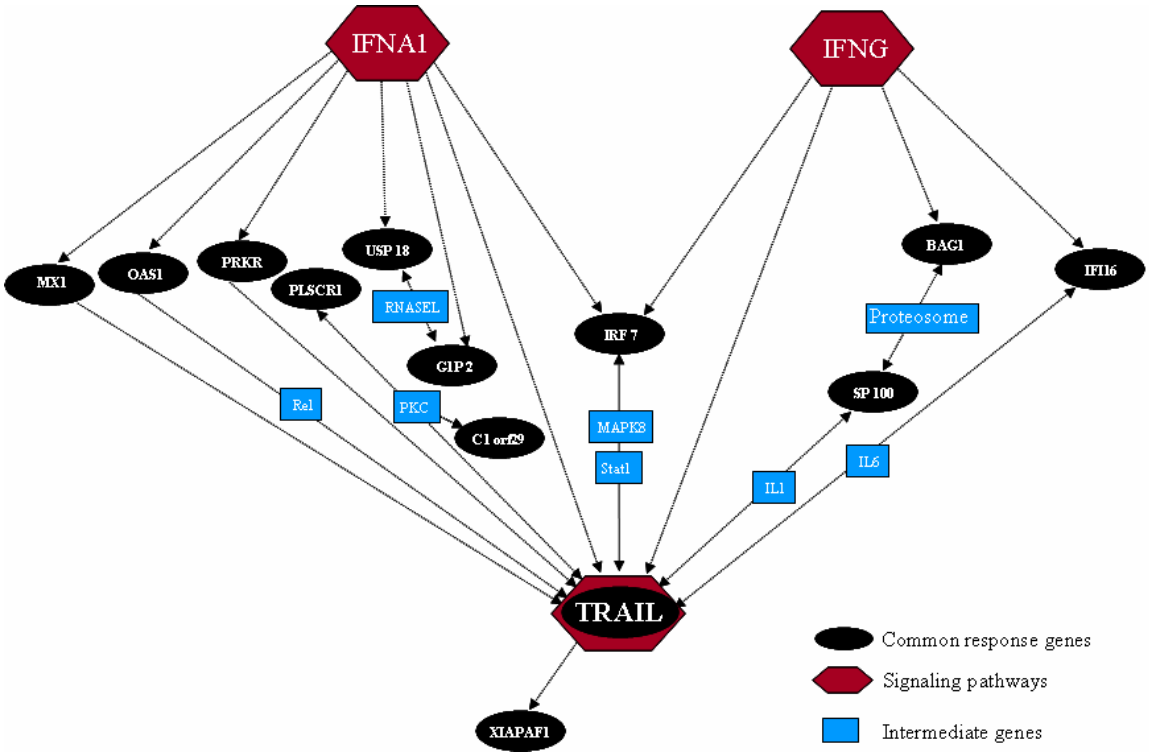


Figure 2. TRAIL is potential linker of the majority of the common response genes. The software used generated a Biological Association Network (BAN) of known protein interactions using a proprietary database containing over 140,000 references that are updated automatically on protein interactions obtained from PubMed (NCBI, NIH). The software was set up to identify and display common putative regulator(s) of the common response genes.

We selected RNA viruses (WNV, YFV, Hantaan and SNV) to control for immune response and antiviral genes which might be induced commonly in response to infection with RNA viruses. DNA viruses (vaccinia and EBV) were used as controls for non-specific viral responses. Among the RNA viruses, YFV and SNV, which are also hemorrhagic fever viruses, were used as controls to identify cellular responses that might be involved in regulation or induction of pathology (hemorrhage) during DENV infection. We wanted to infect HUVECs with DENV and other viruses at a dose which might be close to the physiologically relevant dose of infection. Hence, HUVECs were exposed to all the viruses at M.O.I. of 1. We performed qRT-PCR for DENV, WNV, YFV or Hantaan in virus-infected HUVECs to determine if these viruses were able to replicate in HUVECs.

Among the Gene expression analysis of DENV, other RNA viruses and DNA viruses identified 30 genes that were potentially differentially regulated specifically in DENV-infected HUVECs (Fig. 3). Some of the genes listed include C1S (classic complement), kynureninase (kynurenine pathway of L-Trp degradation), SOD2 (protects mitochondria from oxidative stress damage) (174), CARP, IL-1β (pro-inflammatory cytokine), IL8, cathepsin S, TNFSF4 (OX40L) implicated in Th2 priming to Th cells (91), DTR, IL1rL1, tenascin C, SCYB5 (epithelial-derived neutrophil activating, ENA-78) (87), ATP binding protein regulating calcium influx (P2RX4) (232).

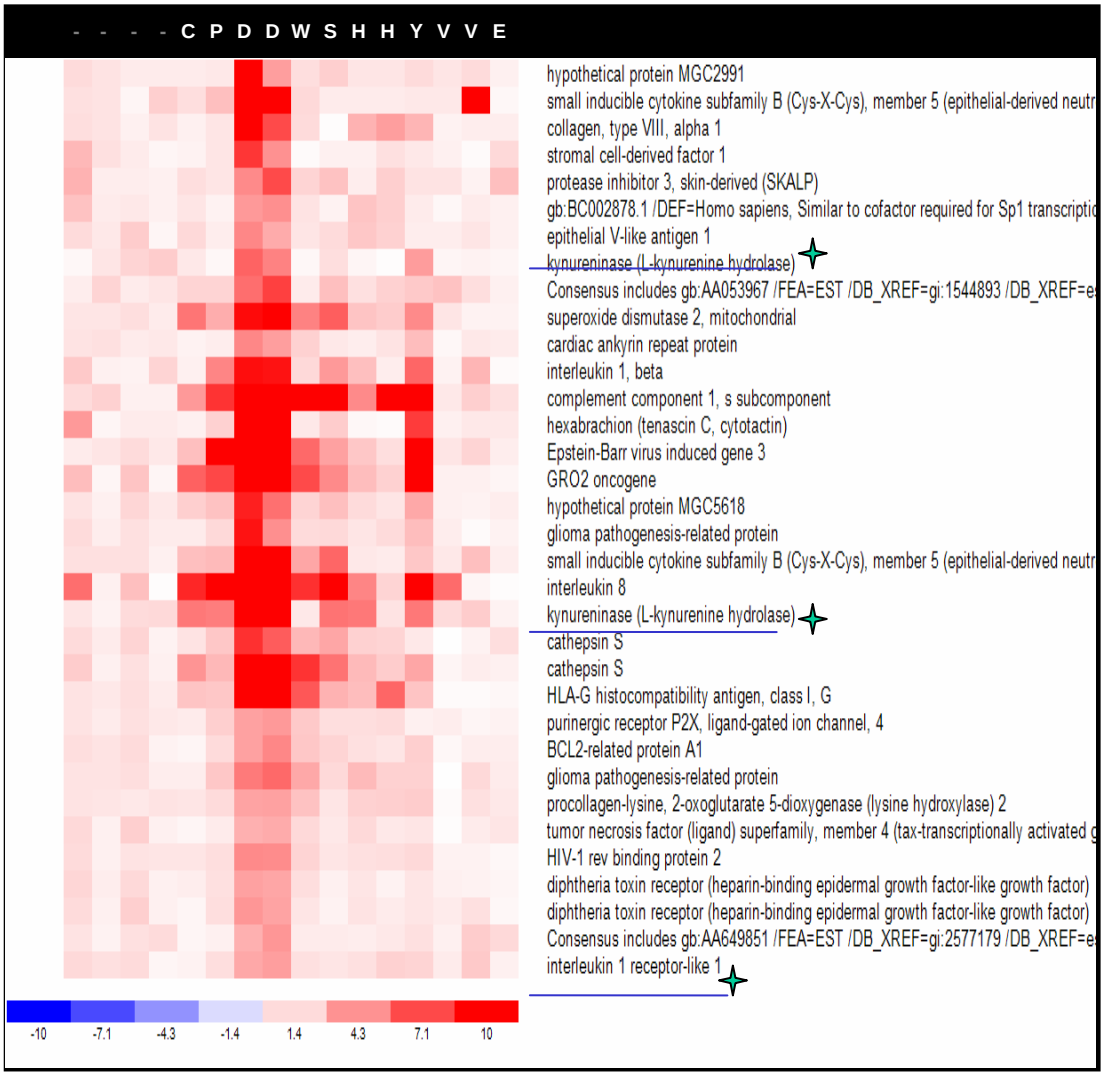


Figure 3. DENV-specific gene expression analysis using HG-U133A Affymetrix GeneChips. Expression levels for the 30 DENV-specific genes in HUVECs exposed for 48 hours to DENV, other RNA viruses or DNA viruses known to infect HUVECs. *In vitro* infection profiles were normalized to C6/36 insect cell supernatant-treated samples or culture media for each cell type independently. Hierarchical cluster analysis used a Pearson correlation. Color indicates relative changes in induction (*n*-fold). On the color scale, dark red represents 20-fold up-regulation, and white indicates no change. Affymetrix microarray analysis was performed using GeneSpring software (Agilent). Each lane in the figure is an independent experiment.

Confirmation of the DENV-specific gene expression analysis

We confirmed an increase in expression for 6 potential DENV-specific response genes to infection of HUVECs and monocytes with DENV *in vitro*. Kynureninase was among the list of genes specifically induced in response to DENV infection in HUVECs. Kynureninase enzyme regulates a downstream step in the production of NAD+ from tryptophan. In literature, studies have focused on the first which is the rate limiting step of the kynurenine pathway of tryptophan degradation. IDO enzyme regulates that initial and rate-limiting step of the tryptophan catabolism pathway. IDO has shown to play a role in T cell proliferation and peripheral tolerance (150, 151). Also, previous studies have found IDO activity to be important for IFNγ mediated antiviral function for viruses (4, 160). Hence, we focused on IDO qRT-PCR expression levels. qRT-PCR analysis confirmed DENV infection induced gene expression levels suggested by Affymetrix analysis (Table 5).

To determine their expression in patient samples we further confirmed specificity of the differential regulation of these genes in PBMCs obtained from DENV (n=3) and OFI (n=1) patients during febrile phase of the infection (data not shown). The preliminary comparison with OFI patient PBMCs was done to control for non-specific activation of these genes due to febrile (most likely viral) illness.

We found that IDO and ST2 gene mRNA were expressed at much higher levels in DENV patient PBMCs as compared to in OFI patient PBMCs (data not shown). Hence, we have identified two potential DENV infection specific genes using genomic analysis of *in vitro* DENV-infected HUVECs.

Gene Name	GeneChip	PCR
	Fold Induction	Fold Induction
C1S	4.4	7
DTR	4.27	1.3
ST2	2.13	1.3
P2RX4	4.15	1.6
SOD2	4.89	3.2
IL8	5.8	2.5
IDO	6.02	20829

Table 5: Confirmation of 7 DENV response genes in HUVECs. Relative induction was calculated with qRT-PCR software (Applied Biosystems). qRT-PCR confirmed induction of the genes following DENV-infection suggested by the GeneChip results in the HUVECs for 4 out of the 7 genes.

Expression levels of seven DENV infection inducible genes were validated by qRT-PCR.

III.3. Investigation of the Susceptibility of Muscle Satellite Cells to DENV

The cause of myalgia during DENV infection is still unknown. One possible hypothesis for the myalgia and musculoskeletal pain frequently observed during DENV infection is that muscle cells are infected by DENV. Muscle satellite cells are precursor skeletal muscle cells. Satellite cells are resident, proliferative cells found in the skeletal muscle. These cells express muscle specific markers like neural adhesion molecule (NCAM) or CD56 and Desmin (19, 72) and will spontaneously fuse to form mature, contractile, skeletal muscle. In addition, they their proliferative capacity makes them an ideal model for *in vitro* infection studies on skeletal muscle. Hence, to determine whether DENV can infect primary muscle cells, we exposed human muscle satellite cells to DENV *in vitro*.

Purity of muscle satellite cells

Desmin and CD56 are both considered reliable markers for muscle satellite cells among cells cultured from skeletal muscle (165, 204, 239). Fibroblasts, which co-propagate in cell culture, do not express CD56 (6). Hence, we used CD56 as a marker to determine the purity of muscle satellite cells cultured *in vitro*. Approximately 15-30% of the live gated cells did not stain positive for CD56 (Fig. 4).

DENV can infect and produce infectious progeny in muscle satellite cells

To determine whether these cells could serve as a host for DENV infection, we exposed them to DENV *in vitro* and determined their susceptibility to a productive DENV infection.

Primary human muscle satellite cells were infected with DENV2 strain New Guinea C (NGC), DENV1 strain Hawaii, DENV4 strain 814669 at M.O.I. of 2, for 24 and 48 hours and stained for DENV antigen. Similar proportions of muscle satellite cells stained positive for DENV2 antigen at the 24 hour (16.9±4.2%, n=3) and 48 hour (9.5±3.4%, n=3) time-points (Fig. 5A and 5B). Since DENV2 NGC represents a prototype strain of DENV we investigated whether DENV1 and DENV4 strains could also productively infect muscle satellite cells.

Muscle satellite cells also stained positive for DENV1 (6.6±2.7%, n=2) and DENV4 (21.5±12.3%, n=2) antigens (Fig. 5B) at levels comparable to DENV2 as determined by flow cytometry. These results demonstrate that muscle satellite cells are susceptible to infection with multiple DENV serotypes, *in vitro*.

We also demonstrated the direct presence of DENV antigen in muscle satellite cells using immunofluorescence microscopy. Cells were grown on microscope cover slips overnight and then infected with DENV2 at an MOI of 2. After 48 hours, cells were fixed and stained for DENV and desmin. Desmin and DAPI double-positive cells clearly stained positive for DENV antigen in the cytoplasm (Fig. 6).

To determine whether DENV-infected muscle satellite cells produced infectious DENV progeny we performed plaque assay on DENV-infected muscle satellite cell supernatants. High levels of infectious DENV progeny (10^4-10^6 pfu/ml) were

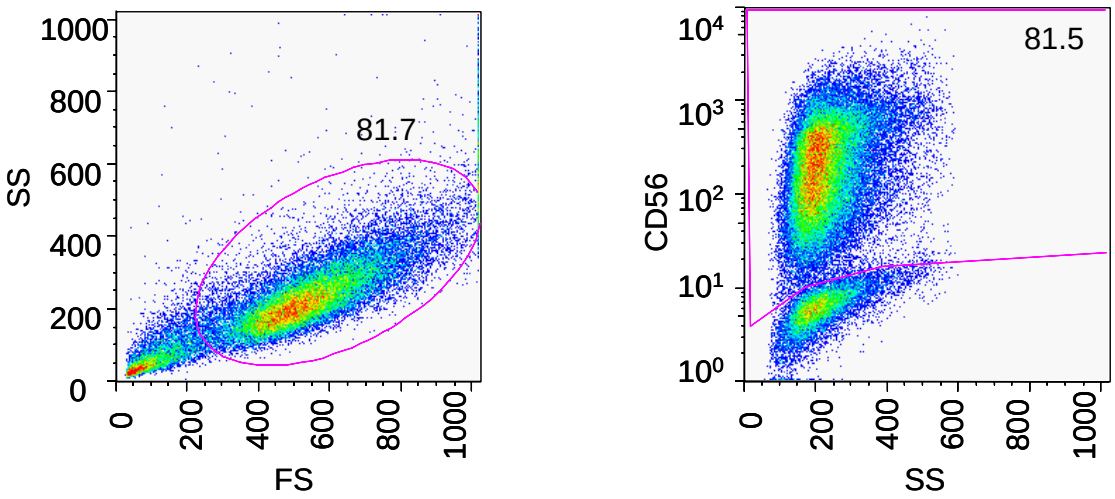
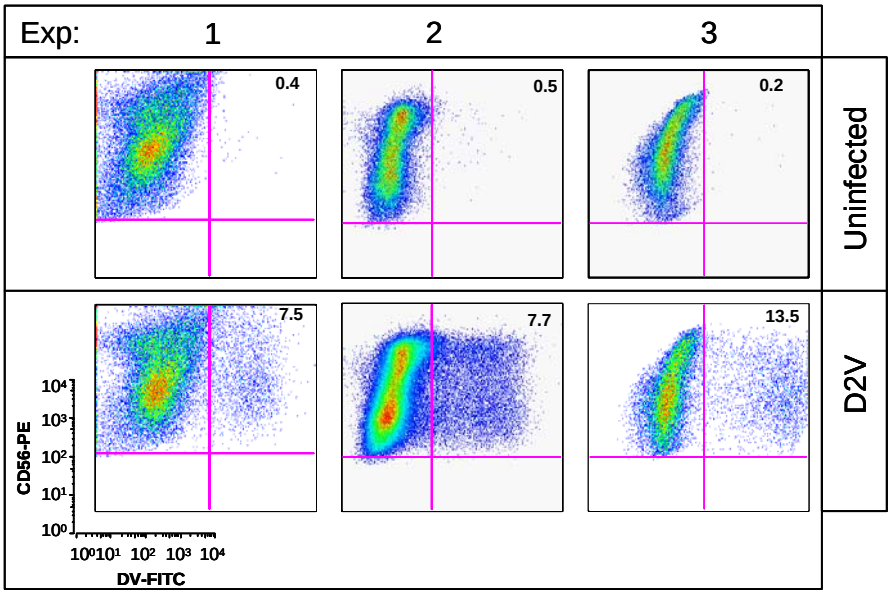


Figure 4. Gating of live cells and CD56-positive population. Forward scatter (FS) and side scatter (SS) plot was used to initially gate on live cells. Next the live gated cells were gated on CD56 Ab to select the muscle satellite cells for further flow cytometry analysis.

A



B

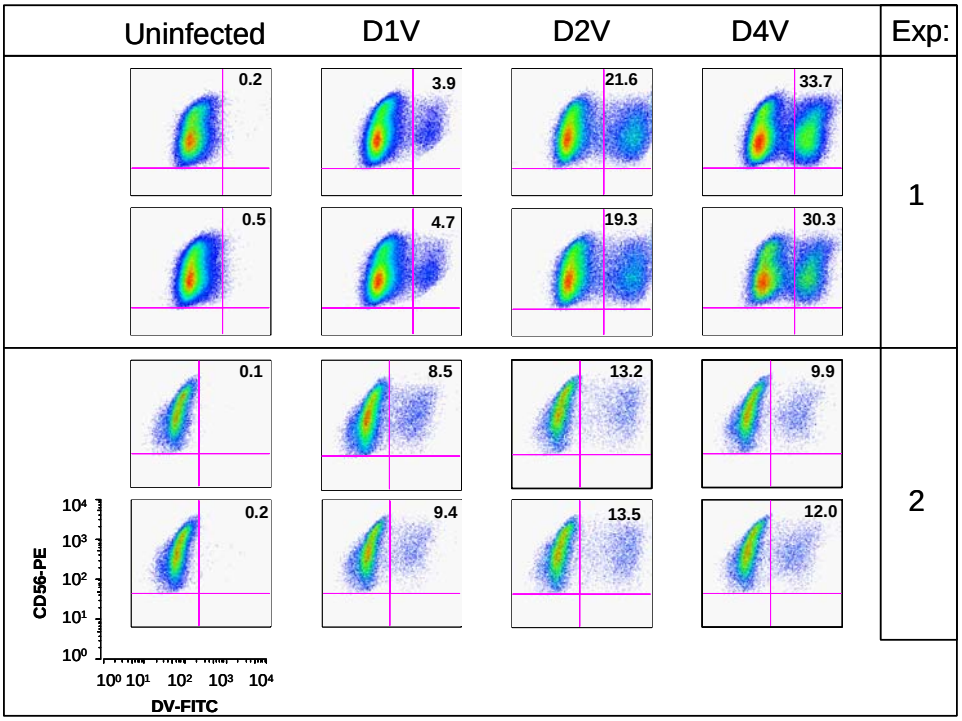


Figure 5. Detection of dengue virus (DENV) in primary human muscle satellite cells by flow cytometry. (A) Primary muscle satellite cells obtained from three donors were infected with DENV2 for 48 hours at M.O.I. of 2. Both uninfected and DENV-infected cells were stained with anti-DENV-FITC (Chemicon). (B) Primary muscle satellite cells obtained from two donors were infected with DENV1, DENV2 and DENV4 for 24 hours at M.O.I. of 2. Each infection was performed in duplicate.

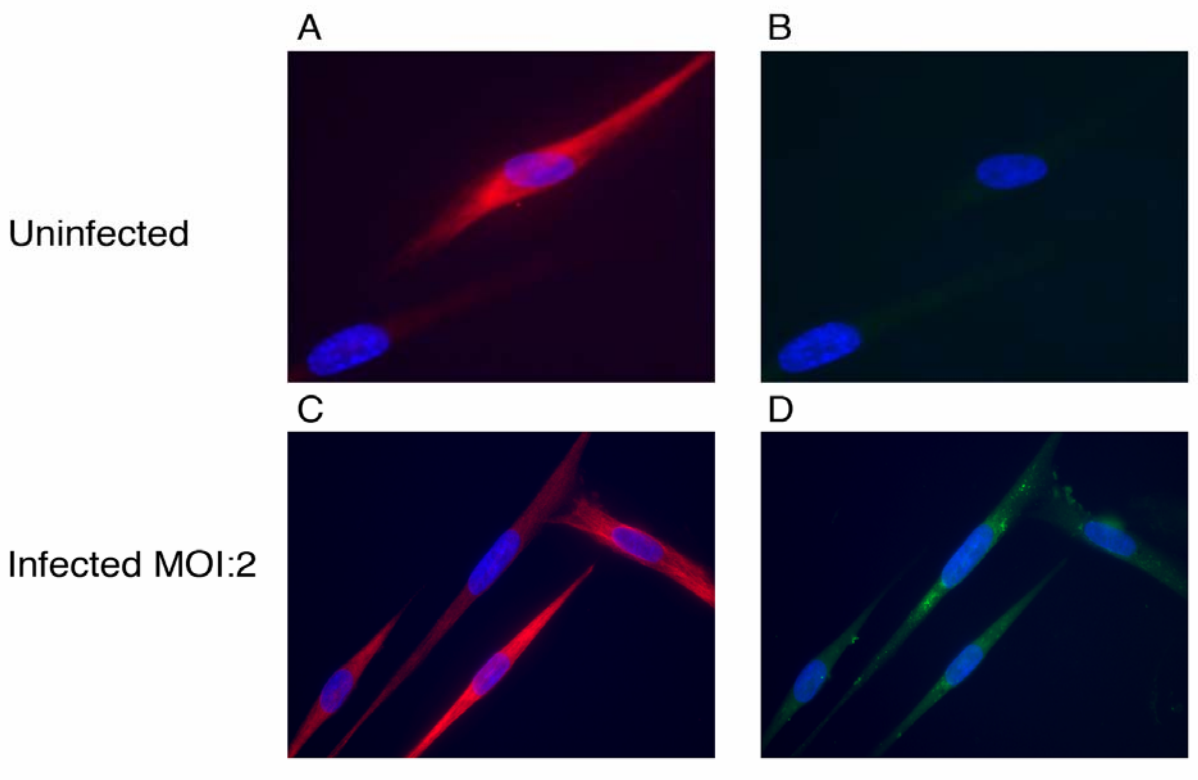


Figure 6. Immunofluorescence microscopy of muscle satellite cells using 20X objective. (A-D) Nuclei are visualized using DAPI staining, which is shown in blue in all four images. A and B represent uninfected cells. A, desmin (red). B, dengue (green) localization absent. C and D represent cells infected with DENV at MOI of 2: C, desmin (red) is seen in all four cells within the field of view. D, all four muscle satellite cells are positively stained with anti-dengue antibody (green) indicating DENV infection.

detected in the supernatant from both DENV2- and DENV4-infected muscle satellite cells by plaque assays (Fig. 7).

Chapter Summary

One aim of this section of the thesis was to apply gene expression analysis of multiple DENV-infected primary human cell types to identify genes of interest for further study. We applied a two pronged approach for identification of genes of significance for regulation or identification of DENV infection.

The common gene expression profile of 23 genes detected in DENV-infected monocytes, B cells and HUVECs identified TRAIL, one of the common response genes, as a potential common linker of the type-I and type-II IFN response genes (PathwayArchitect, Stratagene). In addition, we identified potential endothelial cell DENV infection-specific response genes in HUVECs infected *in vitro*. Among the DENV-specific response genes confirmed in PBMCs from acute phase of the disease in OFI and DENV patients we identified ST2 and IDO genes as potential biomarkers for DENV infection.

Another aim of this section of the thesis was to determine the capacity of DENV to infect primary muscle satellite cells. Flow cytometry analysis and immunofluorescence microscopy experiments showed that DENV can infect primary muscle satellite cells. Further, large amounts of infectious DENV progeny were released by primary muscle satellite cell cultures as detected by plaque assay.

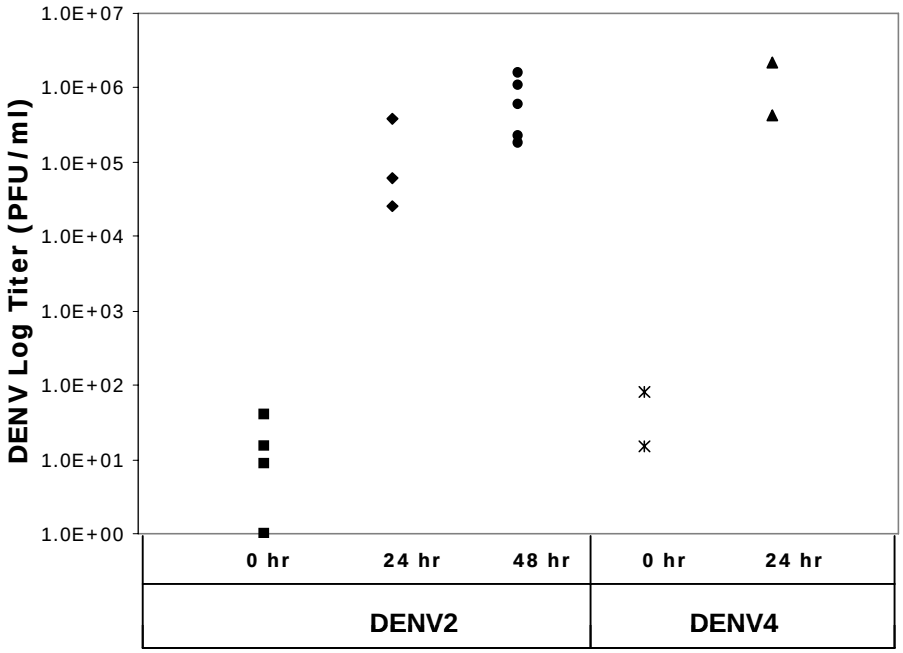


Figure 7. Muscle satellite cells produce infectious DENV progeny. Muscle satellite cells were infected with DENV for 0, 24 and 48 hours at M.O.I. of 2. At each time point plaque assay was performed to determine infectious DENV progeny. Experiments performed for DENV2 infection at zero hour (n=4), 24 hour (n=3) and 48 hour (n=5). Experiments performed for DENV4 infection at zero hour (n=2) and 24 hour (n=2).

Chapter IV

TRAIL is involved in the antiviral and the anti-inflammatory response of primary human cells to DENV infection

The common gene expression profile data from Chapter III identified TRAIL as a potential common linker of the type-I and type-II IFN-inducible genes. TRAIL, a member of the TNF superfamily, has been shown in previous studies to have antiviral and anti-tumor activity(22, 26, 102, 133, 171, 184, 187, 220, 223, 238). TRAIL exhibits potent antiviral activity against influenza, CMV and other viruses (22) (89, 188). In addition, Secchiero et al. has shown that TRAIL can inhibit the adhesion of leukocytes to TNFα- and IL1β- activated HUVECs and HAECs (185).

Hence, in this section of the thesis we determined the pattern of expression of TRAIL following DENV infection and whether TRAIL played a role in regulating DENV infection and pro-inflammatory genes in primary human cells.

TRAIL mRNA induction in DCs, Mo and B cells

In Chapter III, we observed that the expression of TRAIL mRNA was induced in DENV-infected primary human cells. Using real-time PCR, we determined TRAIL mRNA expression levels in monocytes, B cells and DCs infected with DENV, *in vitro*. TRAIL mRNA was induced in DENV-infected monocytes, B cells, and DCs (Fig. 8). The induction of TRAIL mRNA was similar in all the cell types tested.

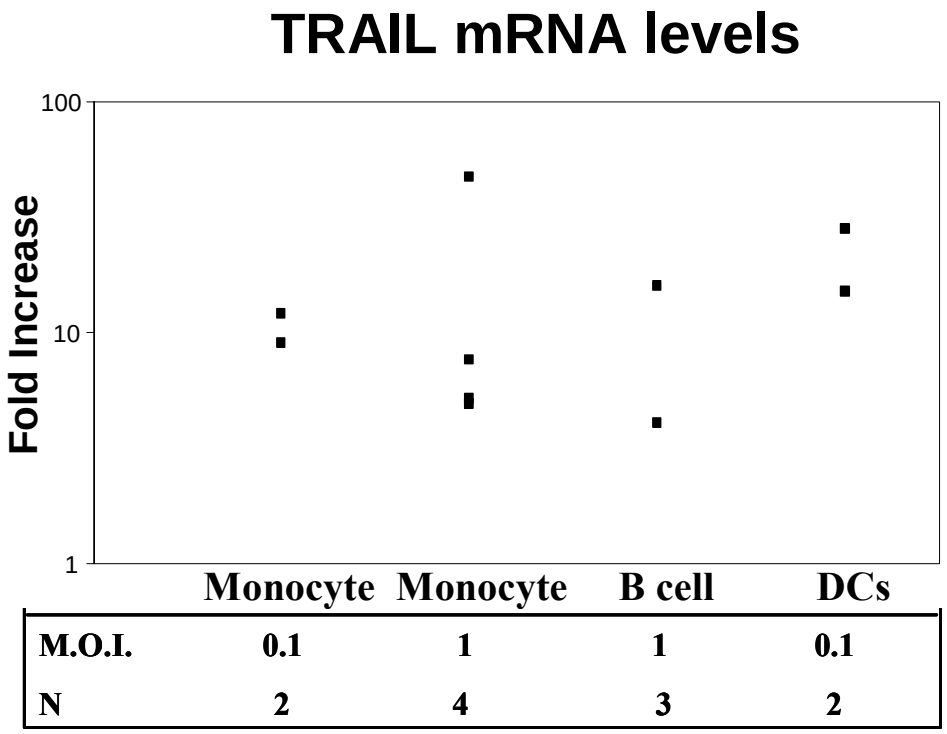


Figure 8. TRAIL mRNA induction in DENV-infected cells. Monocytes, B cells, and DCs were infected with DENV at MOI of 0.1 and / or 1 PFU/ml for 48 hours. TRAIL mRNA expression was quantified by qRT-PCR on total RNA extracts. β-actin mRNA was used as a control probe. Data shown are representative of multiple (N) independent experiments.

TRAIL protein is produced by DENV-infected cells

Cells can regulate protein levels through other mechanisms and mRNA levels may not reflect protein levels (13, 15, 128, 144). Hence, we wanted to confirm that DENV infection induced an increase in TRAIL protein levels. TRAIL protein is capable of initiating its function through the engagement of its death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5) (44). Previous studies have shown that TRAIL protein can be secreted by the cell or localize inside (cytoplasm) or on the surface of the cell (56, 147, 224).

Initially, we determined whether TRAIL expression was induced at the protein level in cell culture supernatants and cell lysates from DENV-infected monocytes and cell culture supernatants from DENV-infected DCs. Monocytes and DCs are the most accepted cellular targets for DENV infection and replication, *in vivo*. No TRAIL protein was detected in the supernatant of DENV-infected monocytes and DCs by ELISA, most likely due to the small amounts of TRAIL present at the timepoint when the assay was performed and at the amount of cells in the culture (one million cells per ml at 0.1 MOI for 48 hours of infection). However, we detected larger amounts of TRAIL protein, 180.93 ± 101.9 pg/ml (mean ± standard deviation [SD]; $n = 3$), in DENV-infected monocyte cell lysates compared to uninfected monocyte lysate at 48 hours after infection with DENV *in vitro* at 1 MOI (Fig. 9A). TRAIL protein levels were below the level of detection (ELISA) in uninfected monocytes. These results indicated that TRAIL protein is localized inside the cell or on the surface of cells following DENV infection.

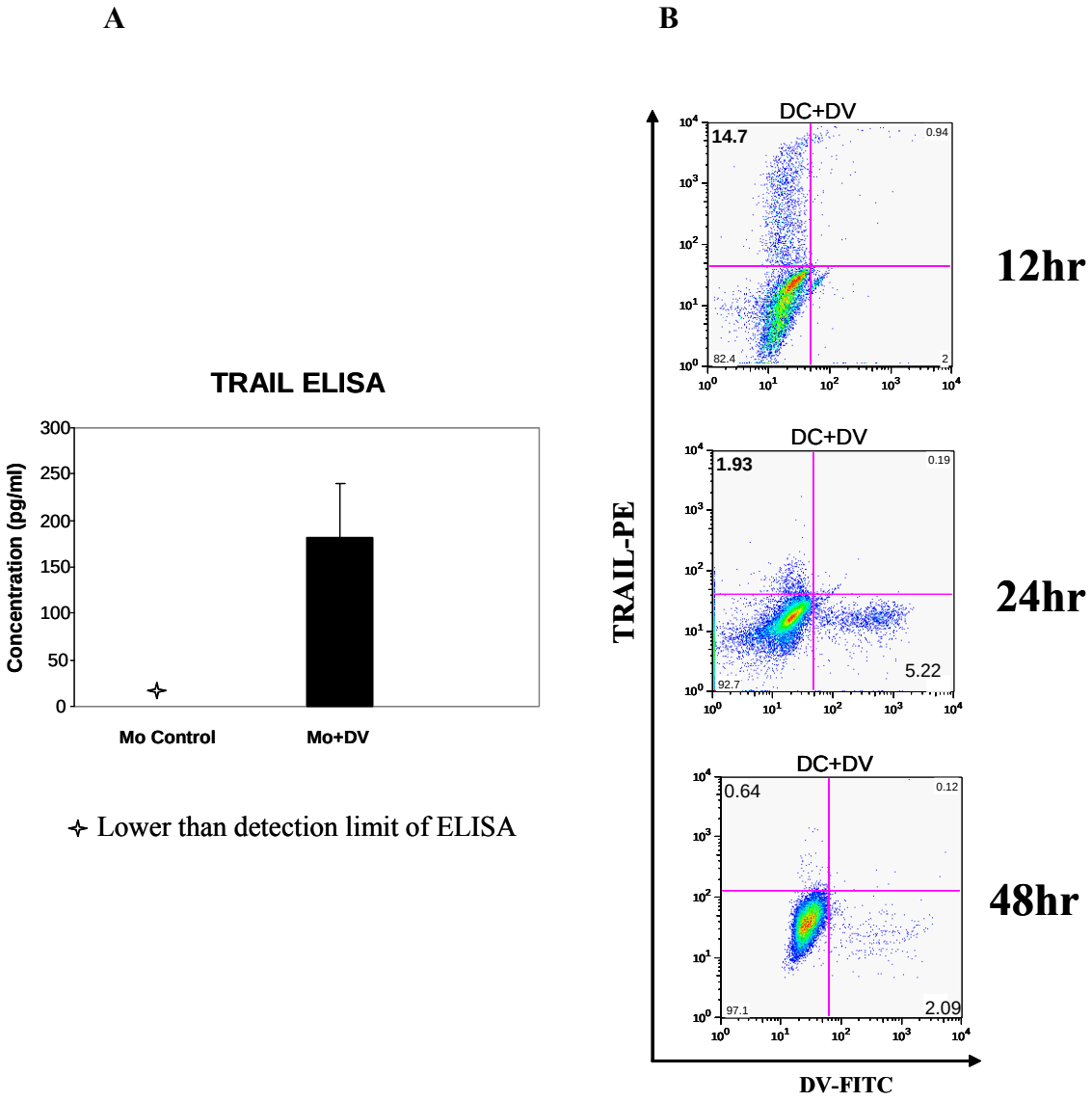


Figure 9. Intracellular TRAIL protein levels were measured (A) in cell lysates by ELISA, and (B) by intracellular staining. (A) Monocytes (Mo) were infected with DENV at an MOI of 1 PFU/cell and then cultured for 48 hours. Levels of TRAIL protein were quantified in cell lysates using ELISA (R&D Systems). Data shown are representative of three experiments. (B) DCs were infected with DENV for 12, 24, and 48 h at an MOI of 0.1. Cells were treated with brefeldin A for the last 8 h of each time point. TRAIL protein was detected by flow cytometry using TRAIL-PE (BD Biosciences). DENV antigen was stained using the DENV-FITC Ab (2H2 Ab). Data shown are representative of two experiments.

In vitro, DENV-infected primary human monocytes do not stain well for DENV antigen unlike DENV-infected monocyte derived dendritic cells, which stain clearly for DENV antigen. Hence, to determine whether DENV-infected or bystander (uninfected) cells of the same culture flask were able to express TRAIL, we analyzed DENV-infected DCs by flow cytometry. We did not detect TRAIL protein on the surface of DENV-infected monocytes and DCs (data not shown), suggesting that TRAIL protein detected in DENV-infected cells was localized inside the cell. Hence, we performed intracellular staining assay on DENV-infected DCs to identify the cells producing TRAIL protein following DENV infection.

Increased intracellular TRAIL protein was detected at 12 hours but the TRAIL protein levels decreased to much lower levels by the 24- or 48-hour time points after infection, with less than 2% of the cells expressing TRAIL (Fig. 9B). However, we could not detect DENV antigen expression at the 12 hour timepoint postinfection. Hence, we cannot conclude whether TRAIL was expressed in DENV-infected or uninfected (bystander) cells or both. The lack of TRAIL detection at 24 and 48 hour post-infection indicates that TRAIL expression is transient in DCs. Very few DCs were positive for DENV antigen at the 12 hour timepoint postinfection when the TRAIL protein levels were high. One possible explanation for this finding is that, since TRAIL is an early expressed gene, it is possibly an upstream regulator of cellular DENV response.

The DENV antigen-positive cells did not express TRAIL protein. An explanation for this finding is that DENV infection might inhibit expression of TRAIL protein. TRAIL has previously been shown to function as an antiviral

molecule against CMV, ECMV, influenza and other viruses (89, 184). Thus, these data lead us to speculate that DENV might block TRAIL expression to suppress the antiviral response against DENV.

In summary, DENV infection induced TRAIL protein in cells was both transient and early, 12 hours postinfection.

TRAIL functions as an antiviral molecule against DENV

(A) Blocking of endogenous TRAIL enhances DENV replication *in vitro*

To determine if TRAIL produced in response to DENV infection was involved in the antiviral response against DENV, we determined the effect of TRAIL neutralizing antibody (Catalog# 550912, BD Biosciences) on DENV levels in infected cells. IFNα is a strong inducer of soluble and cell surface expression of TRAIL in monocytes and B cells (56). Since we were using primary monocytes and B cells to determine the effect of blocking TRAIL protein on DENV levels, the IFNα-mediated increase in TRAIL expression was utilized to titrate the TRAIL neutralizing antibody. Titration of TRAIL neutralizing antibody was performed against IFNα (100 U/ml) treatment in primary monocytes to determine the concentration of the antibody required to neutralize the surface bound TRAIL protein (56). The titration experiment found that the anti-TRAIL Ab at 50ug/ml concentration was able to block binding of the fluorescent Ab to all surface bound TRAIL protein (data not shown). TRAIL neutralizing antibody at the titrated concentration was used to block TRAIL function in DENV-infected monocytes, B cells, and HUVECs.

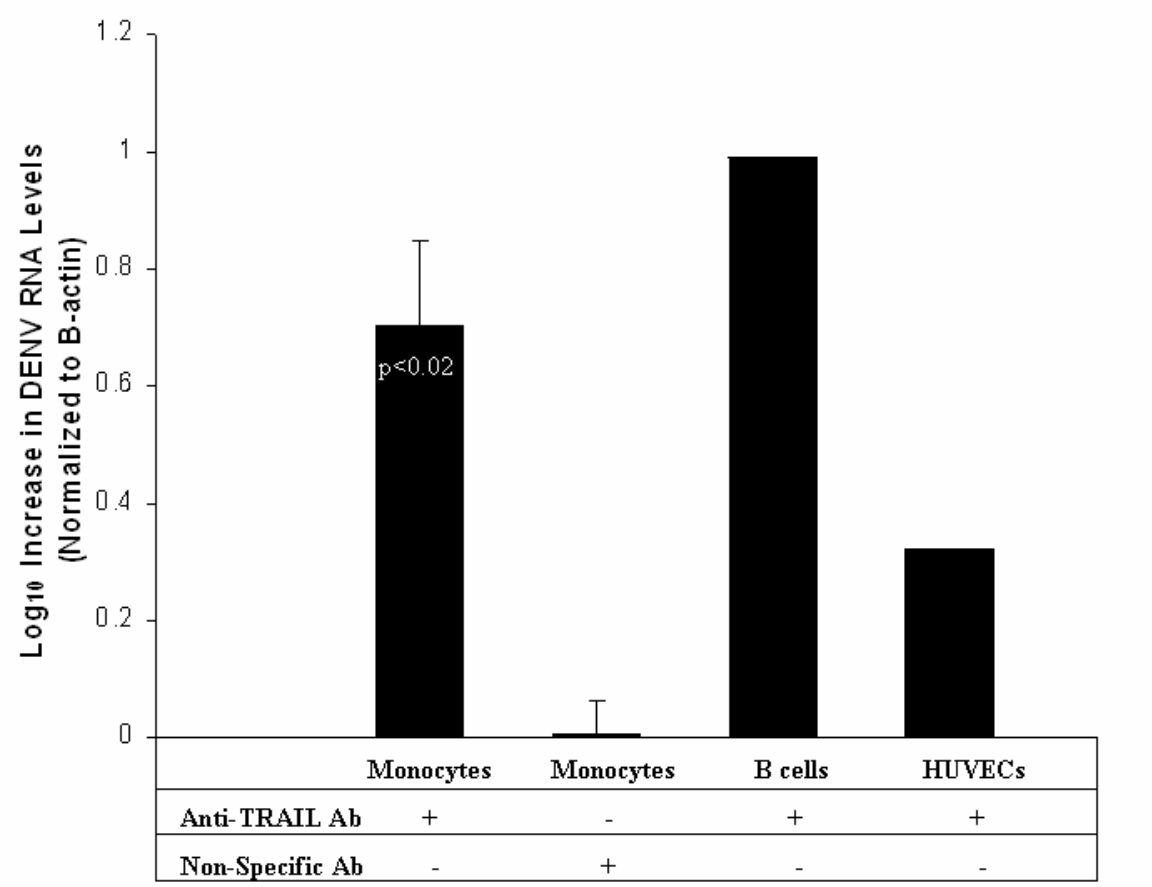


Figure 10. TRAIL blocking monoclonal antibody increases DENV replication. Monocytes, B cells, and HUVECs were infected with DENV at an MOI of 1 PFU/cell and then cultured for 48 hours. TRAIL blocking antibody (50 ng/ml) was added 24 hours prior to infection with DENV. Intracellular DENV RNA levels were quantified by qRT-PCR. β-Actin mRNA was used as a control probe. The data presented are the means of five experiments for monocytes and two experiments each for B cells and HUVECs. Results for nonspecific antibody-treated monocytes are the means of three experiments (± SD). TRAIL blocking Ab increased DENV levels ($p<0.02$) as compared to non-specific Ab in monocytes.

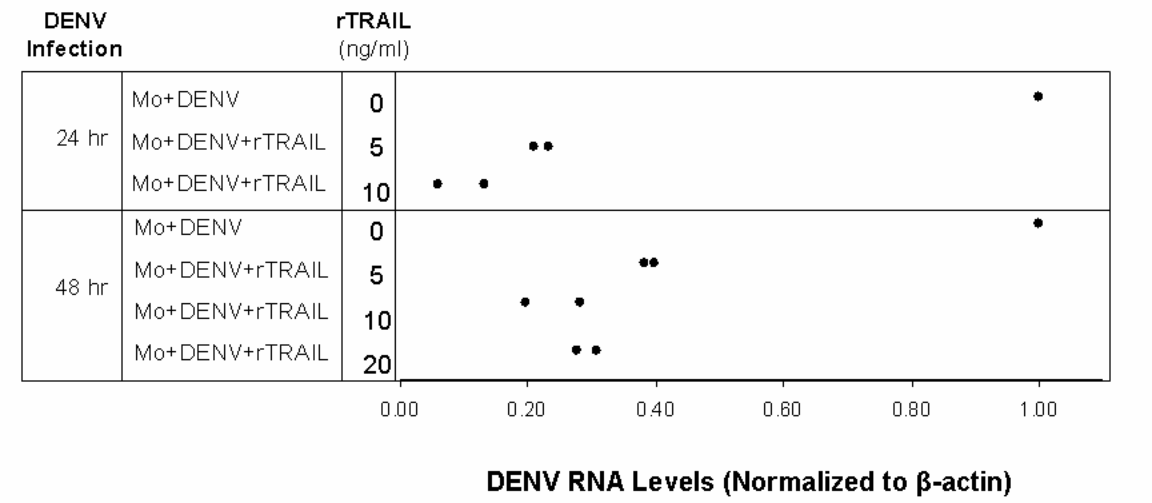
At 48 h postinfection, monocytes incubated with anti-TRAIL showed a 0.7 ± 0.3 (mean ± SD) log increase in DENV RNA levels relative to untreated, or to control antibody-treated cells ($P < 0.02$, unpaired Student's *t* test; $n = 5$). In duplicate experiments, an average 0.99 and 0.32 log increase in DENV RNA was observed in B cells and HUVECs, respectively (Fig. 10). Irrelevant antibody treatment did not affect DENV RNA levels in monocytes (0.008 ± 0.09 log increase in DENV RNA). Results from the antibody blocking experiments show that TRAIL protein has antiviral activity against DENV *in vitro*.

(B) Addition of Exogenous recombinant TRAIL

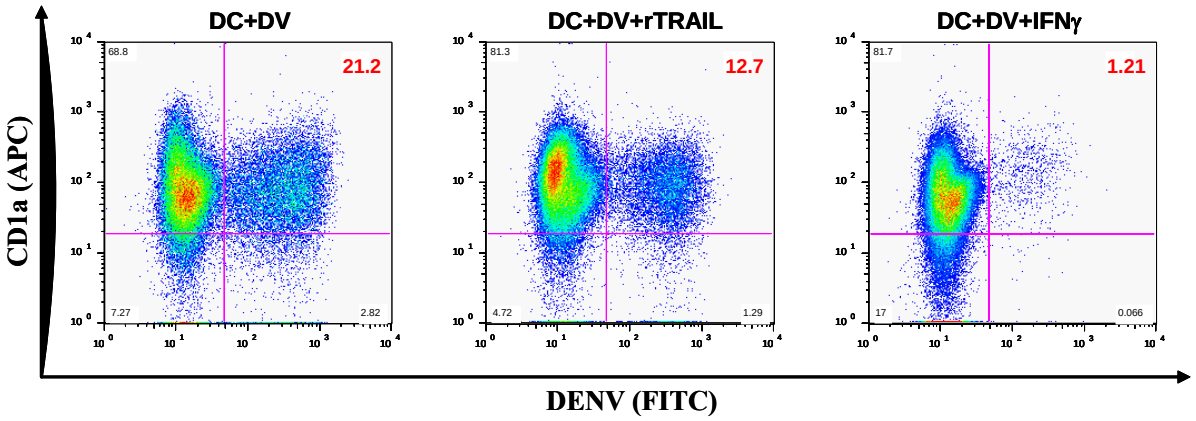
Monocytes and dendritic cells were treated with recombinant TRAIL to determine whether exogenous rTRAIL (Catalog# SE721-0100, Biomol and (Catalog# 616374, Merck) was able to inhibit DENV replication. Inhibition of DENV RNA levels and antigen was used as a read-out of lower levels of DENV in monocytes and dendritic cells, respectively.

Monocytes pretreated with 5 to 20 μg/ml of rTRAIL (Catalog# 616374, Merck) showed a 0.5 and 1 log decrease in DENV RNA copy number after 24 and 48 hours, respectively (Fig. 11A). We also determined inhibition of DENV infection in DCs 48 hours postinfection. Since DCs are more efficiently infected by DENV than the other cells used, we were able to measure the percent DENV infected cells by flow cytometry. DCs treated with rTRAIL (20 μg/ml) showed a decrease of 67.5% ± 22.9% (mean ± SD) in DENV (n=9) antigen-positive cells (data not shown). IFNγ (500 U/ml), which has previously been shown to inhibit DENV in DCs (78), inhibited

A


DENV Infection
rTRAIL (ng/ml)
24 hr
Mo+DENV
Mo+DENV+rTRAIL
Mo+DENV+rTRAIL
0
5
10
48 hr
Mo+DENV
Mo+DENV+rTRAIL
Mo+DENV+rTRAIL
Mo+DENV+rTRAIL
0
5
10
20
0.00
0.20
0.40
0.60
0.80
1.00
DENV RNA Levels (Normalized to β-actin)

B


DC+DV
DC+DV+rTRAIL
DC+DV+IFNγ
CD1a (APC)
DENV (FITC)
68.8
21.2
7.27
2.82
81.3
12.7
4.72
1.29
81.7
1.21
17
0.066

C

Time-point	Experiment #	DENV Antigen Positive DCs		
		DENV	DENV+rTRAIL	DENV+IFNγ
48 hours	1	21.2	12.7	1.2
	2	20.7	0.6	0.5
	3	1.6	1.0	0.8
	4	1.9	0.7	0.4
	5	8.7	4.7	0.5
	6	8.0	1.4	0.4
	7	1.9	0.1	0.1
	8	6.8	1.3	1.1
	9	2.9	1.0	1.6

Figure 11. rTRAIL treatment inhibits DENV infection. (A) Monocytes were infected with DENV at an MOI of 0.1 PFU/cell and then cultured for 24 and 48 hours. Monocytes were pretreated with rTRAIL 24 hours prior to infection with DENV. Intracellular DENV RNA levels were quantified by qRT-PCR. DENV RNA levels are obtained by applying the formula: DENV+rTRAIL / DENV for each dose of rTRAIL at each timepoint. β-actin mRNA was used as a control probe. Results are the means of two experiments. (B, C) DCs were pretreated with rTRAIL for 24 hours followed by infection with DENV2 or (C) DENV3 at an MOI of 0.1. At the 48-h time point the cells were stained intracellularly for DENV antigen (2H2 monoclonal antibody). (B) Data presented from one representative experiment of nine for DENV performed. (C) Data from all experiments are presented for cells treated with rTRAIL (20 μg/ml) DENV (n = 9) or IFNγ (500 U/ml) (n = 9). Data are presented as percent DENV antigen positive DCs following DENV infection or DENV infection and rTRAIL or IFNγ treatment. FITC, fluorescein isothiocyanate; APC, allophycocyanin.

DENV antigen-positive cells by 81.5% ± 20.3% (mean ± SD) (n=9) (data not shown). Percentage of DENV antigen positive DCs following infection and rTRAIL and IFNγ treatments are shown in Figure 11B and Figure 11C.

Overall, the data from experiments using TRAIL inhibition by antibody and addition of exogenous rTRAIL demonstrate that TRAIL inhibits DENV infection in primary human cells, *in vitro*.

TRAIL inhibits production of DENV progeny virus

Previous experiments showed that TRAIL can lower DENV RNA levels and DENV antigen expression in multiple cell types (Figure 10 and Figure 11). Next, we performed plaque assay on the dendritic cell culture supernatants to measure the effect of rTRAIL on production of infectious DENV progeny.

DENV infectious progeny was significantly reduced ($P < 0.007$, paired Student's *t* test; $n = 5$) in the supernatant of DENV-infected DCs treated with rTRAIL (Figure 12). The range of inhibition was from 0.9 to 2.8 logs. Hence, TRAIL can inhibit infection and replication of DENV in primary human cells *in vitro*.

TRAIL antiviral function in DCs is independent of apoptosis

A majority of previously published reports have found that TRAIL functions by inducing apoptosis of tumor cells and cell lines (26, 102, 133, 223, 238). However, most primary cells are resistant to TRAIL-mediated apoptosis (171, 184). TRAIL has also been shown to either inhibit viral infections or induce viral infection induced pathology during measles virus (219), cytomegalovirus (175) (205), reovirus (42) and

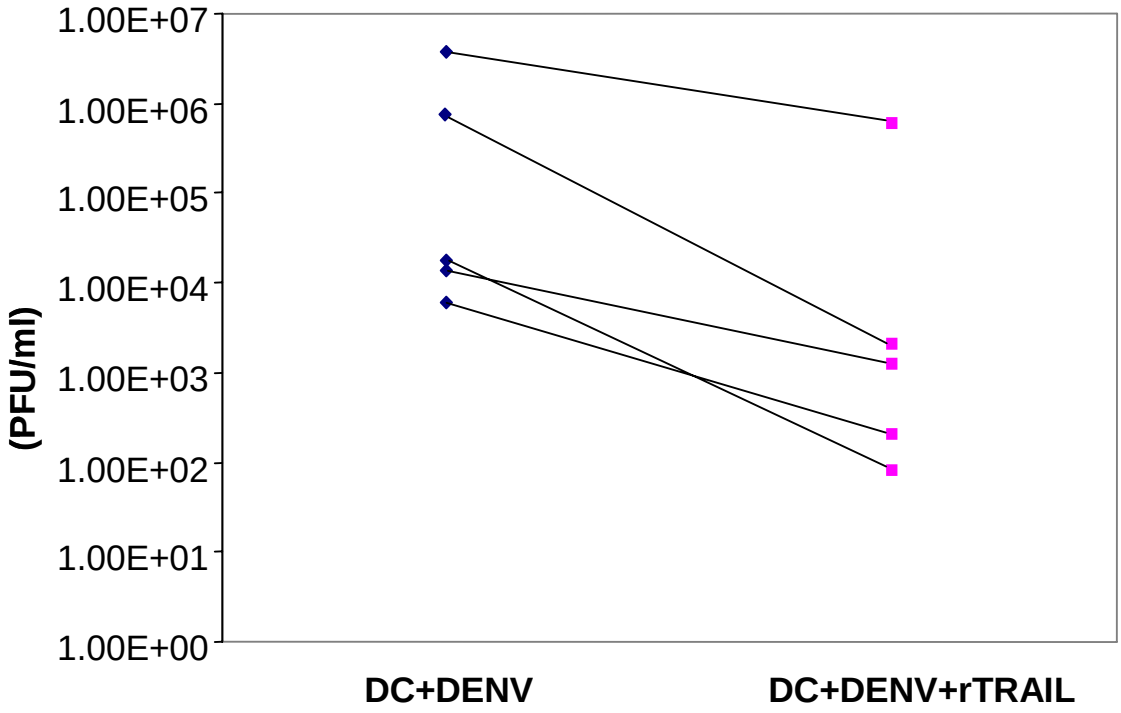
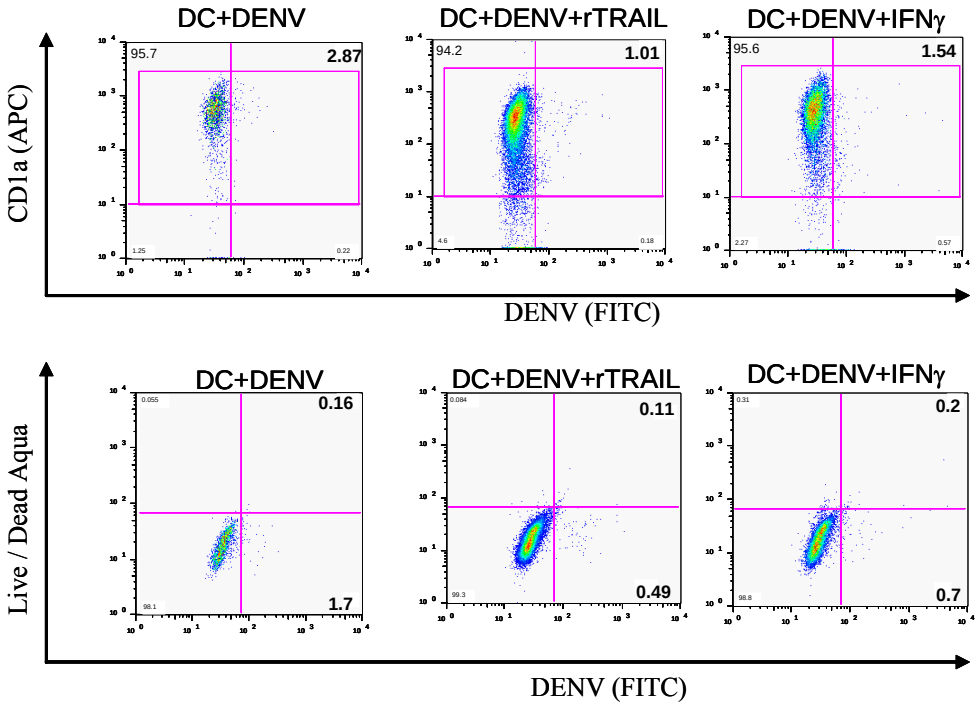


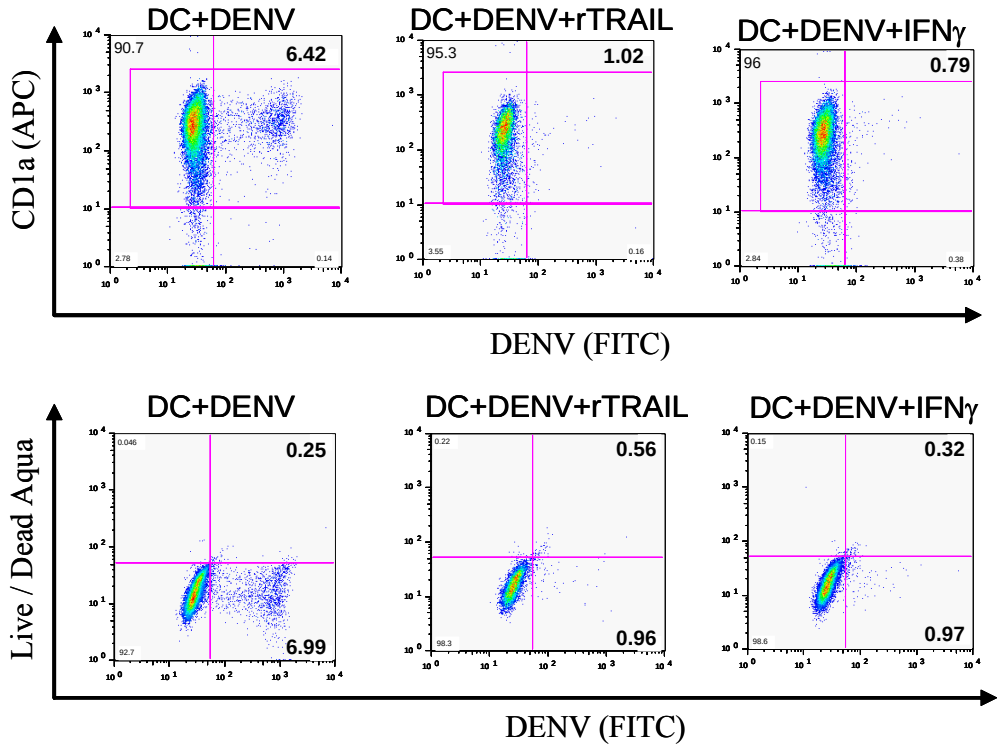
Figure 12. rTRAIL reduces levels of DENV progeny in DENV-infected DCs. DCs pretreated with rTRAIL for 24 hours were infected with DENV for 48 hours at an MOI of 0.1. At the end of the experiment culture supernatants were collected and stored at –70°C. DENV titers were determined by plaque assay on LLC-MK2 cell monolayers. Inhibition of DENV titers was observed in rTRAIL-treated DENV-infected DC supernatants. Results for five independent experiments are shown.

(A) Live / Dead Aqua

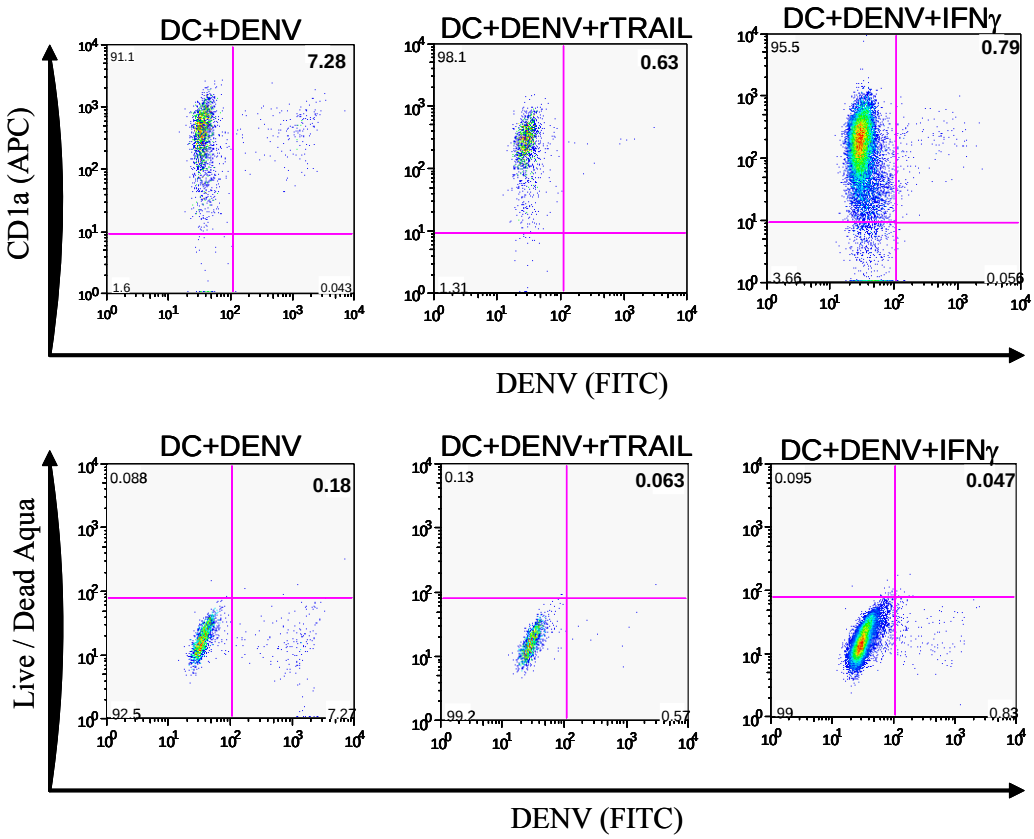
12 hours, n=2



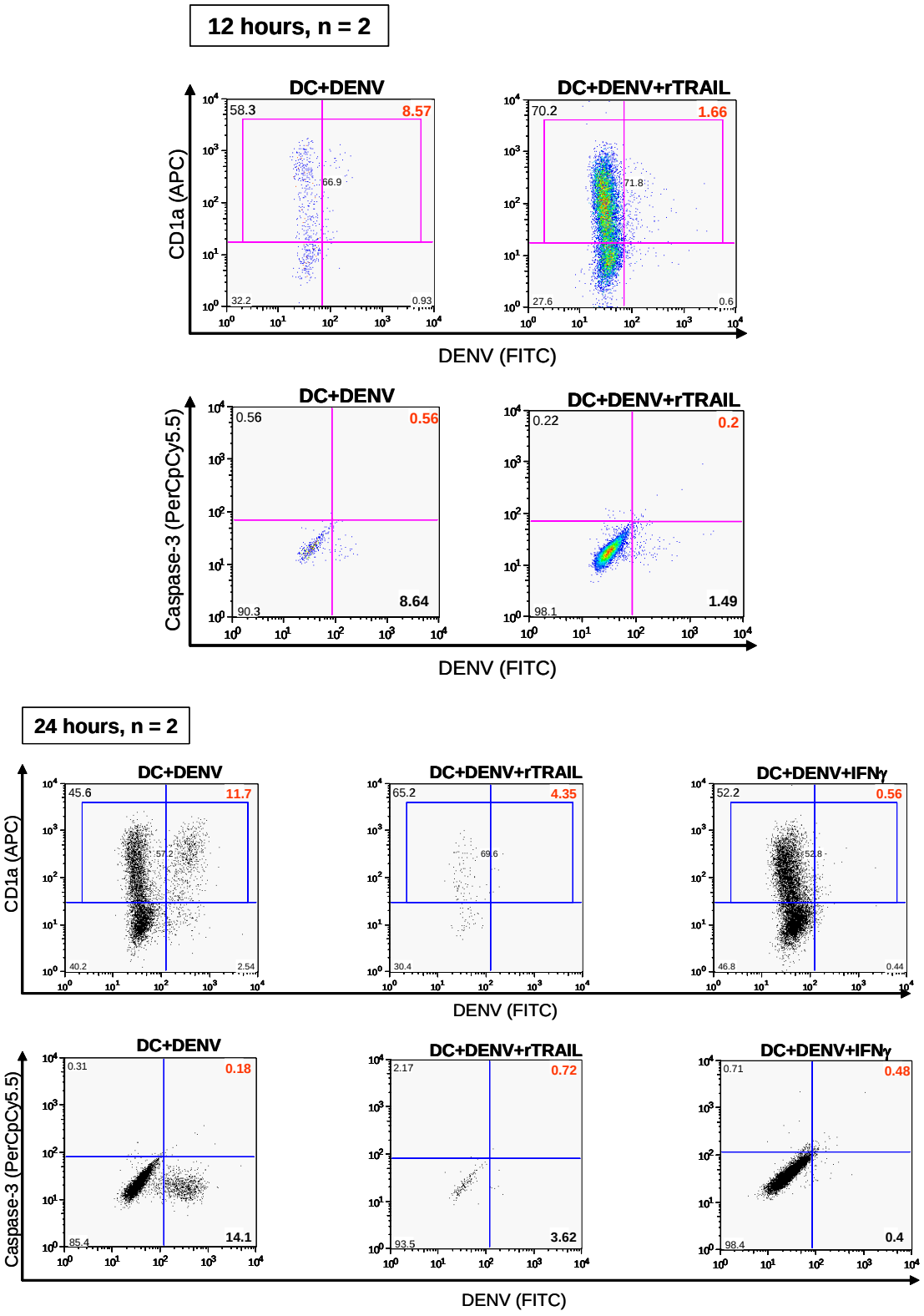
24 hours, n=2



48 hours, n=4


DC+DENV
DC+DENV+rTRAIL
DC+DENV+IFNγ
CD1a (APC)
91.1
7.28
1.6
0.043
98.1
0.63
1.31
95.5
0.79
3.66
0.056
DENV (FITC)
DC+DENV
DC+DENV+rTRAIL
DC+DENV+IFNγ
Live / Dead Aqua
0.088
0.18
92.5
7.27
0.13
0.063
99.2
0.57
0.095
0.047
99
0.83
DENV (FITC)

(B) Active Caspase-3



(C) Cleaved (25kDa) PARP-1

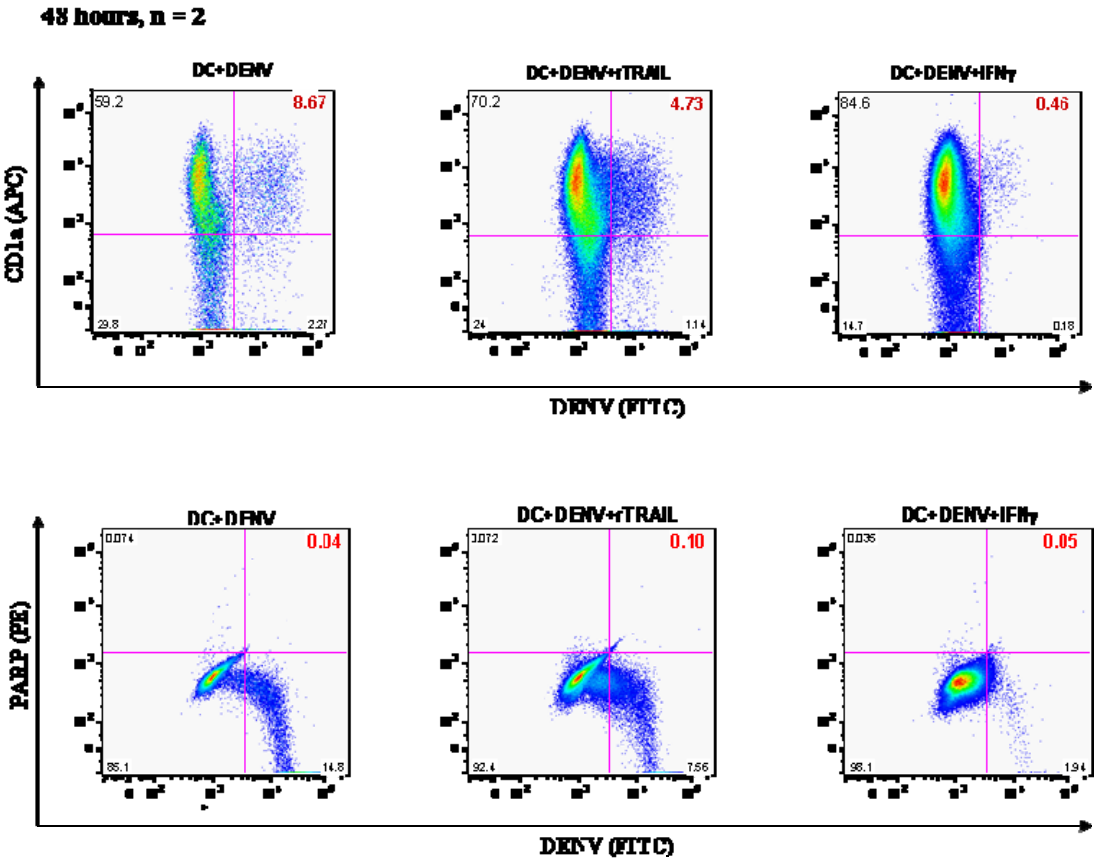


Figure 13. Absence of apoptosis in rTRAIL-treated DENV-infected DCs. DCs were pretreated overnight with rTRAIL as described in Materials and Methods, followed by infection with DENV for 48 hours at an MOI of 1. Live/Dead Aqua and Caspase-3 fluorescence was used to identify apoptotic DCs at 12 hours (A) and 24 hours (B). Live/Dead Aqua, Caspase-3 and PARP-1 fluorescence was determined at 48 hours (C) after rTRAIL treatment and DENV infection. Data from a representative experiment are shown for each time point. FITC, fluorescein isothiocyanate; APC, allophycocyanin. DCs treated with camptothecin B (2 mM/ml) for 4 hours were used as a positive control for caspase-3 stain at each time point (data not shown).

adenovirus (205) infection in an apoptosis-dependent manner. Hence, we investigated whether rTRAIL-mediated inhibition of DENV was dependent on apoptosis of DENV-infected primary cells. DENV-infected DCs pre-treated with or without rTRAIL were stained for DENV antigen and Live/Dead Aqua dye or early (caspase-3) and late (PARP-1) markers of apoptosis at 12, 24 and 48 hours postinfection.

The decrease in DENV infection of rTRAIL-treated DCs at the 12, 24 and 48 hour timepoints postinfection did not correlate with an increase in staining with active caspase-3, Live Dead Aqua dye (Catalog#L34957, Invitrogen), or cleaved PARP-1 proteins, which respectively detect early to late stages of apoptosis (Fig. 13 A, B, C). In some of the experiments, for Live/Dead Aqua and Active caspase-3 stain, fewer cells are seen in the flow cytometry plots due to unequal allocation of DCs to stain for controls and not as a result of fewer cells at the end of the experiment. I did count dendritic cells at the start and end of few experiments and found no decrease in DCs following rTRAIL or IFNγ treated DENV-infected DCs as compared to DENV-infected DCs. Camphothecin-B treated DCs stained positive for apoptosis markers suggesting that the DCs used in the experiment were capable of undergoing apoptosis. These data suggest that TRAIL inhibits DENV in an apoptosis-independent manner. The apoptosis-independent inhibition of DENV is novel since TRAIL mediated inhibition of other viruses or TRAIL mediated pathology by viruses like influenza, reoviruses, HIV, measles virus, CMV and EMCV is postulated to be apoptosis-dependent.

TRAIL anti-viral function requires pre-treatment for optimal DENV inhibition

Kumar-Sinha et al. had shown that rTRAIL treatment induced expression of different genes depending on the time period of pre-treatment (108). Therefore, to understand the mechanism of action of rTRAIL-mediated inhibition of DENV we determined the time-point of pre-treatment required for rTRAIL to affect its antiviral function. DCs were treated for 24 hours, 12-15 hours, 30 min or 0 hour before infecting the cells with DENV for 48 hours.

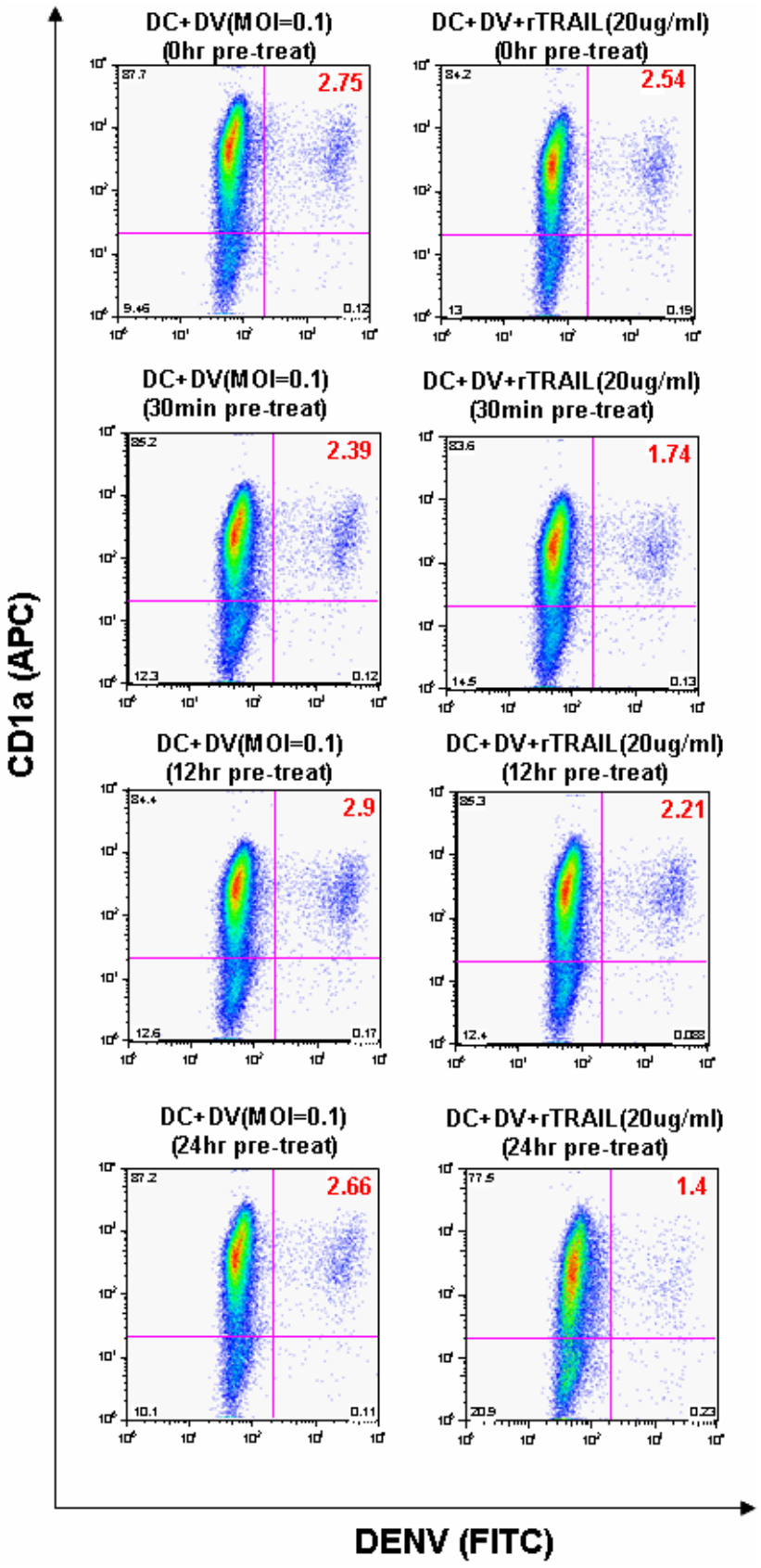
We found that 24 hour, 12-15 hour and 30 min pre-treatment with rTRAIL inhibited the percentage of DENV antigen-positive cells at the 48 hour timepoint after infection (Fig. 14).

TRAIL mRNA induction is dependent on a functional IFNα signaling pathway

IFNα/β can induce expression of TRAIL in many cell types (56, 88). Tyk2, STAT1, JAK1, and IFNαR 2c mutants (U1A, U3A, U4A, and U5A) derived from wild-type fibroblast cells (2fTGH) were used to determine whether the type-I IFN signaling pathway regulates TRAIL mRNA induction during DENV infection. TaqMan qRT-PCR was used to quantify TRAIL mRNA levels.

Unlike the wild-type cells, none of the mutant cell lines showed up-regulation of TRAIL in response to DENV infection (Fig. 15). These data show that type I IFN signaling is necessary for TRAIL mRNA induction.

A)


DC+DV(MOI=0.1)
(0hr pre-treat)
2.75
DC+DV+rTRAIL(20ug/ml)
(0hr pre-treat)
2.54
DC+DV(MOI=0.1)
(30min pre-treat)
2.39
DC+DV+rTRAIL(20ug/ml)
(30min pre-treat)
1.74
DC+DV(MOI=0.1)
(12hr pre-treat)
2.9
DC+DV+rTRAIL(20ug/ml)
(12hr pre-treat)
2.21
DC+DV(MOI=0.1)
(24hr pre-treat)
2.66
DC+DV+rTRAIL(20ug/ml)
(24hr pre-treat)
1.4
CD1a (APC)
DENV (FITC)

B)

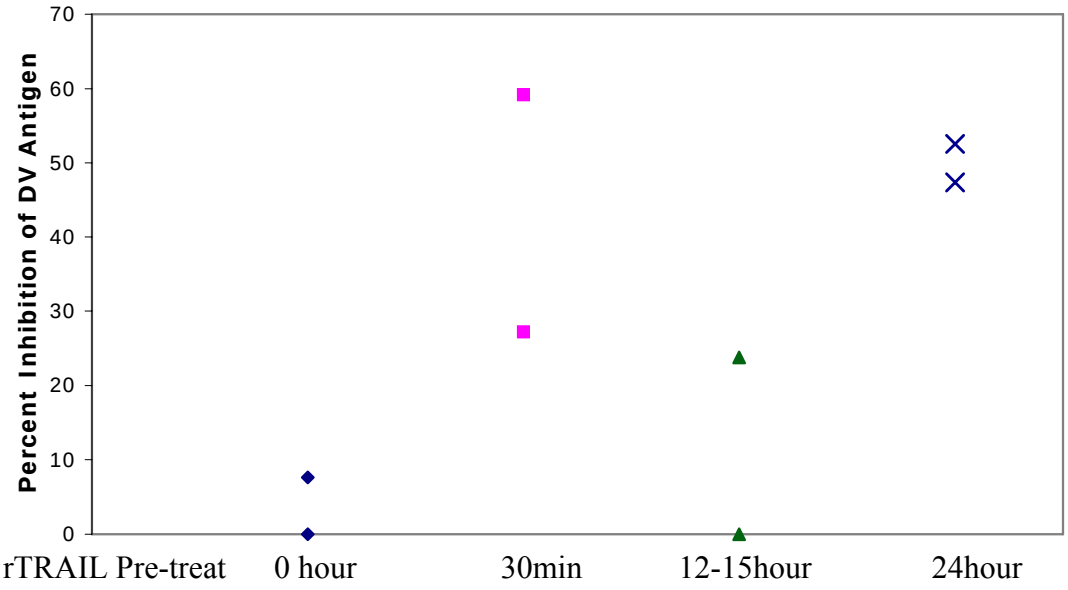


Figure 14. Effect of pre-treatment of DC with rTRAIL for various time periods prior to DENV infection. DCs were pre-treated with rTRAIL for 24 hours, 12-15 hours, 30 min or 0 hours before infection with DENV. At 48 hour after infection, cells were stained for DENV antigen (DENV-FITC, 2H2 Ab). (A) Data from one representative experiment showing percent DENV antigen positive cells. (B) Data are presented as percent inhibition of DENV antigen at 48 hours postinfection in rTRAIL pre-treated cells as compared to DENV-infected cells. Results for the two independent experiments performed are shown.

TRAIL anti-viral function requires signaling through the IFNα-receptor

The type-I IFN signaling pathway is important for control of DENV infection in studies in mice and *in vitro* experiments (51, 52, 194). We found that DENV infection-mediated TRAIL mRNA induction was dependent on the type-I IFN signaling pathway. Hence, we tested whether antiviral activity of rTRAIL was also dependent on a functional type-I IFNR signaling pathway. When DENV-infected DCs were pre-treated with type-I IFN receptor blocking Ab (anti-IFNαR Ab) along with rTRAIL, most of the rTRAIL-mediated antiviral effect was inhibited (Fig. 16). In separate experiments, we confirmed that antiIFNαR Ab blocked the inhibition of DENV by exogenous IFNα (300U/ml) pre-treatment. These results suggest that rTRAIL-mediated inhibition of DENV was partly dependent on a functional type-I IFNR pathway. antiIFNαR Ab treatment did not completely block the antiviral activity of rTRAIL against DENV, which suggests that type-I IFN-independent mechanisms also contribute to the rTRAIL-mediated inhibition of DENV infection.

Moreover, data from the representative experiment in Figure 16 shows that antiIFNαR Ab treatment of either DENV-infected cells or DENV-infected cells treated with rTRAIL increased DENV antigen positive cells by 50% as compared to both DENV-infected cells and DENV-infected cells treated with rTRAIL. Hence, another possible explanation of the results from Figure 16 is that the decrease in DENV antigen following antiIFNαR Ab treatment of rTRAIL treated cells was due to inhibition of the antiviral effect of type-I IFN, but not rTRAIL, against DENV. This hypothesis suggests that rTRAIL inhibits DENV by a type-I IFN independent mechanism.

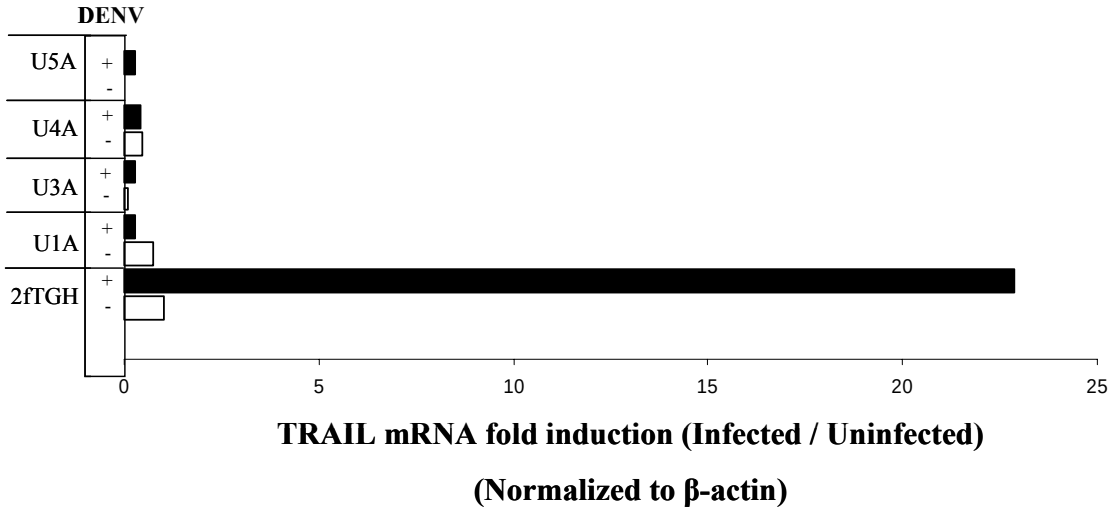
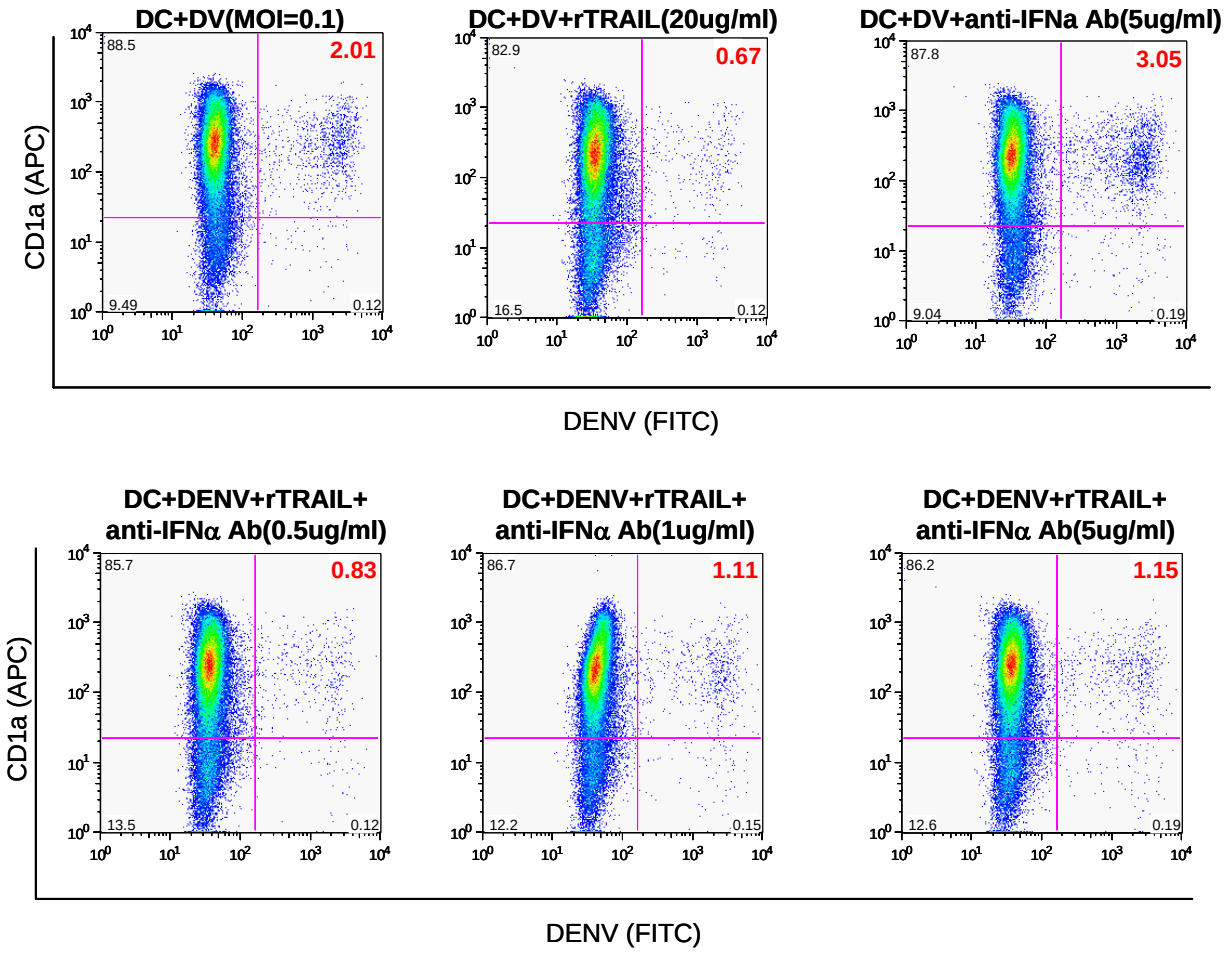


Figure 15. TRAIL induction in response to DENV infection is type-I IFN dependent. 2fTGH (wild type) and U1A, U3A, U4A, and U5A (type-I IFN mutant) fibroblast cells were infected with DENV at an MOI of 1 PFU/cell and then cultured for 48 h. TRAIL mRNA levels were quantified by qRT-PCR analysis. β-actin mRNA, a constitutively expressed protein, was used as a control probe. Results are representative of two independent experiments.

A)


DC+DV(MOI=0.1)
DC+DV+rTRAIL(20ug/ml)
DC+DV+anti-IFNa Ab(5ug/ml)
CD1a (APC)
88.5
2.01
9.49
0.12
82.9
0.67
16.5
0.12
87.8
3.05
9.04
0.19
DENV (FITC)
DC+DENV+rTRAIL+ anti-IFNα Ab(0.5ug/ml)
DC+DENV+rTRAIL+ anti-IFNα Ab(1ug/ml)
DC+DENV+rTRAIL+ anti-IFNα Ab(5ug/ml)
CD1a (APC)
85.7
0.83
13.5
0.12
86.7
1.11
12.2
0.15
86.2
1.15
12.6
0.19
DENV (FITC)

B)

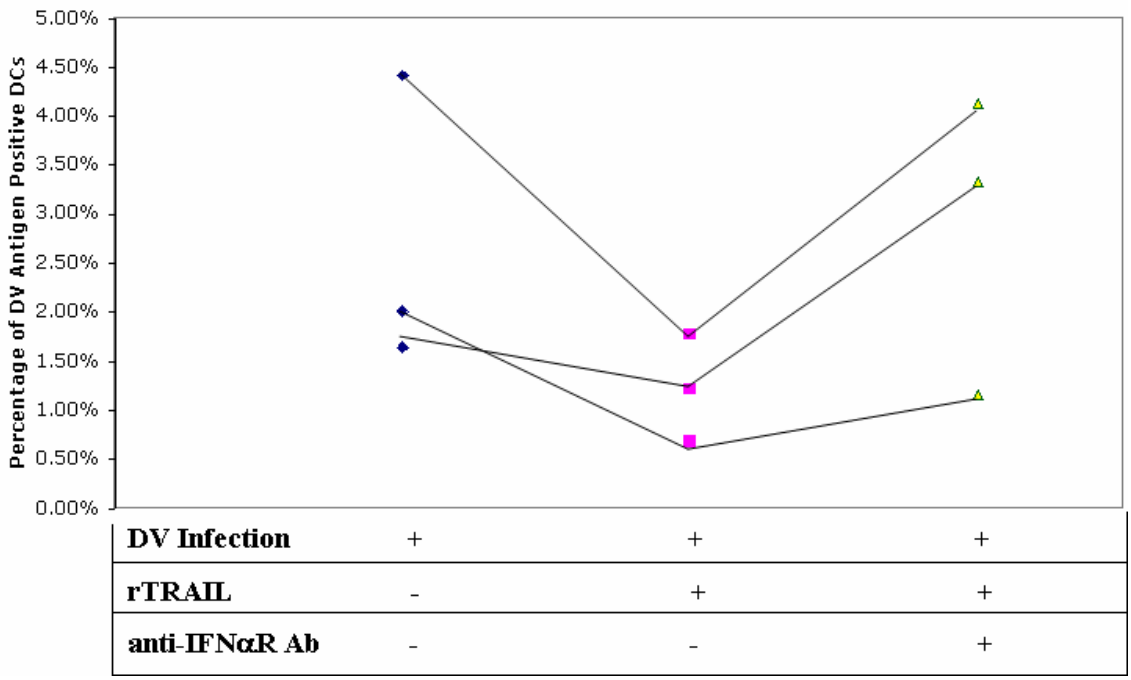


Figure 16. Type-I IFN receptor blocking Ab inhibits rTRAIL-mediated antiviral activity against DENV in DCs. DCs were pretreated with anti-IFNαR Ab (5ug/ml) for 30 hours before infection with DENV. Six hours post anti-IFNαR Ab treatment, rTRAIL (20ug/ml) was added to the cell culture. DCs were infected with DENV at an MOI of 0.1 for 48hours. Cells were stained intracellularly for DENV antigen (2H2 monoclonal antibody). (**A**) Data from one representative experiment is shown. (**B**) Data are presented as percentage of DENV positive DCs from three independent experiments(n=3).

TRAIL suppresses the production of pro-inflammatory cytokines and chemokines in DENV-infected dendritic cells

Activation and expansion of serotype cross-reactive memory T cells during secondary DENV infections alters the T cell phenotype to production of inflammatory rather than cytototoxic T cell proteins (16, 145). Elevated levels of Th1 pro-inflammatory cytokines (IFNγ, TNFα, IL6, IL-1β, IL-2) and various chemokines (IL-8, MIP-1β, MCP1, RANTES) have been detected in dengue infected patients' serum / plasma, and the expression of some of these mediators seems to be related to the severity of the disease (37, 63, 64, 96, 121, 176). Hence, treatment of DENV infection should involve regulation of the pro-inflammatory cytokine and chemokine response along with inhibition of DENV titers to prevent DENV infection mediated immunopathogenesis.

A recent study by Secchiero et al. showed that TRAIL inhibits the TNFα- and IL1β-induced adhesion of leukocytes of HUVECs and HAECs (185). The inhibition of the leukocyte adhesion to HUVECs and HAECs was not due to interference of NFκB mediated up-regulation of ICAM1, VCAM-1 and E-Selectin-1. However, the inhibitory effect of TRAIL was due to the down-modulation of the expression of TNFα-induced chemokines MCP-2 and IP-10 (185). Our laboratory found that levels of MCP-2 and IP-10 were significantly higher in sera from patients with dengue during the acute stage of the disease (I Bosch, unpublished data). In addition, TNFα, ITAC, IL-6, MIP-1β, IP-10, IL-8 and IL1β are among the pro-inflammatory chemokines and cytokines elevated during DENV infection in patients (61, 64, 80, 81, 96, 121, 176, 222). TRAIL was earlier found to be induced following DENV

infection in primary human cells, *in vitro*. Hence, we wanted to investigate whether rTRAIL might also function to downregulate the expression of MCP-2, IP-10 and maybe other pro-inflammatory cytokines and chemokines induced by DENV infection during acute phase of the disease, *in vivo*. To address this hypothesis, we studied the effect of rTRAIL pre-treatment on DENV-induced expression of pro-inflammatory genes, MCP-2, IP-10, IL-6, TNFα, ITAC and IL-8. Several of these proteins have been implicated in the immune-mediated increase in vascular permeability observed in patients with DHF (81, 176, 222). To investigate whether the effect of rTRAIL treatment was specific to pro-inflammatory gene expression, we also included antiviral genes IFNα, MDA-5 and IRF7 in the list of genes being tested for the level of expression in rTRAIL treated DENV-infected cells. Inhibition of DENV RNA levels was used as a positive control for the effect of rTRAIL treatment.

rTRAIL pre-treatment effectively suppressed the DENV infection-mediated increase in mRNA expression levels of the pro-inflammatory genes, MCP-2, MIP-1β, IP-10, IL-6 and TNFα (Fig. 17).

As compared to DENV-infected DCs, rTRAIL treatment inhibited IP-10 (90.5%), MCP-2 (84.6%), MIP-1β (67.4%), TNFα (61.3%), IL-6 (85.2%) and, to a lesser extent, IL-8 (29.2%) (Fig. 17). The two antiviral genes tested showed some suppression in expression: MDA-5 (53.3%) and little suppression of IRF-7 (34.4%) (Fig. 17). The suppression of cytokines and chemokines that can induce vascular permeability (TNFα), coagulation (IL-6) and recruit activated leukocytes (IP-10, MCP2 and MIP-1β), suggests a role for TRAIL in suppressing the pro-inflammatory response to DENV infection.

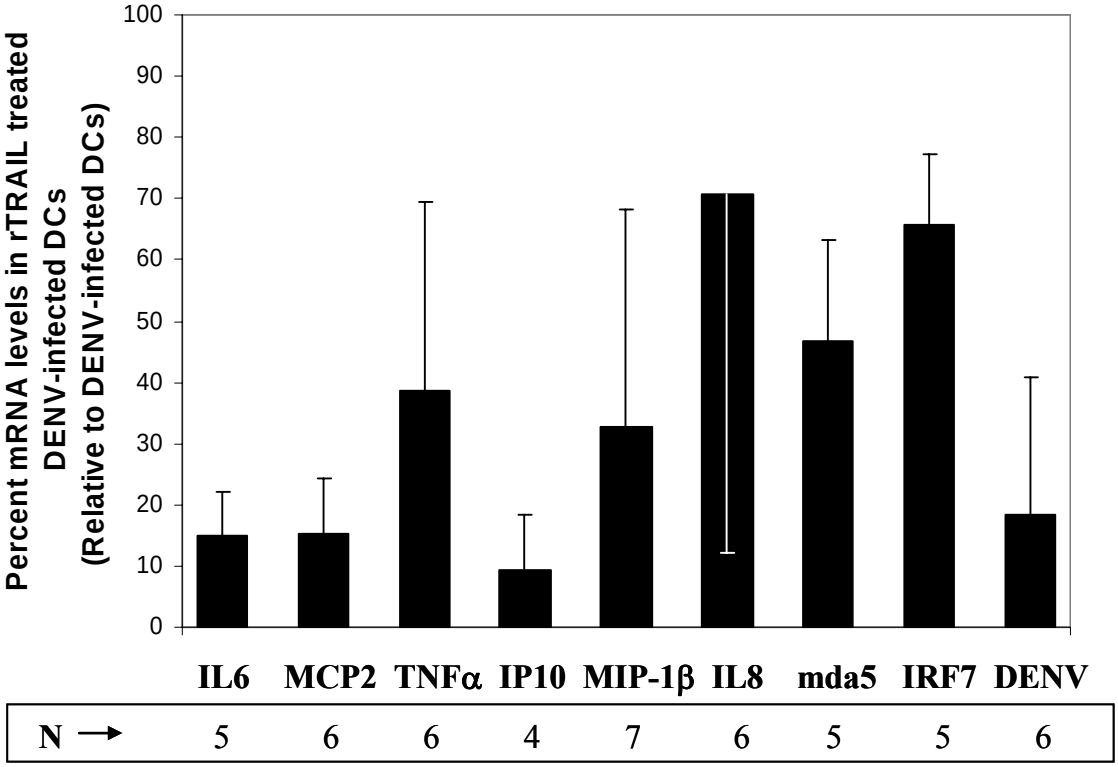


Figure 17: Percent mRNA levels in rTRAIL treated DENV-infected DCs compared to DENV-infected DCs. mRNA relative quantification using the $2^{-\Delta\Delta Ct}$ method (131); β-actin was used as an endogenous control (Gene whose expression levels are fairly constant at any time inside the cell). Mean values ± standard error of mean are presented. N indicates the number of independent experiments.

Next, we determined if the decrease in expression of the pro-inflammatory cytokines and chemokines was also seen at the protein level. Hence, we further analyzed the levels of MCP-2, IP-10, IL-6, ITAC and IFNα protein in cell culture supernatants from DENV-infected and rTRAIL pre-treated DENV-infected DCs at the 48 hours time point.

All the proteins tested were detected at significantly lower levels in supernatants from rTRAIL pre-treated DENV-infected DCs as compared to supernatants from DENV-infected DCs, confirming the mRNA expression data (Fig. 18). Thus, rTRAIL treatment inhibits both expression and secretion of pro-inflammatory chemokines and cytokines in response to DENV infection *in vitro*. Taken together, these data suggest that TRAIL could function as a negative regulator of pro-inflammatory genes induced by DENV-infected DCs.

TRAIL protein levels in Dengue patient serum

TRAIL was induced at mRNA and protein level following DENV infection, *in vitro*. In addition, TRAIL exerted both, antiviral function against DENV and suppressed pro-inflammatory cytokines and chemokines induced by DENV infection in primary human cells, *in vitro*. Based on this *in vitro* data we hypothesize that TRAIL might have potential to treat DENV infection, *in vivo*. Hence, we wanted to determine and compare levels of TRAIL protein in DENV patient serum. We also determined the level of TRAIL in OFI patient serum to know how specific TRAIL expression was for DENV infection.

TRAIL levels in serum from DENV-infected patients (N=19) were increased during the febrile days of the disease and dropped close to normal levels after

defervescence (Fig. 19). We found statistically significant differences in TRAIL serum levels between DENV-infected patients and OFI (N=4) during the febrile days of the disease ($p<0.02$).

There were no significant differences between TRAIL levels in serum from OFI and healthy donors after the febrile phase of the disease.

Interestingly, kinetics of TRAIL serum levels found in this study seems to co-relate with DENV levels in DENV patients based on previously published clinical reports. The kinetics of TRAIL protein levels in dengue patient serum lends support to our previously published antiviral activity against DENV in human primary cells.

Chapter Summary

The results from Chapter IV identified TRAIL as a potential common linker of the type-I and type-II common response genes. Initial experiments performed in this section of the thesis demonstrate that TRAIL expression is induced in monocytes, B cells and DCs following DENV infection and this TRAIL protein expression is early (12 hour postinfection), transient atleast in dendritic cells. Experiments where either endogenous TRAIL protein was blocked using a TRAIL neutralizing Ab or exogenous rTRAIL was added to the cell culture showed that TRAIL has potent antiviral activity against DENV, atleast *in vitro*. We found that rTRAIL inhibited DENV in a novel apoptosis-independent manner which has not been demonstrated for TRAIL mediated inhibition of any other virus. Using wild type fibroblast cells (2fTGH) and type-I IFN pathway mutant cells derived from 2fTGH cells we investigated whether increased TRAIL mRNA expression was dependent on a

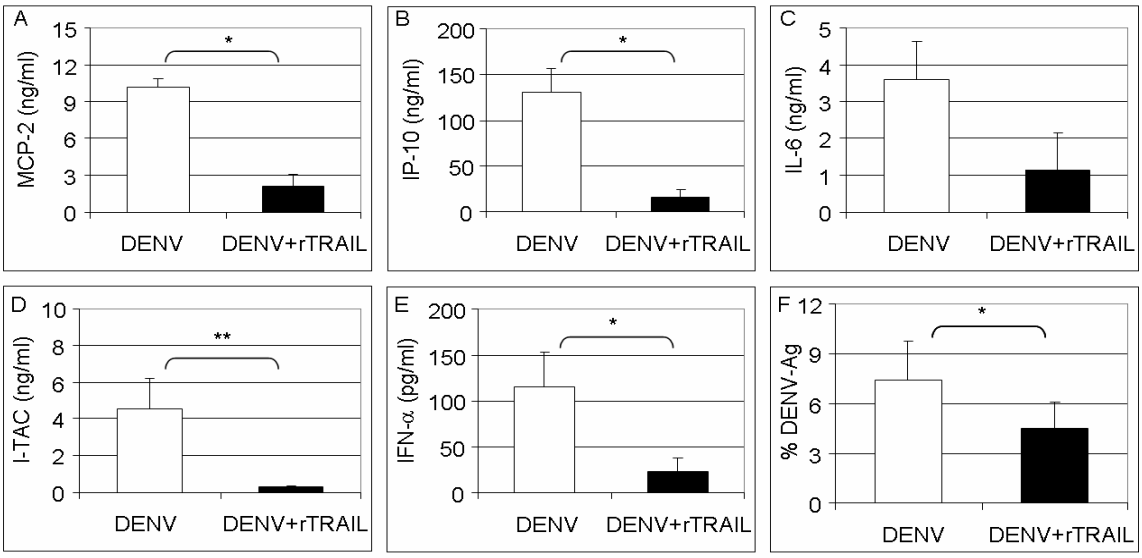


Figure 18. Effect of rTRAIL pre-treatment on MCP-2, IP-10, ITAC, IL-6 and IFNα levels in culture supernatants from DENV-infected DCs. DCs were incubated with or without rTRAIL (20 ug/ml) for 24 hours and then infected with DENV at M.O.I. of 0.1 for 48 hours. Levels of (A) MCP-2, (B) IP-10, (C) IL-6, (D) ITAC, and (E) IFNα in supernatants from DENV-infected DC (white bars) and rTRAIL-pretreated DENV-infected cells (black bars) at 48 hours post-infection were determined by commercial ELISA (N=3-7). Errors bars represent standard error of the mean for each treatment. Mann–Whitney analysis showed significant differences for MCP-2 ($p<0.05$); IP-10 ($p<0.02$); ITAC ($p<0.01$); IFNα ($p<0.02$) when comparing DENV-infected versus rTRAIL pre-treated / DENV-infected DCs. (F) Percentage of DENV antigen-positive DC in DENV-infected (white bars) and DENV-infected + rTRAIL (black bars) cultures determined by flow cytometry. Paired t-test analysis showed a significant difference ($p<0.03$).

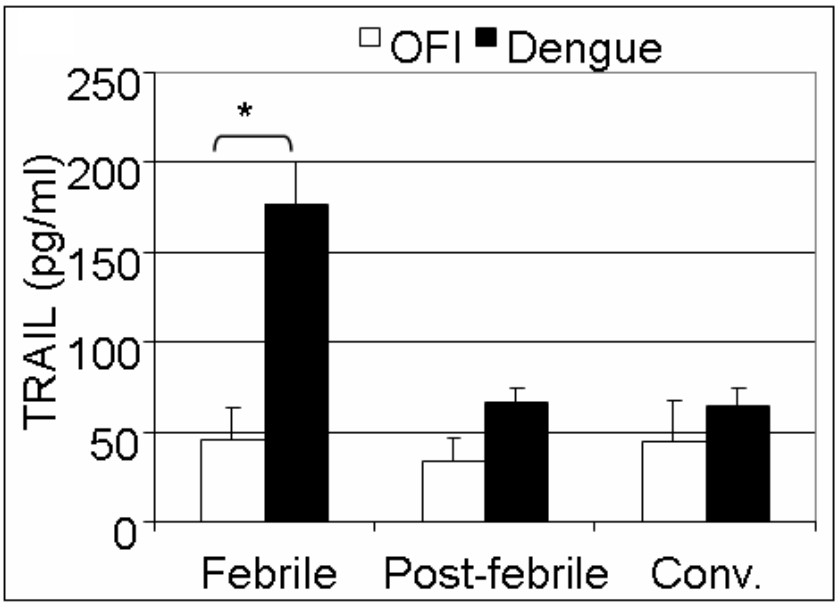


Figure 19. Serum TRAIL levels in DENV (black bars) and OFI (white bars) patients. TRAIL protein levels were determined during three phases of the disease: febrile, post-febrile and convalescence (Conv.) in both, OFI and DENV patient samples. Results are expressed as a mean ± standard error of mean. The mean TRAIL level in sera of healthy donors was 33.7 ± 8.8 (N=8) pg/ml. Mann–Whitney statistical analysis showed significant differences in TRAIL serum levels between OFI and dengue during febrile phase of the disease ($p<0.02$). Conv.: convalescence.

functional type-I IFN signaling pathway. We found that a functional type-I IFN signaling pathway was required for DENV infection induced TRAIL expression. Adding IFNαR blocking Ab to the rTRAIL treated cells infected with DENV lead to an increase in DENV antigen levels. This data suggests that either most of the antiviral activity of rTRAIL is type-I IFN signaling pathway dependent or IFNαR blocking Ab blocked type-I IFN mediated antiviral activity against DENV without affecting the antiviral effect of rTRAIL against DENV.

Pro-inflammatory cytokines (IL-6, TNFα) and chemokines (MCP-2, IP-10, MIP-1β, ITAC) elevated in response to DENV infection in DCs were suppressed both at mRNA and protein level following rTRAIL treatment. Elevated serum levels of TRAIL protein were also detected in DENV patients during febrile phase of the disease as compared to OFI patient and healthy donor serum samples.

Chapter V

Identification of sST2 and IDO as biomarkers for DENV infection and role of IDO activity in IFNγ - mediated inhibition of DENV infection

Potential DENV-specific response genes were confirmed in DENV-infected HUVECs, *in vitro* and in dengue patient and OFI patient PBMCs isolated during the acute phase of the disease. The OFI and dengue patient analysis confirmed that ST2 and IDO genes were induced to a greater extent in DENV patient PBMCs as compared to OFI patient PBMCs.

We focused the experiments in this section of the thesis on confirming specificity (biomarker) of their upregulation in DENV patient serum and to understand their function in relation to DENV infection.

sST2 and ST2L mRNA levels in DENV-infected HUVECs

One of the genes found to be upregulated in the "common" gene expression response to DENV infection was ST2. However, multiple forms of ST2 are expressed via alternative mRNA splicing, and the Affymetrix GeneChip probe hybridized to the mRNA of both soluble (sST2) and cell surface bound (ST2L) isoforms. Hence, we hypothesized that both or either one of these two isoforms could be responsible for detection of higher ST2 mRNA in samples tested. To address this hypothesis, we analyzed the expression of sST2 and ST2L mRNAs in DENV-infected HUVECs by semi-quantitative RT-PCR. We designed forward and reverse primers specific either to the sST2 or the ST2L isoform of the gene.

We found that soluble ST2 mRNA transcripts were much more abundant in DENV-infected HUVECs as compared to uninfected HUVECs at the 48hour timepoint of infection (Fig. 20). ST2L mRNA was also slightly differentially expressed. However, no ST2L protein was detected on the surface of DENV-infected HUVECs at the 48 hour timepoint by flow cytometry (data not shown). The result that sST2 isoform expression is induced in DENV-infected HUVECs suggests but does not confirm that the upregulated ST2 mRNA detected in PBMCs from DENV-infected patients was the sST2 isoform.

sST2 protein levels in DENV patient serum

The data in HUVECs showed that sST2 isoform is upregulated in response to DENV infection. Cytokines such as IL-1β, TNFα, IL-6, which are elevated in DENV patient serum are known to induce sST2 expression (123, 164, 207). Moreover, TNFα and IL-6 are significantly higher in DENV-infected patients with symptoms of DHF/DSS (64, 81).

To determine if production of sST2 was induced specifically in DENV patients, we tested dengue patient serum for levels of sST2 protein during the acute stage of the disease.

We found a statistically significant increase in serum sST2 levels in DENV patients during the acute stage of the disease (Fig. 21A). The serum levels of sST2 were similar to levels in OFI patient serum by the convalescent day. The highest levels of

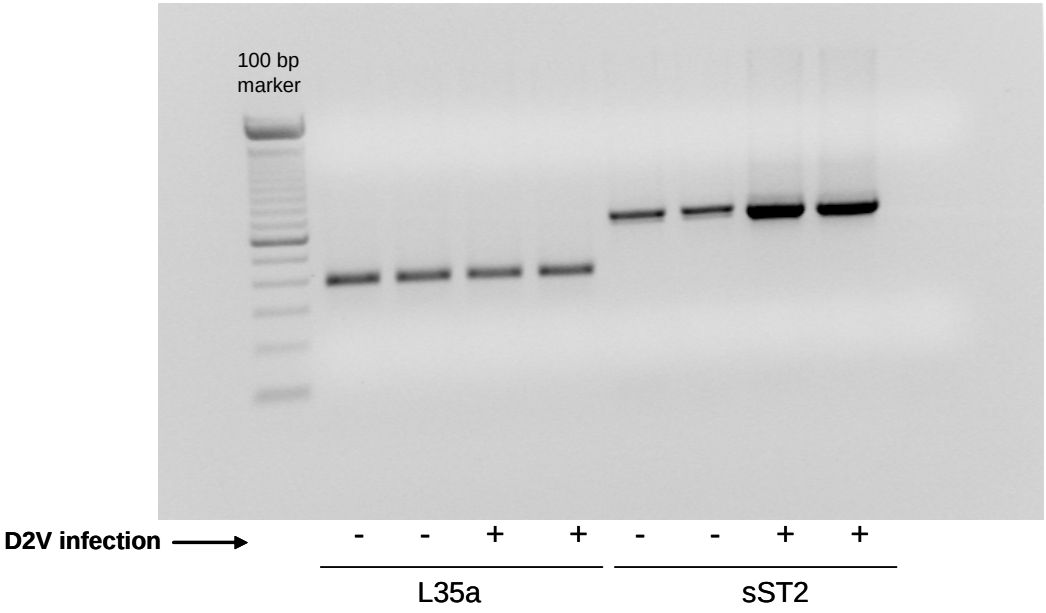
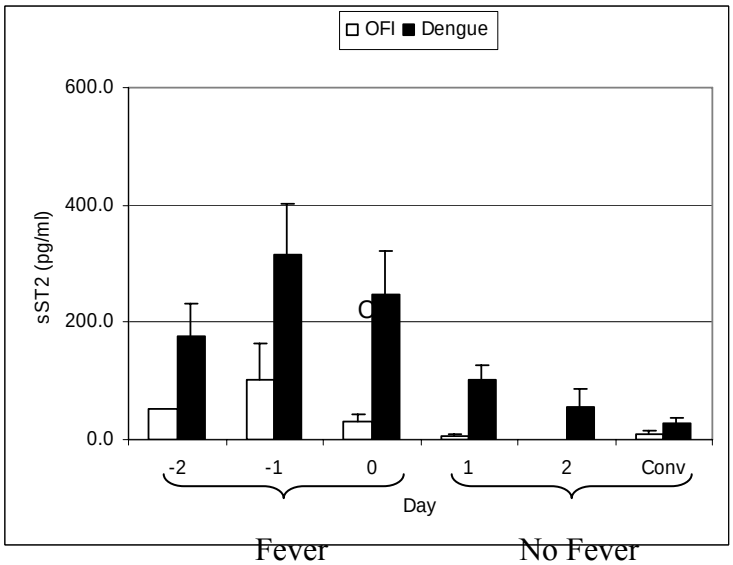


Figure 20. Soluble ST2 is transcriptionally induced in DENV-infected HUVECs, *in vitro*. HUVECs were infected with DENV at MOI of 1. After 48 hours of infection RNA was extracted from cells and sST2 and ribosomal protein L35a mRNAs were amplified using semi-quantitative RT-PCR. The first 2 lanes for both genes represent control medium (C6/36, insect cell supernatant) and the 3rd and 4th lane represent two DENV-infected HUVEC sample. Results from two independent experiments are shown.

A



B

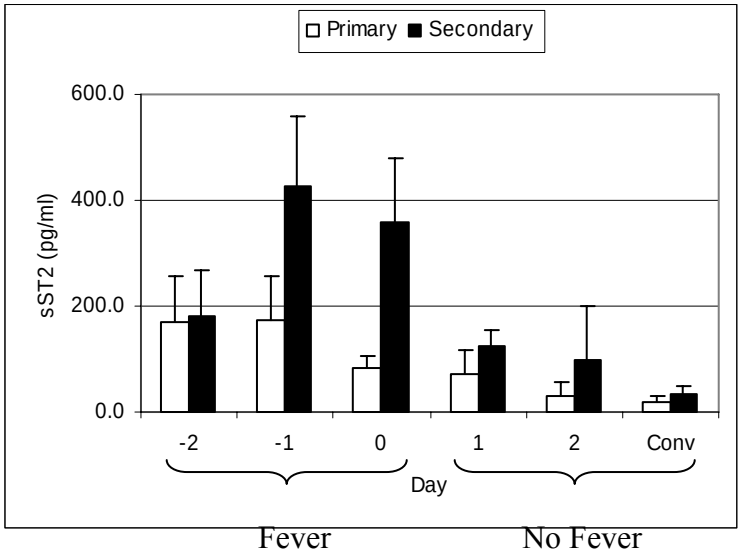


Figure 21. Soluble ST2 (sST2) levels in serum from patients during the course of the disease were quantified by ELISA. Fever day 0 is the day of defervescence. Fever days -2 and -1 are febrile days. Fever days +1 and +2 are post-febrile days; conv is the convalescent day. Upper panel: Other Febrile Illness (OFI) (N=11) and dengue patients (N=24); significant differences were found in sST2 levels between OFI and dengue patients on fever days -1 ($p<0.01$) and 0 ($p<0.01$). Lower panel: dengue patients classified as Primary (N=10) or Secondary (N=13) infections; significant differences were found in sST2 levels between primary and secondary infections on fever days -1 ($p<0.04$) and 0 ($p<0.03$). sST2 levels in the serum from normal donors: 15.9±4.4 pg/ml (N=14). Non-parametric statistical analysis using Mann-Whitney U test was utilized. Bars represent mean values, with error bars showing SD.

sST2 were detected on fever days –1 and 0, suggesting that sST2 may be a marker for DENV infection in patients during the late phase of the infection. Among dengue patients, higher sST2 levels were detected during secondary as compared to primary dengue infections (Fig. 21B) suggesting that sST2 might be a potential biomarker for secondary dengue infections.

IDO mRNA levels in uninfected, DENV-infected and IFNγ treated + DENV-infected DCs

IFNγ is known to induce IDO mRNA gene expression. We confirmed whether the induction of IDO mRNA was due to DENV infection or IFNγ or both using the DC model for DENV infection. DENV infection alone induced IDO expression in DCs (Table 6). Moreover, IFNγ pre-treatment increased the expression of IDO gene after DENV infection (Table 6).

All the DCs were infected with DENV at an MOI of 0.1. Infection was stopped at the 48 hour timepoint and RNA was extracted from cells. IDO mRNA levels were quantified using qRT-PCR. Data from three experiments are presented. Error bars represent standard error of the mean.

Increased IDO activity in dengue patient serum

IFNγ has been shown to induce IDO activity to mediate part of its antiviral function (4, 160). IDO mediated tryptophan depletion also plays a role in the immune-modulatory function in APC - T cell interactions (138, 150). Furthermore, higher IFNγ levels have been documented in dengue patients by multiple studies. We

Experiment No.	**IDO mRNA Fold Induction**	
	DC+DV	DV+DV+IFNγ
1	2	14
2	14	75
3	306	2851

Table 6. IFNγ treatment substantially increases the DENV infection mediated expression of IDO. Monocyte-derived DCs were generated as mentioned in Chapter II. DCs were either pre-treated or not with IFNγ (500U/ml) for 12-16 hours. Results for three independent experiments are shown.

hypothesized that the higher systemic IFNγ levels would increase IDO activity in DENV patients. Hence, we determined IDO activity in patient serum. IDO enzyme catalyzes the metabolism of tryptophan to kynurenine, the rate-limiting step in the kynurenine pathway of tryptophan degradation. Determining the tryptophan quotient [(kynurenine/tryptophan)*100], which is an indirect method to demonstrate IDO enzyme activity has been extensively used to identify increased IDO activity.

Increased IDO activity was detected in dengue patient serum as compared to OFI patient serum (Fig. 22). The levels of tryptophan were significantly lower in dengue patient serum during the acute, post-febrile and convalescent stages of the disease which might be a result of higher IDO activity and lower intake of nutrition due to weakness, vomiting and nausea. Kynurenine levels were significantly increased during the febrile stage of the disease, but decreased during the post-febrile stage and were similar to kynurenine levels in OFI patients by the convalescent day. The IDO activity was significantly higher in dengue patient serum during all phases tested. However, the significance and level of IDO activity was much higher during the acute and post febrile phase of the disease. The results indirectly show that IDO enzyme activity was strongly induced in dengue patients.

IFNγ-mediated antiviral function is partly dependent on IDO activity

The function of IDO is to metabolize tryptophan, an essential amino acid, to kynurenine. IFNγ treatment has been previously reported to induce IDO gene expression and to utilize IDO activity as a part of its antiviral function (4, 160, 172).

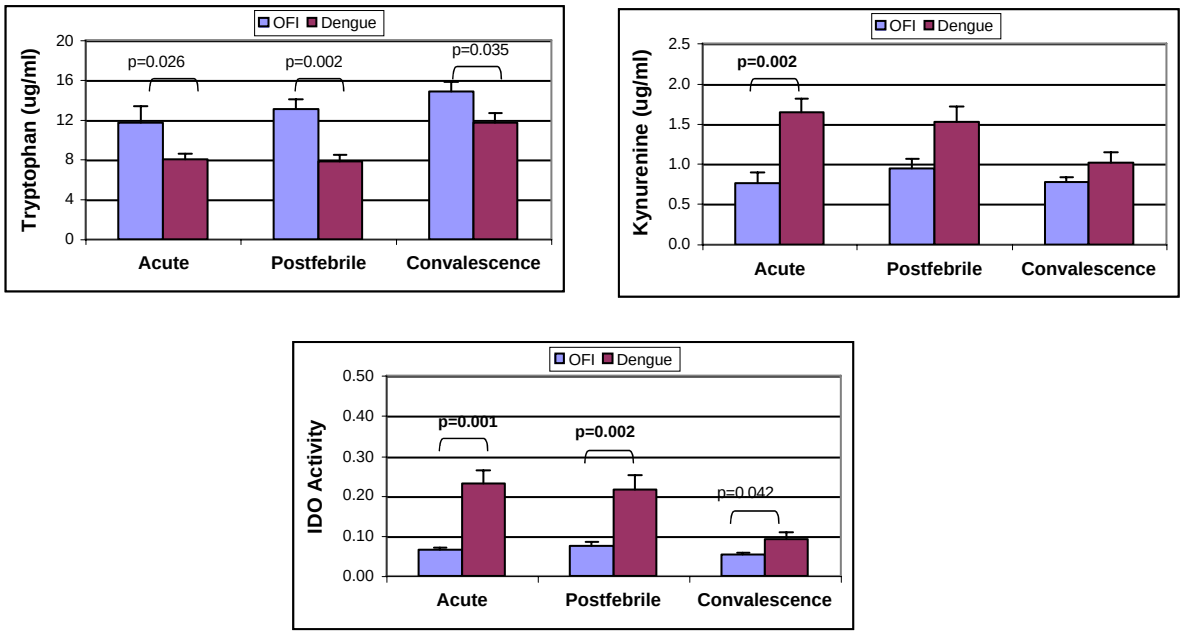


Figure 22.Tryptophan and kynurenine levels in serum from OFI (N=5) and dengue patients (N=6) during acute, post-febrile and convalescent days were measured using mass spectrometry. Bars represent mean values (-/+ SEM). Values for healthy donors (N=5) are: tryptophan: 13.24 (±0.85) ug/ml and kynurenine: 0.66 (±0.09) ug/ml. Unpaired t-test analysis to compare OFI vs. dengue was performed. All the DENV patients in this study were clinically defined as DF by WHO criteria. IDO activity = (kynurenine / tryptphan)*100.

We hypothesized that IFNγ utilizes IDO enzyme activity as part of its antiviral activity against DENV infection. Hence, we tested the role of IDO activity in the IFNγ-mediated inhibition of DENV using the DC model. Treatment with 1-methyl tryptophan (1-MT), a synthetic analogue of tryptophan was used to block IDO activity (172, 206).

IFNγ inhibited DENV infection as shown by a reduction in antigen-positive cells, consistent with previously published results. 1-MT treatment restored an average 66% of DENV RNA levels inhibited by IFNγ treatment (Fig. 23). This data indicate that IFNγ activates IDO activity as a part of its antiviral function.

	DC+DENV	DC+DENV + IFNγ	DC+DENV+IFNγ + 1-MT
	Fold Decrease in DENV RNA Levels		
Experiment # 1	1	471.9	103.9
Experiment # 2	1	58.4	9.8
Experiment # 3	1	3.8	1.2
Experiment # 4	1	108	30

Figure 23. Inhibition of IDO activity reduces the IFNγ-mediated antiviral function against DENV. DCs were infected with DENV with or without pre-treatment with IFNγ (500U/ml) and 1-MT (1mM) for 12-16 hours before infection. All the DCs were infected with DENV at an MOI of 0.1 for 48 hours. Intracellular DENV RNA levels were quantified by qRT-PCR. β-actin mRNA was used as a control probe. Data are presented as inhibition of DENV RNA levels. Results for four independent experiments are shown.

Discussion

Identification of antiviral and biomarker genes against dengue virus

The goal of the present project was to identify cellular responses to DENV infection by means of studying gene expression and identify (i) potential protein biomarkers of DENV infection and (ii) potential antiviral proteins. Specifically, we hypothesized that there is a set of cellular genes, which is necessary to clear DENV in susceptible human cells. We initially set out to identify gene expression changes induced in primary human cells by DENV infection using global gene expression analysis. The analysis identified a common gene expression profile of 23 differentially regulated genes in DENV-infected peripheral blood mononuclear cell subsets (B cells and monocytes) and HUVECs. TRAIL, one of the common response genes, was identified as a potential common linker between the IFNα and IFNγ signaling pathway inducible common response genes, was selected for further study.

Endothelial cells play a central role in the clinical presentation of the symptoms of DHF/DSS, such as increased vascular permeability, hypovolemic shock, bleeding, circulatory failure and haemoconcentration. *In vitro* and findings in mice have shown that endothelial cells are targets for DENV infection (7, 36, 225). *In vivo*, lung vascular endothelial cells and sinusoidal endothelial cells have stained positive for DENV antigen (92). With the goal of identifying biomarkers for DENV infection, we initially focused on understanding the effect of direct DENV infection on HUVECs. Gene expression analysis in DENV-infected HUVECs was compared to

gene expression analysis in cells infected with other RNA viruses (WNV, YFV, Hantaan and SNV). Global gene expression analysis identified a set of 30 genes potentially differentially regulated in DENV-infected HUVECs as compared to HUVECs infected with the other RNA viruses mentioned above. Among the genes that were confirmed for differential expression, IDO and ST2 were found to be specifically regulated in PBMCs from DENV-infected patients as compared to OFI patient PBMCs and were selected for further study.

TRAIL Gene:

(i) Expression

(ii) Antiviral function

(iii) Pro-inflammatory gene inhibition

(i) TRAIL Expression

Gene expression analysis in DENV-infected HUVECs, B cells and monocytes identified a set of 23 common response genes. Among the 23 common response genes, 11 genes, other than TRAIL were confirmed for their differential expression due to the unavailability of TaqMan qRT-PCR primers and probes for the other 11 common response genes. (Table 3). The induction of TRAIL mRNA expression by DENV infection was confirmed in multiple DENV-permissive primary human cells. Upregulation of TRAIL expression was also confirmed at the protein level in DENV-infected cells. We were unable to detect secreted TRAIL protein in the supernatant from DENV-infected primary monocytes and dendritic cells, but TRAIL protein

levels were highly induced in lysates of DENV-infected monocyte. We used DC to determine the pattern of expression of TRAIL protein following DENV infection; DENV-infected dendritic cells were double-stained for DENV antigen and TRAIL protein by cell surface and intracellular cytokine staining (ICS) assay. However, using flow cytometry we were unable to detect TRAIL on the surface of monocytes or DCs following DENV infection. One possible explanation for these results is rapid internalization of TRAIL protein, or due to its storage and release inside cytoplasmic vesicles (147). While uninfected DCs cultures did not express any detectable TRAIL protein, TRAIL expression was detected in infected cultures early but transient following DENV infection. The upregulation of TRAIL protein expression occurred early (at 12 hours post infection) and was transient, with reduced staining at 24 and 48 hours post-infection. In summary, based on our data, TRAIL mRNA and protein are upregulated early and transiently after DENV infection in DENV-infected cells.

One possible explanation for the lack of TRAIL expression in infected cells, compared to bystander cells, is the direct effect that the virus may have on signaling in the infected cell. IFNα is a known strong inducer of TRAIL transcription, while DENV has been shown to inhibit IFNα signaling in infected cells (95, 152, 153). Thus, TRAIL expression may not have been detected in DENV-infected DCs due to inhibition of IFNα signaling.

We used wild-type (2fTGH) and type I IFN mutant (U1A (TYK2), U3A (STAT1), U4A (JAK1), and U5A(IFNR-2.2) human fibroblast cell lines (132, 137, 169) to determine whether the increased TRAIL expression induced by DENV infection was IFNα-dependent. Increased TRAIL mRNA expression was detected

only in wild type 2fTGH cells, showing that a functional IFNα signaling pathway was required for the induction of TRAIL expression following DENV infection. This finding supports the hypothesis that blocking of IFN type I receptor signaling by DENV blocked the induction of TRAIL gene expression.

Recently, Matsuda et al. reported that TRAIL protein secreted by HepG2 cells after DENV infection was partly responsible for apoptosis of uninfected HepG2 cells (136). Global gene expression analysis in DENV-infected HepG2 cells and primary cells (DCs, monocytes, B cells, and HUVECs) performed in our laboratory has shown a distinct set of differentially regulated genes in HepG2 versus primary human cells (Fig. 1B). Unlike primary cells, TRAIL mRNA levels were not up-regulated in DENV-infected HepG2 cells. We believe that DENV infection of primary cells may better reflect a physiological response. In the previous study of Matsuda et al., 80% of DENV-infected HepG2 cells stained positive for cell death (136), raising the possibility that the TRAIL protein detected in the supernatant of DENV-infected cells was not secreted but resulted from cell death. In contrast, DCs infected with DENV showed minimal staining with the apoptosis detection dye (Live/Dead Aqua) or other markers of apoptotic cells (PARP-1 and caspase-3).

One possible explanation of the IFNαR blocking experiments is that majority of the *in vitro* antiviral activity of TRAIL against DENV is IFNαR-dependent. In addition, DENV infection-induced TRAIL gene expression was dependent on a functional IFNα signaling pathway, *in vitro*. Hence, we wanted to know whether the kinetics of TRAIL expression followed the known pattern of IFNα expression levels in DENV patients (124). TRAIL protein expression was determined in febrile,

postfebrile and convalescence samples of serum from DF patients. The kinetics of TRAIL serum levels correlated with IFNα and DENV levels reported in previous studies (124); TRAIL protein levels peaked during the febrile phase of the disease and returned to baseline levels by the post-febrile phase of the disease.

(ii) Anti-viral function of TRAIL

In vitro and *in vivo* studies have demonstrated tumoricidal activity of TRAIL without significant toxicity toward normal cells or tissues (171, 184). TRAIL has been shown to mediate antiviral functions *in vivo* in mouse models of influenza and encephalomyocarditis virus infection (89). Influenza viral clearance was prolonged in mice injected with anti-TRAIL antibody. TRAIL expressed by NK cells was crucial to limit encephalomyocarditis virus replication *in vivo* (184).

To determine the effect of TRAIL on DENV infection, we treated monocytes, B cells and HUVECs with anti-TRAIL Ab and monocytes and DCs with rTRAIL prior to infection with DENV. Multiple assays demonstrated that TRAIL is a potent antiviral molecule against DENV. Increase in DENV RNA levels following treatment with anti-TRAIL Ab indicates that TRAIL has antiviral activity against DENV and this activity is non-redundant. The reduction in DENV RNA levels suggests that TRAIL inhibits viral RNA production, which can affect protein synthesis. The lower detection of DENV antigen and reduction in plaque forming units per milliliter represents decrease in DENV infection and infectious viral progeny production, respectively. rTRAIL was procured from five different commercial sources- Merck (Catalog# 616374 & Catalog# 616375), UB Biological (Catalog# T8180), Alexis

(Catalog# 201-073-C020) and Biomol- and tested for antiviral activity against DENV. Of the five rTRAILs tested, rTRAIL obtained from Merck (Cat# 616375, discontinued) and Biomol (Catalog# SE-721) were functionally active in our study. The ability to induce apoptosis in cell lines like U937 cells or Jurkat cells is the only biological activity assay performed for all the commercially available recombinant TRAIL proteins including those tested in this study. rTRAIL treatment did not induce apoptosis of DENV-infected or bystander cells in this study. Recent studies have shown that rTRAIL also triggers apoptosis-independent functions in cells. We believe that the five commercially available rTRAILs might each signal differentially downstream after binding to the TRAIL-Rs, two of which (Biomol and Merck) induce genes involved in the antiviral activity of rTRAIL against DENV. In addition, the rTRAIL from Biomol and Merck have been used extensively to study TRAIL function (126, 142, 167).

The concentration of Biomol rTRAIL (5 to 20 μg/ml) that was able to inhibit DENV replication in this study was similar to the concentration used in studies demonstrating TRAIL-induced apoptosis of tumor cells (2, 26). In the literature, TRAIL anti-cancer function has been associated with its ability to induce apoptosis (2, 3, 25, 26). To determine whether TRAIL inhibited DENV by an apoptosis-dependent or -independent mechanism, we stained DENV-infected DCs pre-treated with TRAIL for Live/Dead Aqua, active Caspase-3 and cleaved PARP-1 protein. Live/Dead Aqua dye can permeate only into cells with compromised membranes (dead or dying cells). The dye can discriminate between live and dead cells by irreversibly binds to free amines, which are much more abundant inside rather than on

the cell surface. The extrinsic and intrinsic pathways of cell death converge at the activation of an effector enzyme, caspase-3. Caspase-3 is an early detection marker for cell death. PARP-1 mediates Poly(ADP-ribosyl)ation and PARP-1 activity represents an important pathway in the restoration of cellular integrity (86, 90). PARP-1 protein is cleaved by caspase-3 into two smaller fragments and detection of these fragments of PARP-1 protein is considered a sensor for early DNA damage (122). The presence of cleaved PARP-1 is widely used as an diagnostic marker of apoptosis in many cell types (63, 101). These studies showed neither increased DC apoptosis in DENV-infected DC cultures nor apoptosis of DENV antigen-positive cells. Thus, we conclude that exogenous recombinant TRAIL inhibited DENV-infection in DCs by a novel apoptosis-independent mechanism. We hypothesized that TRAIL might be inhibiting DENV replication by acting as an antiviral agent and not by inducing apoptosis.

TRAIL treatment has previously been shown to induce a different cohort of IFNα/β inducible genes, including transcriptional induction of IFNβ mRNA, at various timepoints following recombinant TRAIL treatment (108) suggesting a cross-talk with the IFNα/β signaling pathway. If this finding was true for the effect of rTRAIL treatment on DENV infection, time-point of rTRAIL pre-treatment might also affect the antiviral activity of rTRAIL against DENV. Hence, we performed a time course of rTRAIL pre-treatment to determine whether rTRAIL antiviral activity against DENV was dependent on the timepoint of pre-treatment. The data indicate that 24hour pre-treatment with rTRAIL produced the maximal and reproducible inhibition of DENV. Our earlier experiment on antigen staining of DENV-infected

DCs had shown that only bystander cells expressed TRAIL protein following DENV infection. Hence, one possible explanation for the requirement of 24 hour pre-treatment to inhibit maximal level of DENV could be that bystander cells require between 12-15 to 24 hours to express and secrete known antiviral cellular proteins which act upon DENV-infected cells. A previous study showed that at least 16 hour pre-treatment of cancer cells with rTRAIL was required to induce IFNβ transcription (108). Since we required 24 hours' pre-treatment to observe maximal inhibition of DENV replication, it is possible that the antiviral activity of rTRAIL against DENV involves IFNα/β activity.

IFNs enhance expression of TRAIL, while, on the other hand, TRAIL treatment can enhance expression of IFN-inducible genes like IFITM1, IFIT1, STAT1, LGal3BP, and PRKR as well as IFNβ itself (108). The molecular cross-talk and functional synergy observed between the TRAIL and IFN signaling pathways is not limited to the genes involved in apoptosis and may have implications for the physiologic role and mechanism of action of TRAIL protein. Data presented previously in this thesis has shown that DENV infection-induced TRAIL mRNA expression is dependent on a functional IFNα signaling pathway. To understand if the IFNα signaling pathway played a role in the antiviral activity of TRAIL, we pre-treated DENV-infected cells with rTRAIL in the presence of IFNαR-blocking antibody. We observed an increase in DENN antigen following treatment with IFNαR-blocking Ab. One possible explanation for this finding is that part of the exogenous rTRAIL-mediated antiviral activity against DENV is dependent on IFNαR. This hypothesis supports the model that rTRAIL induces IFNα/β as a part of

its antiviral response against DENV. Based on the *in vitro* and *in vivo* data we speculate that TRAIL is involved in limiting DENV spread *in vivo*.

(iii) Suppression of pro-inflammatory cytokine production by TRAIL

Many studies have implicated the involvement of T cells in the pathogenesis of DHF/DSS (112, 145, 168). Cross-reactive T cell activation, proliferation and release of pro-inflammatory cytokines and chemokines that can directly affect the endothelial cells inducing plasma leakage is a widely-proposed model for DHF/DSS following DENV-infection (145, 146). Higher circulating levels of sCD4, sCD8, IFNγ, IL-2, TNFα, sTNFR, IL8, IL6, MIF and MCP1 have been detected in sera of patients with dengue (112). Also, DHF has been associated with higher serum levels of pro-inflammatory cytokines and chemokines like IL-2, TNFα, IL6, IL8, MCP1 than DF (37, 63, 64, 96, 121, 176).

Dendritic cells regulate initiation and activation of both the innate and adaptive immune responses. In addition, DCs are the principal antigen-presenting cells that regulate activation of NK cells and naïve and memory T cells (203). *In vivo*, DCs are important targets for DENV infection (230). Palmer et al. (166) and Libraty et al. (125) have further demonstrated that phenotypic changes occur in both uninfected bystander DCs and DENV-infected DCs in the same cell culture, suggesting that DENV alters DC activation and maturation. Hence, DCs are a good *in vitro* primary human cell model, which can be used to study effects of rTRAIL treatment on DENV infection-induced changes in cellular gene expression.

Secchiero et al. (185) has recently shown that recombinant TRAIL completely

abrogated the TNFα-mediated increase in leukocyte adhesion to HUVECs by selectively downregulating the expression of IP-10 and MCP-2. This TRAIL treatment mediated modulation of leukocyte/endothelial cell adhesion did not interfere with the TNFα and IL-1β induced expression of VCAM-1, ICAM-1, E-selectin and NFκB genes. Therefore, we determined whether TRAIL was able to suppress expression of MCP-2, IP-10, IL-6, TNFα, MIP-1β and IL-8, pro-inflammatory chemokines and cytokines induced in response to DENV infection in DCs. We found that TRAIL significantly suppressed expression of MCP-2, IP-10, IL-6, TNFα and MIP-1β mRNA levels induced as a result of DENV infection in DCs. We also demonstrated the suppression by rTRAIL of MCP-2, IP-10, ITAC, and IL-6 protein secretion from DENV-infected DCs. Taken together, these data indicate that TRAIL can inhibit DENV replication and expression of pro-inflammatory genes. Some of the genes suppressed by rTRAIL treatment (IP-10, ITAC, MIP-1β TNFα and IL-6) have also been detected at higher levels in serum of dengue patients; of particular interest, TNFα and IL-6 serum levels correlate with the severity of dengue disease.

One theoretical approach to treating DENV immunopathology is to find a molecule with the capacity to both inhibit viral replication and regulate pro-inflammatory cytokine production. *In vivo* studies still need to be performed to establish the antiviral and anti-inflammatory functions of rTRAIL; however, we believe that the *in vitro* data suggest that TRAIL might be a therapy with dual beneficial function.

ST2 Gene:

(i) Expression

(ii) Biomarker for DENV infection

(iii) Biomarker for Primary versus Secondary DENV

(i) ST2 Expression

Alternative splicing of the ST2 gene generates three mRNAs, corresponding to a longer membrane-anchored form (ST2L), a shorter released form (sST2) and a membrane bound variant form (ST2V) (21, 215, 235). We detected increased ST2 mRNA in DENV-infected cells, *in vitro* and dengue patient PBMCs, by quantitative RT-PCR. However, the TaqMan primer and probe used for this assay was not specific to any of the three isoforms of ST2 gene. To identify if the specific isoforms, sST2 or ST2L, were upregulated following DENV infection, we performed semi-quantitative RT-PCR analysis using primers specific for these two isoforms in DENV-infected HUVECs. The sST2 isoform of the ST2 gene was found to be more abundant and also clearly upregulated in DENV-infected HUVECs. The ST2L isoform was weakly expressed in uninfected HUVECs and slightly induced at the mRNA level in DENV-infected HUVECs. However, no cell surface expression of ST2L was detected on HUVECs following DENV infection. We did not analyze ST2V mRNA and cell surface levels in HUVECs. In summary, the sST2 isoform of the ST2 gene was upregulated in DENV-infected HUVECs *in vitro*. These data do not conclusively prove that only the mRNA for sST2 isoform was upregulated in DENV patient PBMCs. Evidence of increased sST2 levels in sera of dengue patients further suggests

that the increased expression of ST2 gene mRNA identified in DENV patient PBMCs of dengue patients was of the sST2 isoform. However, further studies will be needed to determine ST2L and ST2V expression levels on the surface of PBMCs isolated from dengue patients during the acute phase of the disease.

(ii) Biomarker for DENV infection

sST2 has previously been proposed as a biomarker for heart failure (227). To determine the specificity of the increase in sST2 protein levels in patients with dengue, we compared sST2 levels in dengue patient serum to levels in serum of patients with other febrile illnesses (OFI). We found higher levels of sST2 protein in serum from DENV-infected patients as compared to serum from OFI patients. Serum levels of sST2 protein were elevated at the end of the febrile stage of the disease, reaching peak levels between fever day -1 and fever day 0 followed by decrease to normal levels by convalescence day. These data from a small number of dengue patients indicate that serum sST2 levels are a possible marker for DENV infection.

(iii) Biomarker for Primary versus Secondary DENV

Most of the patients in the clinical study were diagnosed as having DF, by serology. Since DF patients composed the majority of our cohort, we analyzed the data on sST2 levels according to antibody titers, which allowed us to classify cases as primary or secondary DENV infections. Higher levels of sST2 protein were found in patients with secondary infections than in those with primary infections. Currently HI titers are used to differentiate between primary and secondary DENV infections (41).

Hence, serum levels sST2 is a possible marker for DENV infection and a promising candidate as a biomarker for secondary dengue infections. However, further tests need to be performed to determine the specificity and sensitivity of serum sST2 detection test. Overall, a larger clinical study needs to be performed to confirm that sST2 is a marker for (i) DENV infection and (ii) secondary DENV infection. We also do not know which cells produce sST2 protein *in vivo* during DENV infections.

Reports have suggested that sST2 protein regulates both the pro-inflammatory response as well as the Th2 immune response. Soluble ST2 reduced IL-33 induced production of IL-4, IL-5 and IL-13 in splenocytes (75). In addition, sST2 treatment of bone marrow-derived and alveolar macrophages can inhibit LPS induced pro-inflammatory cytokines IL6, IL-12 and TNFα (164, 207). Soluble ST2 treatment was able to decrease TNFα levels elevated during ischemia reperfusion injury (characterized by local and systemic inflammation) (59). sST2 could act as a regulator of inflammation through a mechanism that involves the inhibition of Toll-like receptor 4 signaling by sequestration of MyD88 and Mal adapter proteins (27) or inhibition of NFκB activation by decreasing IκB degradation (209). Also, *in vivo* experiments have shown that sST2 protein was able to attenuate the production of pro-inflammatory cytokines IL-1β, TNFα, IL-6, and IL-12 in the murine collagen-induced arthritis model (123).

During secondary DENV infections, serotype-cross-reactive memory T cells from a previous infection prevail over the naïve T cells specific for the infecting serotype (55, 146). The activation of cross-reactive T cells may lead to suboptimal clearance of the virus and an excessive production of soluble mediators mimicking

"original antigenic sin" (145). High levels of pro-inflammatory cytokines like TNFα and IL-6 that have been found in dengue patients (8, 80, 81) can induce sST2 expression (109, 208). In addition, DENV-infected monocytes and PBMC supernatant can induce vascular permeability (33, 50). The Fagundes et al. study also demonstrated that high dose of sST2 inhibited vascular permeability and hemorrhage (clinical symptoms also associated with DHF/DSS) in ischemia reperfusion injury induced intestine and lungs compared to control IgG treatment (59). Hence, we propose that elevated sST2 levels found in dengue patients could be an indication of immune hyperactivation as well as a mechanism to down-regulate the pro-inflammatory cytokine response and maybe downregulate vascular permeability and hemorrhage associated with severe DENV infections.

Overall, the findings in this study show a transient elevation of sST2 protein levels in serum of DENV-infected patients around the time of defervescence, as compared to sST2 protein levels in OFI patients. Results show that in our patients, levels of sST2 protein in serum were higher in patients with secondary infections. Severe clinical manifestations (DHF/DSS) correlate with secondary infections. Hence, understanding the molecular mechanism(s) involved in the regulation and biological effects of sST2 protein in DENV infections deserves further investigation including exposure of DENV-infected endothelial cells to pro-inflammatory cytokines or supernatant from DENV-infected monocytes and PBMCs in the presence of sST2 to determine the effect of sST2 protein on vascular permeability.

IDO Gene:

(i) Expression

(ii) Biomarker for DENV infection

(iii) Antiviral function

(i) IDO Expression

IDO was another gene whose mRNA expression levels were higher in PBMCs from patients with dengue as compared to patients with OFI. Previous studies have shown that increased IDO activity plays a role in IFNγ-mediated inhibition of viruses (160). Heseler et al. (77). In addition, IFNγ is a potent inducer of IDO expression (180). We were interested in studying the function of IDO in IFNγ-mediated inhibition of DENV infection (or replication) in DCs. We found increased expression of IDO mRNA in DENV-infected DCs. The increase in IDO mRNA expression associated with DENV infection was further enhanced by IFNγ treatment. Thus, the data on IDO gene expression presented in this thesis indicate that both DENV infection and IFNγ can induce IDO gene expression in DENV-infected DCs.

(ii) Biomarker for DENV infection

Traditionally recognized for its role in pregnancy, transplantation, autoimmunity and neoplasia, the immunoregulatory enzyme IDO has been found to have potential in the control of virus infection and inflammation (67, 76, 138). IDO-dependent tryptophan depletion has been reported as an antiviral mechanism used by IFNγ to regulate viral progeny (160). Increased IDO activity might also have a

beneficial role to regulate inflammation and protect peripheral cells like endothelial cells and hepatocytes from effector T cell-mediated cytotoxicity. IDO expressed in antigen-presenting cells was essential to protect allogenic liver graft from T cell attack, suggesting a role of IDO as a immunosuppressive molecule (127). Another study by Thebault P et al. demonstrated the essential role of IFNγ from regulatory CD4+CD25+ T cells to induce IDO expression in graft endothelial cells to induce tolerance to MHC mismatched rat cardiac allograft (212).

IDO is ubiquitously distributed in mammalian tissues and cells (105). It catalyzes the initial and rate-limiting step in the catabolism of L-tryptophan along the kynurenine pathway (210). Determination of tryptophan and kynureneine levels is an indirect method of determining IDO enzyme activity (150). This indirect method was used to measure levels of IDO activity in sera from patients with dengue or OFI as well as healthy donor serum samples. We detected higher serum IDO activity in DENV-infected patients as compared to patients with OFI and healthy donors. Hence, we present evidence that increased serum IDO activity is a biomarker for DENV infection.

The increase in IDO activity was statistically significant during the febrile, post-febrile and convalescence (7-10 days after defervescence) phases of the disease. The levels of IDO activity and the differences in IDO activity levels between DENV and OFI patients were greater during the febrile ($p<0.001$) and post-febrile ($p<0.002$) phases of the disease as compared to the convalescence phase ($p<0.05$). DENV viremia titers decrease rapidly at the end of the febrile phase of the disease (fever

day-1 to fever day +1), while the resolution of immune response occurs in the post-febrile phase of the disease (124).

These data suggest that the factors involved in activation of IDO gene expression are at their peak during the febrile and post-febrile phase of the disease but that the factors have not completely returned to normal levels by 7-10 days after defervescence. IFNγ is the most potent known inducer of IDO expression (12). Other cytokines such as IFNα/β, TNFα and IL-1β alone cannot induce IDO expression. However, TNFα and IL-1β synergistically enhance IDO expression induced by IFNγ (12). IFNγ, IFNα/β, TNF-α and IL-1β are detected at higher levels, than healthy donors, systemically in DENV patients. Levels of each cytokine return to normal values on different days in the post-febrile phase of the disease, which might contribute to the elevated IDO activity detected even on the convalescence day (8, 81, 124).

Another conclusion can be made from the levels of tryptophan and kynurenine detected in DENV patient serum. Significantly lower tryptophan levels were detected on all days in the dengue patient samples as compared to OFI patients. However, significantly higher kynurenine levels were detected only during the acute phase in dengue serum samples. IDO catalyzes the conversion of tryptophan to its stable metabolite kynurenine. The circulating levels of tryptophan might be lower in dengue patients due to higher IDO enzyme activity and lower oral intake of nutrients due to nausea, vomiting and weakness, clinical symptoms associated with DENV disease. However, since IDO catalyzes the conversion of tryptophan to its stable metabolite kynurenine, higher kynureneine metabolite levels will correlate with the level of IDO

activity. Hence, kynurenine levels might be a better indicator of IDO enzyme activity than IDO activity in DENV patient serum. If kynureneine levels are considered an indicator of IDO activity, there is a significant increase in IDO enzyme activity in DENV patients only during the acute phase of the disease. This hypothesis would suggest that the factor/s that induces IDO expression is suppressed after the febrile phase of the disease.

Interestingly, the days of increase in serum kynurenine levels correlate with an increase in IFNγ levels and decrease in DENV viremia in DENV infected patients with DF (124), suggesting that IFNγ and not DENV might activate IDO gene expression in DENV-infected patients. IFNγ has been shown to be critical in the control of DENV replication in mouse and *in vitro* studies (125, 194, 230). Based on the importance of IDO activity in the antiviral activity of IFNγ against DENV *in vitro* and the correlation between the increase of IFNγ and significantly higher kynurenine levels, I speculate that IDO activity might play an important role in the antiviral activity of IFNγ against DENV *in vivo*.

(iii) Antiviral function

IFNγ-inducible IDO activity has been shown to play a role in regulating viruses (160). Heseler et al. (77) showed that 1-methyl-tryptophan (an analog of L-Tryptophan that is a competitive inhibitor of IDO activity (31) inhibited the anti-parasitic effect of IFNγ and IL-1 in an *in vitro* model of Toxoplasma gondii infection. IDO enzyme was also shown to play an important role in the antiviral activity of IFNγ against measles virus and in epithelial, endothelial and astroglial cells and herpes

simplex virus replication in HeLa cells (4, 160). Recent studies have found that the inhibition of T cell activation by IDO might be due to tryptophan starvation and/or production of T cell inhibitory metabolite of the kynurenine pathway (18, 105). We found significant lower levels of L-Tryptophan during the febrile and post-febrile stages of dengue illness and higher levels of L-Kyn during the febrile stage. The prevailing hypothesis is that the depletion of L-Tryptophan provokes a "starvation state" that results in inhibition of the growth of micro-organisms *in vitro* (32).

Wu et al. have reported that IFNγ can inhibit DENV infection of DCs (78, 125). Hence, we used DENV-infected DC to study the role of IDO in the antiviral mechanism mediated by IFNγ. Our results confirmed the observation that IFNγ inhibits DENV infection of DCs. Moreover, the IFNγ-mediated antiviral activity against DENV was significantly reduced in 1-MT-treated DCs. These results suggest that IDO activity plays an important role in the antiviral function of IFNγ against DENV, at least in DCs.

IDO and one of its kynurenine pathway metabolites produced by monocytes and DCs have been shown to inhibit T cell proliferation and activation (18, 143, 150). We did not study the effect of increased IDO activity in DENV-infected DCs on T cell proliferation and activation. However, DENV-infected DCs have been reported to be poor stimulators of allogeneic T cells (79, 135). One study observed that the proliferative responses of PBMCs to stimulation with DENV antigens, PHA, anti-CD3 or tetanus toxoid were significantly decreased during acute DENV illness (135). The decrease in the T cell proliferation could be reversed by addition of accessory cells from a normal donor, suggesting that the deficit lay with the antigen-presenting

cells from the dengue patients. The possible role of IDO in the suppression of T cell proliferation in these models deserves further investigation.

Satellite Muscle Cells Are Targets for DENV Infection

Dengue virus (DENV) is a mosquito-borne flavivirus that causes an acute febrile disease in humans characterized by musculoskeletal pain, heachache, rash and leucopenia. Few autopsy studies have been performed to date with the goal to identify cellular targets of DENV infection and none of these studies have looked at muscle cells for the presence of DENV antigen or RNA. The cause for muscle affection in dengue patients is not understood. A recent study on Chikungunya virus, a virus which also causes persistent skeletomuscular pain and myalgia, was found to infect muscle satellite cells in acutely infected individuals (165). In my thesis, we performed flow cytometry and immunofluorescence microscopy to demonstrate that primary human muscle satellite cells are highly susceptible to infection by multiple DENV serotypes. We further demonstrated that DENV replicates in muscle cells, based on plaque assays showing high levels of DENV progeny in culture supernatants. These data demonstrate that DENV infects and replicates in human muscle satellite cells, which might play a role in myalgia observed during DENV infection in patients. However, first a clinical study should determine whether skeletal / cardiac muscle cells are targets for DENV *in vivo*.

Conclusion

Global gene expression analysis was applied in this thesis to identify genes important in the response of primary human cells to DENV infection. We hypothesized that dentification of endogenous antiviral proteins that regulate DENV infection would help in understanding and treating the disease. Type-I and type-II IFNs are considered critical in the regulation of DENV based on *in vitro* and mouse studies (51, 52, 78, 152, 153, 193). This thesis identified TRAIL, a type-I IFN-inducible gene, and IDO, a type-II IFN-inducible gene, as playing important roles in Type-I and type-II IFN-mediated antiviral function against DENV, respectively.

The main challenge in treating dengue patients that present with severe clinical manifestations is that the symptoms of plasma leakage develop abruptly during the immediate post-febrile phase of the disease. Secondary DENV infections are associated with a markedly increased risk for DHF/DSS. However, there are no known biomarkers that can distinguish (i) secondary from primary infection or (ii) patients who are going to develop the severe form of the disease. This thesis identified sST2 and IDO as two biomarkers for DENV infection. In addition, sST2 is the first biomarker that can distinguish secondary from primary DENV infection.

Muscle affection is a frequent clinical symptom observed in dengue patients. In this thesis we found that DENV virus can infect and replicate in muscle satellite cells. Results from this section of the thesis strongly suggest that future clinical studies should be performed to determine whether muscle cells are targets for DENV, *in vivo*.

The ultimate goal of these studies was to identify (1) Potential targets to treat DENV infection and its immunopathology (2) Potential dengue infection biomarkers. Based on our results using global gene expression analysis, we believe that using the common response gene profile in the pathogen-susceptible cells and DENV-specific response gene profile in the cell appropriate for that particular disease model is a successful strategy that can be applied to identify important cellular response genes to other pathogens.

References

1. 2003. Joint WHO HQ/SEAROP/WPRO meeting on DengueNet implementation in South-East Asia and the Western Pacific, Kuala Lumpur, 11-13 December 2003. Wkly Epidemiol Rec **78:**346-7.
2. **Abdollahi, T., N. M. Robertson, A. Abdollahi, and G. Litwack.** 2003. Identification of interleukin 8 as an inhibitor of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in the ovarian carcinoma cell line OVCAR3. Cancer Res **63:**4521-6.
3. **Abdollahi, T., N. M. Robertson, A. Abdollahi, and G. Litwack.** 2005. Inhibition of TRAIL-induced apoptosis by IL-8 is mediated by the p38-MAPK pathway in OVCAR3 cells. Apoptosis **10:**1383-93.
4. **Adams, O., K. Besken, C. Oberdorfer, C. R. MacKenzie, O. Takikawa, and W. Daubener.** 2004. Role of indoleamine-2,3-dioxygenase in alpha/beta and gamma interferon-mediated antiviral effects against herpes simplex virus infections. J Virol **78:**2632-6.
5. **Agrawal, D. K., P. Tandon, U. C. Chaturvedi, and A. Kumar.** 1978. Biochemical study of certain enzymes and metabolites of the carbohydrate metabolism in the skeletal muscle of the dengue virus-infected mice. J Gen Virol **40:**399-408.
6. **Allen, R. E., C. J. Temm-Grove, S. M. Sheehan, and G. Rice.** 1997. Skeletal muscle satellite cell cultures. Methods Cell Biol **52:**155-76.
7. **Anderson, R., A. D. King, and B. L. Innis.** 1992. Correlation of E protein binding with cell susceptibility to dengue 4 virus infection. J Gen Virol **73 (Pt 8):**2155-9.
8. **Avila-Aguero, M. L., C. R. Avila-Aguero, S. L. Um, A. Soriano-Fallas, A. Canas-Coto, and S. B. Yan.** 2004. Systemic host inflammatory and coagulation response in the Dengue virus primo-infection. Cytokine **27:**173-9.
9. **Avirutnan, P., N. Punyadee, S. Noisakran, C. Komoltri, S. Thiemmeca, K. Auethavornanan, A. Jairungsri, R. Kanlaya, N. Tangthawornchaikul, C. Puttikhunt, S. N. Pattanakitsakul, P. T. Yenchitsomanus, J. Mongkolsapaya, W. Kasinrerk, N. Sittisombut, M. Husmann, M. Blettner, S. Vasanawathana, S. Bhakdi, and P. Malasit.** 2006. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J Infect Dis **193:**1078-88.
10. **Avirutnan, P., L. Zhang, N. Punyadee, A. Manuyakorn, C. Puttikhunt, W. Kasinrerk, P. Malasit, J. P. Atkinson, and M. S. Diamond.** 2007. Secreted NS1 of dengue virus attaches to the surface of cells via interactions with heparan sulfate and chondroitin sulfate E. PLoS Pathog **3:**e183.
11. **Azeredo, E. L., L. M. De Oliveira-Pinto, S. M. Zagne, D. I. Cerqueira, R. M. Nogueira, and C. F. Kubelka.** 2006. NK cells, displaying early activation, cytotoxicity and adhesion molecules, are associated with mild dengue disease. Clin Exp Immunol **143:**345-56.

12. **Babcock, T. A., and J. M. Carlin.** 2000. Transcriptional activation of indoleamine dioxygenase by interleukin 1 and tumor necrosis factor alpha in interferon-treated epithelial cells. Cytokine **12:**588-94.
13. **Badri, K. R., S. Modem, H. C. Gerard, I. Khan, M. Bagchi, A. P. Hudson, and T. R. Reddy.** 2006. Regulation of Sam68 activity by small heat shock protein 22. J Cell Biochem **99:**1353-62.
14. **Balmaseda, A., S. N. Hammond, M. A. Perez, R. Cuadra, S. Solano, J. Rocha, W. Idiaquez, and E. Harris.** 2005. Short report: assessment of the World Health Organization scheme for classification of dengue severity in Nicaragua. Am J Trop Med Hyg **73:**1059-62.
15. **Barth, S., T. Pfuhl, A. Mamiani, C. Ehses, K. Roemer, E. Kremmer, C. Jaker, J. Hock, G. Meister, and F. A. Grasser.** 2008. Epstein-Barr virus-encoded microRNA miR-BART2 down-regulates the viral DNA polymerase BALF5. Nucleic Acids Res **36:**666-75.
16. **Beaumier, C. M., A. Mathew, H. S. Bashyam, and A. L. Rothman.** 2008. Cross-reactive memory CD8(+) T cells alter the immune response to heterologous secondary dengue virus infections in mice in a sequence-specific manner. J Infect Dis **197:**608-17.
17. **Becerra, A., R. V. Warke, N. de Bosch, A. L. Rothman, and I. Bosch.** 2008. Elevated levels of soluble ST2 protein in dengue virus infected patients. Cytokine **41:**114-20.
18. **Belladonna, M. L., U. Grohmann, P. Guidetti, C. Volpi, R. Bianchi, M. C. Fioretti, R. Schwarcz, F. Fallarino, and P. Puccetti.** 2006. Kynurenine pathway enzymes in dendritic cells initiate tolerogenesis in the absence of functional IDO. J Immunol **177:**130-7.
19. **Belles-Isles, M., R. Roy, G. Dansereau, M. Goulet, B. Roy, J. P. Bouchard, and J. P. Tremblay.** 1993. Rapid selection of donor myoblast clones for muscular dystrophy therapy using cell surface expression of NCAM. Eur J Histochem **37:**375-80.
20. **Bera, A. K., R. J. Kuhn, and J. L. Smith.** 2007. Functional characterization of cis and trans activity of the Flavivirus NS2B-NS3 protease. J Biol Chem **282:**12883-92.
21. **Bergers, G., A. Reikerstorfer, S. Braselmann, P. Graninger, and M. Busslinger.** 1994. Alternative promoter usage of the Fos-responsive gene Fit-1 generates mRNA isoforms coding for either secreted or membrane-bound proteins related to the IL-1 receptor. EMBO J **13:**1176-88.
22. **Boonstra, R., and L. Bowman.** 2003. Demography of short-tailed shrew populations living on polychlorinated biphenyl-contaminated sites. Environ Toxicol Chem **22:**1394-403.
23. **Bosch, I., H. Melichar, and A. B. Pardee.** 2000. Identification of differentially expressed genes from limited amounts of RNA. Nucleic Acids Res **28:**E27.
24. **Bosch, I., K. Xhaja, L. Estevez, G. Raines, H. Melichar, R. V. Warke, M. V. Fournier, F. A. Ennis, and A. L. Rothman.** 2002. Increased production of interleukin-8 in primary human monocytes and in human epithelial and endothelial cell lines after dengue virus challenge. J Virol **76:**5588-97.

25. **Bouralexis, S., D. M. Findlay, G. J. Atkins, A. Labrinidis, S. Hay, and A. Evdokiou.** 2003. Progressive resistance of BTK-143 osteosarcoma cells to Apo2L/TRAIL-induced apoptosis is mediated by acquisition of DcR2/TRAIL-R4 expression: resensitisation with chemotherapy. Br J Cancer **89:**206-14.
26. **Bouralexis, S., D. M. Findlay, and A. Evdokiou.** 2005. Death to the bad guys: targeting cancer via Apo2L/TRAIL. Apoptosis **10:**35-51.
27. **Brint, E. K., D. Xu, H. Liu, A. Dunne, A. N. McKenzie, L. A. O'Neill, and F. Y. Liew.** 2004. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. Nat Immunol **5:**373-9.
28. **Brunner, M., C. Krenn, G. Roth, B. Moser, M. Dworschak, E. Jensen-Jarolim, A. Spittler, T. Sautner, N. Bonaros, E. Wolner, G. Boltz-Nitulescu, and H. J. Ankersmit.** 2004. Increased levels of soluble ST2 protein and IgG1 production in patients with sepsis and trauma. Intensive Care Med **30:**1468-73.
29. **Bryant, P. A., D. Venter, R. Robins-Browne, and N. Curtis.** 2004. Chips with everything: DNA microarrays in infectious diseases. Lancet Infect Dis **4:**100-11.
30. **Butthep, P., S. Chunhakan, K. Tangnararatchakit, S. Yoksan, K. Pattanapanyasat, and A. Chuansumrit.** 2006. Elevated soluble thrombomodulin in the febrile stage related to patients at risk for dengue shock syndrome. Pediatr Infect Dis J **25:**894-7.
31. **Cady, S. G., and M. Sono.** 1991. 1-Methyl-DL-tryptophan, beta-(3-benzofuranyl)-DL-alanine (the oxygen analog of tryptophan), and beta-[3-benzo(b)thienyl]-DL-alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase. Arch Biochem Biophys **291:**326-33.
32. **Carlin, J. M., Y. Ozaki, G. I. Byrne, R. R. Brown, and E. C. Borden.** 1989. Interferons and indoleamine 2,3-dioxygenase: role in antimicrobial and antitumor effects. Experientia **45:**535-41.
33. **Carr, J. M., H. Hocking, K. Bunting, P. J. Wright, A. Davidson, J. Gamble, C. J. Burrell, and P. Li.** 2003. Supernatants from dengue virus type-2 infected macrophages induce permeability changes in endothelial cell monolayers. J Med Virol **69:**521-8.
34. **Chan, F. K.** 2007. Three is better than one: pre-ligand receptor assembly in the regulation of TNF receptor signaling. Cytokine **37:**101-7.
35. **Chaturvedi, U. C., A. K. Kapoor, A. Mathur, D. Chandra, A. M. Khan, and R. M. Mehrotra.** 1970. A clinical and epidemiological study of an epidemic of febrile illness with haemorrhagic manifestations which occurred at Kanpur, India, in 1968. Bull World Health Organ **43:**281-7.
36. **Chen, H. C., F. M. Hofman, J. T. Kung, Y. D. Lin, and B. A. Wu-Hsieh.** 2007. Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage. J Virol **81:**5518-26.
37. **Chen, L. C., H. Y. Lei, C. C. Liu, S. C. Shiesh, S. H. Chen, H. S. Liu, Y. S. Lin, S. T. Wang, H. W. Shyu, and T. M. Yeh.** 2006. Correlation of serum

levels of macrophage migration inhibitory factor with disease severity and clinical outcome in dengue patients. Am J Trop Med Hyg **74:**142-7.

38. **Chen, Y., T. Maguire, R. E. Hileman, J. R. Fromm, J. D. Esko, R. J. Linhardt, and R. M. Marks.** 1997. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat Med **3:**866-71.
39. **Cheriyath, V., K. B. Glaser, J. F. Waring, R. Baz, M. A. Hussein, and E. C. Borden.** 2007. G1P3, an IFN-induced survival factor, antagonizes TRAIL-induced apoptosis in human myeloma cells. J Clin Invest **117:**3107-17.
40. **Chou, A. H., H. F. Tsai, Y. Y. Wu, C. Y. Hu, L. H. Hwang, P. I. Hsu, and P. N. Hsu.** 2005. Hepatitis C virus core protein modulates TRAIL-mediated apoptosis by enhancing Bid cleavage and activation of mitochondria apoptosis signaling pathway. J Immunol **174:**2160-6.
41. **Clarke, D. H., and J. Casals.** 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am J Trop Med Hyg **7:**561-73.
42. **Clarke, P., S. M. Meintzer, S. Gibson, C. Widmann, T. P. Garrington, G. L. Johnson, and K. L. Tyler.** 2000. Reovirus-induced apoptosis is mediated by TRAIL. J Virol **74:**8135-9.
43. **Clarke, P., and K. L. Tyler.** 2007. Down-regulation of cFLIP following reovirus infection sensitizes human ovarian cancer cells to TRAIL-induced apoptosis. Apoptosis **12:**211-23.
44. **Cretney, E., J. L. McQualter, N. Kayagaki, H. Yagita, C. C. Bernard, I. S. Grewal, A. Ashkenazi, and M. J. Smyth.** 2005. TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L suppresses experimental autoimmune encephalomyelitis in mice. Immunol Cell Biol **83:**511-9.
45. **Daubener, W., B. Spors, C. Hucke, R. Adam, M. Stins, K. S. Kim, and H. Schroten.** 2001. Restriction of Toxoplasma gondii growth in human brain microvascular endothelial cells by activation of indoleamine 2,3-dioxygenase. Infect Immun **69:**6527-31.
46. **Dauer, M., K. Schad, J. Junkmann, C. Bauer, J. Herten, R. Kiefl, M. Schnurr, S. Endres, and A. Eigler.** 2006. IFN-alpha promotes definitive maturation of dendritic cells generated by short-term culture of monocytes with GM-CSF and IL-4. J Leukoc Biol **80:**278-86.
47. **de la Fuente, C., F. Santiago, L. Deng, C. Eadie, I. Zilberman, K. Kehn, A. Maddukuri, S. Baylor, K. Wu, C. G. Lee, A. Pumfery, and F. Kashanchi.** 2002. Gene expression profile of HIV-1 Tat expressing cells: a close interplay between proliferative and differentiation signals. BMC Biochem **3:**14.
48. **Deen, J. L., E. Harris, B. Wills, A. Balmaseda, S. N. Hammond, C. Rocha, N. M. Dung, N. T. Hung, T. T. Hien, and J. J. Farrar.** 2006. The WHO dengue classification and case definitions: time for a reassessment. Lancet **368:**170-3.
49. **Detweiler, C. S., D. B. Cunanan, and S. Falkow.** 2001. Host microarray analysis reveals a role for the Salmonella response regulator phoP in human macrophage cell death. Proc Natl Acad Sci U S A **98:**5850-5.

50. **Dewi, B. E., T. Takasaki, and I. Kurane.** 2008. Peripheral blood mononuclear cells increase the permeability of dengue virus-infected endothelial cells in association with downregulation of vascular endothelial cadherin. J Gen Virol **89:**642-52.
51. **Diamond, M. S., and E. Harris.** 2001. Interferon inhibits dengue virus infection by preventing translation of viral RNA through a PKR-independent mechanism. Virology **289:**297-311.
52. **Diamond, M. S., T. G. Roberts, D. Edgil, B. Lu, J. Ernst, and E. Harris.** 2000. Modulation of Dengue virus infection in human cells by alpha, beta, and gamma interferons. J Virol **74:**4957-66.
53. **Dib, N., R. E. Michler, F. D. Pagani, S. Wright, D. J. Kereiakes, R. Lengerich, P. Binkley, D. Buchele, I. Anand, C. Swingen, M. F. Di Carli, J. D. Thomas, W. A. Jaber, S. R. Opie, A. Campbell, P. McCarthy, M. Yeager, V. Dilsizian, B. P. Griffith, R. Korn, S. K. Kreuger, M. Ghazoul, W. R. MacLellan, G. Fonarow, H. J. Eisen, J. Dinsmore, and E. Diethrich.** 2005. Safety and feasibility of autologous myoblast transplantation in patients with ischemic cardiomyopathy: four-year follow-up. Circulation **112:**1748-55.
54. **Diehl, G. E., H. H. Yue, K. Hsieh, A. A. Kuang, M. Ho, L. A. Morici, L. L. Lenz, D. Cado, L. W. Riley, and A. Winoto.** 2004. TRAIL-R as a negative regulator of innate immune cell responses. Immunity **21:**877-89.
55. **Dong, T., E. Moran, N. Vinh Chau, C. Simmons, K. Luhn, Y. Peng, B. Wills, N. Phuong Dung, L. Thi Thu Thao, T. T. Hien, A. McMichael, J. Farrar, and S. Rowland-Jones.** 2007. High Pro-Inflammatory Cytokine Secretion and Loss of High Avidity Cross-Reactive Cytotoxic T-Cells during the Course of Secondary Dengue Virus Infection. PLoS ONE **2:**e1192.
56. **Ehrlich, S., C. Infante-Duarte, B. Seeger, and F. Zipp.** 2003. Regulation of soluble and surface-bound TRAIL in human T cells, B cells, and monocytes. Cytokine **24:**244-53.
57. **Elankumaran, S., D. Rockemann, and S. K. Samal.** 2006. Newcastle disease virus exerts oncolysis by both intrinsic and extrinsic caspase-dependent pathways of cell death. J Virol **80:**7522-34.
58. **Endy, T. P., A. Nisalak, S. Chunsuttitwat, D. W. Vaughn, S. Green, F. A. Ennis, A. L. Rothman, and D. H. Libraty.** 2004. Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. J Infect Dis **189:**990-1000.
59. **Fagundes, C. T., F. A. Amaral, A. L. Souza, A. T. Vieira, D. Xu, F. Y. Liew, D. G. Souza, and M. M. Teixeira.** 2007. ST2, an IL-1R family member, attenuates inflammation and lethality after intestinal ischemia and reperfusion. J Leukoc Biol **81:**492-9.
60. **Falgout, B., M. Bray, J. J. Schlesinger, and C. J. Lai.** 1990. Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. J Virol **64:**4356-63.

61. **Fink, J., F. Gu, L. Ling, T. Tolfvenstam, F. Olfat, K. C. Chin, P. Aw, J. George, V. A. Kuznetsov, M. Schreiber, S. G. Vasudevan, and M. L. Hibberd.** 2007. Host gene expression profiling of dengue virus infection in cell lines and patients. PLoS Negl Trop Dis **1:**e86.
62. **Fujigaki, S., K. Saito, K. Sekikawa, S. Tone, O. Takikawa, H. Fujii, H. Wada, A. Noma, and M. Seishima.** 2001. Lipopolysaccharide induction of indoleamine 2,3-dioxygenase is mediated dominantly by an IFN-gamma-independent mechanism. Eur J Immunol **31:**2313-8.
63. **Grader-Beck, T., L. Casciola-Rosen, T. J. Lang, R. Puliaev, A. Rosen, and C. S. Via.** 2007. Apoptotic splenocytes drive the autoimmune response to poly(ADP-ribose) polymerase 1 in a murine model of lupus. J Immunol **178:**95-102.
64. **Green, S., D. W. Vaughn, S. Kalayanarooj, S. Nimmannitya, S. Suntayakorn, A. Nisalak, R. Lew, B. L. Innis, I. Kurane, A. L. Rothman, and F. A. Ennis.** 1999. Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. J Infect Dis **179:**755-62.
65. **Green, S., D. W. Vaughn, S. Kalayanarooj, S. Nimmannitya, S. Suntayakorn, A. Nisalak, A. L. Rothman, and F. A. Ennis.** 1999. Elevated plasma interleukin-10 levels in acute dengue correlate with disease severity. J Med Virol **59:**329-34.
66. **Greidinger, E. L., D. K. Miller, T. T. Yamin, L. Casciola-Rosen, and A. Rosen.** 1996. Sequential activation of three distinct ICE-like activities in Fas-ligated Jurkat cells. FEBS Lett **390:**299-303.
67. **Grohmann, U., F. Fallarino, and P. Puccetti.** 2003. Tolerance, DCs and tryptophan: much ado about IDO. Trends Immunol **24:**242-8.
68. **Gubler, D. J.** 2006. Dengue/dengue haemorrhagic fever: history and current status. Novartis Found Symp **277:**3-16; discussion 16-22, 71-3, 251-3.
69. **Gurtner, G. J., R. D. Newberry, S. R. Schloemann, K. G. McDonald, and W. F. Stenson.** 2003. Inhibition of indoleamine 2,3-dioxygenase augments trinitrobenzene sulfonic acid colitis in mice. Gastroenterology **125:**1762-73.
70. **Halstead, S. B.** 1966. Mosquito-borne haemorrhagic fevers of South and South-East Asia. Bull World Health Organ **35:**3-15.
71. **Halstead, S. B.** 1988. Pathogenesis of dengue: challenges to molecular biology. Science **239:**476-81.
72. **Halstead, S. B.** 1992. The XXth century dengue pandemic: need for surveillance and research. World Health Stat Q **45:**292-8.
73. **Halstead, S. B., N. T. Lan, T. T. Myint, T. N. Shwe, A. Nisalak, S. Kalyanarooj, S. Nimmannitya, S. Soegijanto, D. W. Vaughn, and T. P. Endy.** 2002. Dengue hemorrhagic fever in infants: research opportunities ignored. Emerg Infect Dis **8:**1474-9.
74. **Hasegawa, H., Y. Yamada, H. Harasawa, T. Tsuji, K. Murata, K. Sugahara, K. Tsuruda, M. Masuda, N. Takasu, and S. Kamihira.** 2004. Restricted expression of tumor necrosis factor-related apoptosis-inducing ligand receptor 4 in human peripheral blood lymphocytes. Cell Immunol **231:**1-7.

75. **Hayakawa, H., M. Hayakawa, A. Kume, and S. Tominaga.** 2007. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. J Biol Chem **282:**26369-80.
76. **Hayashi, T., L. Beck, C. Rossetto, X. Gong, O. Takikawa, K. Takabayashi, D. H. Broide, D. A. Carson, and E. Raz.** 2004. Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. J Clin Invest **114:**270-9.
77. **Heseler, K., K. Spekker, S. K. Schmidt, C. R. Mackenzie, and W. Daubener.** 2008. Antimicrobial and immunoregulatory effects mediated by human lung cells: role of IFN-gamma-induced tryptophan degradation. FEMS Immunol Med Microbiol.
78. **Ho, L. J., L. F. Hung, C. Y. Weng, W. L. Wu, P. Chou, Y. L. Lin, D. M. Chang, T. Y. Tai, and J. H. Lai.** 2005. Dengue virus type 2 antagonizes IFN-alpha but not IFN-gamma antiviral effect via down-regulating Tyk2-STAT signaling in the human dendritic cell. J Immunol **174:**8163-72.
79. **Ho, L. J., M. F. Shaio, D. M. Chang, C. L. Liao, and J. H. Lai.** 2004. Infection of human dendritic cells by dengue virus activates and primes T cells towards Th0-like phenotype producing both Th1 and Th2 cytokines. Immunol Invest **33:**423-37.
80. **Hober, D., A. S. Delannoy, S. Benyoucef, D. De Groote, and P. Wattre.** 1996. High levels of sTNFR p75 and TNF alpha in dengue-infected patients. Microbiol Immunol **40:**569-73.
81. **Hober, D., L. Poli, B. Roblin, P. Gestas, E. Chungue, G. Granic, P. Imbert, J. L. Pecarere, R. Vergez-Pascal, P. Wattre, and et al.** 1993. Serum levels of tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 beta) in dengue-infected patients. Am J Trop Med Hyg **48:**324-31.
82. **Hoffmann, O., J. Priller, T. Prozorovski, U. Schulze-Topphoff, N. Baeva, J. D. Lunemann, O. Aktas, C. Mahrhofer, S. Stricker, F. Zipp, and J. R. Weber.** 2007. TRAIL limits excessive host immune responses in bacterial meningitis. J Clin Invest **117:**2004-13.
83. **Hori, H., and C. J. Lai.** 1990. Cleavage of dengue virus NS1-NS2A requires an octapeptide sequence at the C terminus of NS1. J Virol **64:**4573-7.
84. **Huang, Y., and M. S. Sheikh.** 2006. TRAIL death receptors and cancer therapeutics. Toxicol Appl Pharmacol.
85. **Huerre, M. R., N. T. Lan, P. Marianneau, N. B. Hue, H. Khun, N. T. Hung, N. T. Khen, M. T. Drouet, V. T. Huong, D. Q. Ha, Y. Buisson, and V. Deubel.** 2001. Liver histopathology and biological correlates in five cases of fatal dengue fever in Vietnamese children. Virchows Arch **438:**107-15.
86. **Ikejima, M., S. Noguchi, R. Yamashita, T. Ogura, T. Sugimura, D. M. Gill, and M. Miwa.** 1990. The zinc fingers of human poly(ADP-ribose) polymerase are differentially required for the recognition of DNA breaks and nicks and the consequent enzyme activation. Other structures recognize intact DNA. J Biol Chem **265:**21907-13.
87. **Imaizumi, T.-a., K. H. Albertine, D. L. Jicha, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman.** 1997. Human Endothelial Cells Synthesize

ENA-78: Relationship to IL-8 and to Signaling of PMN Adhesion. Am. J. Respir. Cell Mol. Biol. **17:**181-192.

88. **Indraccolo, S., U. Pfeffer, S. Minuzzo, G. Esposito, V. Roni, S. Mandruzzato, N. Ferrari, L. Anfosso, R. Dell'Eva, D. M. Noonan, L. Chieco-Bianchi, A. Albini, and A. Amadori.** 2007. Identification of genes selectively regulated by IFNs in endothelial cells. J Immunol **178:**1122-35.
89. **Ishikawa, E., M. Nakazawa, M. Yoshinari, and M. Minami.** 2005. Role of tumor necrosis factor-related apoptosis-inducing ligand in immune response to influenza virus infection in mice. J Virol **79:**7658-63.
90. **Ivana Scovassi, A., and M. Diederich.** 2004. Modulation of poly(ADP-ribosylation) in apoptotic cells. Biochem Pharmacol **68:**1041-7.
91. **Jenkins, S. J., G. Perona-Wright, A. G. Worsley, N. Ishii, and A. S. MacDonald.** 2007. Dendritic cell expression of OX40 ligand acts as a costimulatory, not polarizing, signal for optimal Th2 priming and memory induction in vivo. J Immunol **179:**3515-23.
92. **Jessie, K., M. Y. Fong, S. Devi, S. K. Lam, and K. T. Wong.** 2004. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J Infect Dis **189:**1411-8.
93. **Jindadamrongwech, S., C. Thepparit, and D. R. Smith.** 2004. Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. Arch Virol **149:**915-27.
94. **Johnson, A. J., and J. T. Roehrig.** 1999. New mouse model for dengue virus vaccine testing. J Virol **73:**783-6.
95. **Jones, M., A. Davidson, L. Hibbert, P. Gruenwald, J. Schlaak, S. Ball, G. R. Foster, and M. Jacobs.** 2005. Dengue virus inhibits alpha interferon signaling by reducing STAT2 expression. J Virol **79:**5414-20.
96. **Juffrie, M., G. M. van Der Meer, C. E. Hack, K. Haasnoot, Sutaryo, A. J. Veerman, and L. G. Thijs.** 2000. Inflammatory mediators in dengue virus infection in children: interleukin-8 and its relationship to neutrophil degranulation. Infect Immun **68:**702-7.
97. **Kalayanarooj, S., and S. Nimmannitya.** 2003. Clinical presentations of dengue hemorrhagic fever in infants compared to children. J Med Assoc Thai **86 Suppl 3:**S673-80.
98. **Kalita, J., U. K. Misra, A. Mahadevan, and S. K. Shankar.** 2005. Acute pure motor quadriplegia: is it dengue myositis? Electromyogr Clin Neurophysiol **45:**357-61.
99. **Kaufman, B. M., P. L. Summers, D. R. Dubois, W. H. Cohen, M. K. Gentry, R. L. Timchak, D. S. Burke, and K. H. Eckels.** 1989. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg **41:**576-80.
100. **Kaufman, B. M., P. L. Summers, D. R. Dubois, and K. H. Eckels.** 1987. Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg **36:**427-34.
101. **Kaufmann, S. H., S. Desnoyers, Y. Ottaviano, N. E. Davidson, and G. G. Poirier.** 1993. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res **53:**3976-85.

102. **Kemp, T. J., B. D. Elzey, and T. S. Griffith.** 2003. Plasmacytoid dendritic cell-derived IFN-alpha induces TNF-related apoptosis-inducing ligand/Apo-2L-mediated antitumor activity by human monocytes following CpG oligodeoxynucleotide stimulation. J Immunol **171:**212-8.
103. **Kemp, T. J., A. T. Ludwig, J. K. Earel, J. M. Moore, R. L. Vanoosten, B. Moses, K. Leidal, W. M. Nauseef, and T. S. Griffith.** 2005. Neutrophil stimulation with Mycobacterium bovis bacillus Calmette-Guerin (BCG) results in the release of functional soluble TRAIL/Apo-2L. Blood **106:**3474-82.
104. **King, A. D., A. Nisalak, S. Kalayanrooj, K. S. Myint, K. Pattanapanyasat, S. Nimmannitya, and B. L. Innis.** 1999. B cells are the principal circulating mononuclear cells infected by dengue virus. Southeast Asian J Trop Med Public Health **30:**718-28.
105. **King, N. J., and S. R. Thomas.** 2007. Molecules in focus: indoleamine 2,3-dioxygenase. Int J Biochem Cell Biol **39:**2167-72.
106. **Kliks, S. C., A. Nisalak, W. E. Brandt, L. Wahl, and D. S. Burke.** 1989. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. Am J Trop Med Hyg **40:**444-51.
107. **Kou, Z., M. Quinn, H. Chen, W. W. Rodrigo, R. C. Rose, J. J. Schlesinger, and X. Jin.** 2008. Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV) infection among human peripheral blood mononuclear cells. J Med Virol **80:**134-46.
108. **Kumar-Sinha, C., S. Varambally, A. Sreekumar, and A. M. Chinnaiyan.** 2002. Molecular cross-talk between the TRAIL and interferon signaling pathways. J Biol Chem **277:**575-85.
109. **Kumar, S., M. N. Tzimas, D. E. Griswold, and P. R. Young.** 1997. Expression of ST2, an interleukin-1 receptor homologue, is induced by proinflammatory stimuli. Biochem Biophys Res Commun **235:**474-8.
110. **Kumari, S. R., H. Mendoza-Alvarez, and R. Alvarez-Gonzalez.** 1998. Functional interactions of p53 with poly(ADP-ribose) polymerase (PARP) during apoptosis following DNA damage: covalent poly(ADP-ribosyl)ation of p53 by exogenous PARP and noncovalent binding of p53 to the M(r) 85,000 proteolytic fragment. Cancer Res **58:**5075-8.
111. **Kurane, I., B. L. Innis, S. Nimmannitya, A. Nisalak, A. Meager, and F. A. Ennis.** 1993. High levels of interferon alpha in the sera of children with dengue virus infection. Am J Trop Med Hyg **48:**222-9.
112. **Kurane, I., B. L. Innis, S. Nimmannitya, A. Nisalak, A. Meager, J. Janus, and F. A. Ennis.** 1991. Activation of T lymphocytes in dengue virus infections. High levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2, and interferon-gamma in sera of children with dengue. J Clin Invest **88:**1473-80.
113. **Kurane, I., J. Janus, and F. A. Ennis.** 1992. Dengue virus infection of human skin fibroblasts in vitro production of IFN-beta, IL-6 and GM-CSF. Arch Virol **124:**21-30.

114. **Kurane, I., A. L. Rothman, P. G. Livingston, S. Green, S. J. Gagnon, J. Janus, B. L. Innis, S. Nimmannitya, A. Nisalak, and F. A. Ennis.** 1994. Immunopathologic mechanisms of dengue hemorrhagic fever and dengue shock syndrome. Arch Virol Suppl **9:**59-64.
115. **Kuroiwa, K., T. Arai, H. Okazaki, S. Minota, and S. Tominaga.** 2001. Identification of human ST2 protein in the sera of patients with autoimmune diseases. Biochem Biophys Res Commun **284:**1104-8.
116. **Kuruvilla, J. G., R. M. Troyer, S. Devi, and R. Akkina.** 2007. Dengue virus infection and immune response in humanized RAG2(-/-)gamma(c)(-/-) (RAG-hu) mice. Virology **369:**143-52.
117. **Kwan, W. H., A. M. Helt, C. Maranon, J. B. Barbaroux, A. Hosmalin, E. Harris, W. H. Fridman, and C. G. Mueller.** 2005. Dendritic cell precursors are permissive to dengue virus and human immunodeficiency virus infection. J Virol **79:**7291-9.
118. **Lai, C. J., A. P. Goncalvez, R. Men, C. Wernly, O. Donau, R. E. Engle, and R. H. Purcell.** 2007. Epitope determinants of a chimpanzee dengue virus type 4 (DENV-4)-neutralizing antibody and protection against DENV-4 challenge in mice and rhesus monkeys by passively transferred humanized antibody. J Virol **81:**12766-74.
119. **Lanciotti, R. S., C. H. Calisher, D. J. Gubler, G. J. Chang, and A. V. Vorndam.** 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol **30:**545-51.
120. **Laoprasopwattana, K., D. H. Libraty, T. P. Endy, A. Nisalak, S. Chunsuttiwat, D. W. Vaughn, G. Reed, F. A. Ennis, A. L. Rothman, and S. Green.** 2005. Dengue Virus (DV) enhancing antibody activity in preillness plasma does not predict subsequent disease severity or viremia in secondary DV infection. J Infect Dis **192:**510-9.
121. **Laur, F., B. Murgue, X. Deparis, C. Roche, O. Cassar, and E. Chungue.** 1998. Plasma levels of tumour necrosis factor alpha and transforming growth factor beta-1 in children with dengue 2 virus infection in French Polynesia. Trans R Soc Trop Med Hyg **92:**654-6.
122. **Lazebnik, Y. A., S. H. Kaufmann, S. Desnoyers, G. G. Poirier, and W. C. Earnshaw.** 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature **371:**346-7.
123. **Leung, B. P., D. Xu, S. Culshaw, I. B. McInnes, and F. Y. Liew.** 2004. A novel therapy of murine collagen-induced arthritis with soluble T1/ST2. J Immunol **173:**145-50.
124. **Libraty, D. H., T. P. Endy, H. S. Houng, S. Green, S. Kalayanarooj, S. Suntayakorn, W. Chansiriwongs, D. W. Vaughn, A. Nisalak, F. A. Ennis, and A. L. Rothman.** 2002. Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. J Infect Dis **185:**1213-21.
125. **Libraty, D. H., S. Pichyangkul, C. Ajariyakhajorn, T. P. Endy, and F. A. Ennis.** 2001. Human dendritic cells are activated by dengue virus infection:

enhancement by gamma interferon and implications for disease pathogenesis. J Virol **75:**3501-8.

126. **Lin, Y., A. Devin, A. Cook, M. M. Keane, M. Kelliher, S. Lipkowitz, and Z. G. Liu.** 2000. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. Mol Cell Biol **20:**6638-45.
127. **Lin, Y. C., C. L. Chen, T. Nakano, S. Goto, Y. H. Kao, L. W. Hsu, C. Y. Lai, B. Jawan, Y. F. Cheng, C. Tateno, and K. Yoshizato.** 2007. Immunological role of indoleamine 2,3-dioxygenase in rat liver allograft rejection and tolerance. J Gastroenterol Hepatol.
128. **Liou, L.-Y., R. E. Haaland, C. H. Herrmann, and A. P. Rice.** 2006. Cyclin T1 but not cyclin T2a is induced by a post-transcriptional mechanism in PAMP-activated monocyte-derived macrophages. J Leukoc Biol **79:**388-396.
129. **Lippa, M. S., L. D. Strockbine, T. T. Le, D. G. Branstetter, C. A. Strathdee, and P. M. Holland.** 2007. Expression of anti-apoptotic factors modulates Apo2L/TRAIL resistance in colon carcinoma cells. Apoptosis **12:**1465-78.
130. **Littaua, R., I. Kurane, and F. A. Ennis.** 1990. Human IgG Fc receptor II mediates antibody-dependent enhancement of dengue virus infection. J Immunol **144:**3183-6.
131. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:**402-8.
132. **Lutfalla, G., S. J. Holland, E. Cinato, D. Monneron, J. Reboul, N. C. Rogers, J. M. Smith, G. R. Stark, K. Gardiner, K. E. Mogensen, and et al.** 1995. Mutant U5A cells are complemented by an interferon-alpha beta receptor subunit generated by alternative processing of a new member of a cytokine receptor gene cluster. Embo J **14:**5100-8.
133. **Ma, H., Y. Liu, S. Liu, H. F. Kung, X. Sun, D. Zheng, and R. Xu.** 2005. Recombinant adeno-associated virus-mediated TRAIL gene therapy suppresses liver metastatic tumors. Int J Cancer **116:**314-21.
134. **Malheiros, S. M., A. S. Oliveira, B. Schmidt, J. G. Lima, and A. A. Gabbai.** 1993. Dengue. Muscle biopsy findings in 15 patients. Arq Neuropsiquiatr **51:**159-64.
135. **Mathew, A., I. Kurane, S. Green, D. W. Vaughn, S. Kalayanarooj, S. Suntayakorn, F. A. Ennis, and A. L. Rothman.** 1999. Impaired T cell proliferation in acute dengue infection. J Immunol **162:**5609-15.
136. **Matsuda, T., A. Almasan, M. Tomita, K. Tamaki, M. Saito, M. Tadano, H. Yagita, T. Ohta, and N. Mori.** 2005. Dengue virus-induced apoptosis in hepatic cells is partly mediated by Apo2 ligand/tumour necrosis factor-related apoptosis-inducing ligand. J Gen Virol **86:**1055-65.
137. **McKendry, R., J. John, D. Flavell, M. Muller, I. M. Kerr, and G. R. Stark.** 1991. High-frequency mutagenesis of human cells and characterization of a mutant unresponsive to both alpha and gamma interferons. Proc Natl Acad Sci U S A **88:**11455-9.

138. **Mellor, A. L., and D. H. Munn.** 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol **4:**762-74.
139. **Mentor, N. A., and I. Kurane.** 1997. Dengue virus infection of human T lymphocytes. Acta Virol **41:**175-6.
140. **Miki, T., H. Sun, Y. Lee, A. Tandin, A. M. Kovscek, V. Subbotin, J. J. Fung, and L. A. Valdivia.** 2001. Blockade of tryptophan catabolism prevents spontaneous tolerogenicity of liver allografts. Transplant Proc **33:**129-30.
141. **Miller, J. L., B. J. Dewet, L. Martinez-Pomares, C. M. Radcliffe, R. A. Dwek, P. M. Rudd, and S. Gordon.** 2008. The Mannose Receptor Mediates Dengue Virus Infection of Macrophages. PLoS Pathog **4:**e17.
142. **Mitsiades, N., V. Poulaki, S. Tseleni-Balafouta, D. A. Koutras, and I. Stamenkovic.** 2000. Thyroid Carcinoma Cells Are Resistant to FAS-mediated Apoptosis But Sensitive to Tumor Necrosis Factor-related Apoptosis-inducing Ligand. Cancer Res **60:**4122-4129.
143. **Molano, A., P. A. Illarionov, G. S. Besra, C. Putterman, and S. A. Porcelli.** 2008. Modulation of invariant natural killer T cell cytokine responses by indoleamine 2,3-dioxygenase. Immunol Lett **117:**81-90.
144. **Mole, S., T. Veerapraditsin, M. G. McPhillips, and S. V. Graham.** 2006. Regulation of splicing-associated SR proteins by HPV-16. Biochem Soc Trans **34:**1145-7.
145. **Mongkolsapaya, J., W. Dejnirattisai, X. N. Xu, S. Vasanawathana, N. Tangthawornchaikul, A. Chairunsri, S. Sawasdivorn, T. Duangchinda, T. Dong, S. Rowland-Jones, P. T. Yenchitsomanus, A. McMichael, P. Malasit, and G. Screaton.** 2003. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med **9:**921-7.
146. **Mongkolsapaya, J., T. Duangchinda, W. Dejnirattisai, S. Vasanawathana, P. Avirutnan, A. Jairungsri, N. Khemnu, N. Tangthawornchaikul, P. Chotiyarnwong, K. Sae-Jang, M. Koch, Y. Jones, A. McMichael, X. Xu, P. Malasit, and G. Screaton.** 2006. T cell responses in dengue hemorrhagic fever: are cross-reactive T cells suboptimal? J Immunol **176:**3821-9.
147. **Monleon, I., M. J. Martinez-Lorenzo, L. Monteagudo, P. Lasierra, M. Taules, M. Iturralde, A. Pineiro, L. Larrad, M. A. Alava, J. Naval, and A. Anel.** 2001. Differential secretion of Fas ligand- or APO2 ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells. J Immunol **167:**6736-44.
148. **Morel, J., R. Audo, M. Hahne, and B. Combe.** 2005. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces rheumatoid arthritis synovial fibroblast proliferation through mitogen-activated protein kinases and phosphatidylinositol 3-kinase/Akt. J Biol Chem **280:**15709-18.
149. **Morens, D. M., and S. B. Halstead.** 1987. Disease severity-related antigenic differences in dengue 2 strains detected by dengue 4 monoclonal antibodies. J Med Virol **22:**169-74.
150. **Munn, D. H., E. Shafizadeh, J. T. Attwood, I. Bondarev, A. Pashine, and A. L. Mellor.** 1999. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med **189:**1363-72.

151. **Munn, D. H., M. Zhou, J. T. Attwood, I. Bondarev, S. J. Conway, B. Marshall, C. Brown, and A. L. Mellor.** 1998. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science **281:**1191-3.
152. **Munoz-Jordan, J. L., M. Laurent-Rolle, J. Ashour, L. Martinez-Sobrido, M. Ashok, W. I. Lipkin, and A. Garcia-Sastre.** 2005. Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J Virol **79:**8004-13.
153. **Munoz-Jordan, J. L., G. G. Sanchez-Burgos, M. Laurent-Rolle, and A. Garcia-Sastre.** 2003. Inhibition of interferon signaling by dengue virus. Proc Natl Acad Sci U S A **100:**14333-8.
154. **Murgue, B., O. Cassar, and X. Deparis.** 2001. Plasma concentrations of sVCAM-1 and severity of dengue infections. J Med Virol **65:**97-104.
155. **Musso, T., G. L. Gusella, A. Brooks, D. L. Longo, and L. Varesio.** 1994. Interleukin-4 inhibits indoleamine 2,3-dioxygenase expression in human monocytes. Blood **83:**1408-11.
156. **Nath, P., D. K. Agrawal, and R. M. Mehrotra.** 1982. Ultrastructural changes in skeletal muscles in dengue virus-infected mice. J Pathol **136:**301-5.
157. **Nathan, M. B., and R. Dayal-Drager.** 2006. Recent Epidemiological Trends, the global strategy and public health advances in dengue. WHO TDR/SWG/08.
158. **Navarro-Sanchez, E., P. Despres, and L. Cedillo-Barron.** 2005. Innate immune responses to dengue virus. Arch Med Res **36:**425-35.
159. **Nisalak, A., T. P. Endy, S. Nimmannitya, S. Kalayanarooj, U. Thisayakorn, R. M. Scott, D. S. Burke, C. H. Hoke, B. L. Innis, and D. W. Vaughn.** 2003. Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. Am J Trop Med Hyg **68:**191-202.
160. **Obojes, K., O. Andres, K. S. Kim, W. Daubener, and J. Schneider-Schaulies.** 2005. Indoleamine 2,3-dioxygenase mediates cell type-specific anti-measles virus activity of gamma interferon. J Virol **79:**7768-76.
161. **Oshikawa, K., K. Kuroiwa, K. Tago, H. Iwahana, K. Yanagisawa, S. Ohno, S. I. Tominaga, and Y. Sugiyama.** 2001. Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. Am J Respir Crit Care Med **164:**277-81.
162. **Oshikawa, K., K. Yanagisawa, S. Ohno, S. Tominaga, and Y. Sugiyama.** 2002. Expression of ST2 in helper T lymphocytes of malignant pleural effusions. Am J Respir Crit Care Med **165:**1005-9.
163. **Oshikawa, K., K. Yanagisawa, S. Tominaga, and Y. Sugiyama.** 2002. Expression and function of the ST2 gene in a murine model of allergic airway inflammation. Clin Exp Allergy **32:**1520-6.
164. **Oshikawa, K., K. Yanagisawa, S. Tominaga, and Y. Sugiyama.** 2002. ST2 protein induced by inflammatory stimuli can modulate acute lung inflammation. Biochem Biophys Res Commun **299:**18-24.
165. **Ozden, S., M. Huerre, J. P. Riviere, L. L. Coffey, P. V. Afonso, V. Mouly, J. de Monredon, J. C. Roger, M. El Amrani, J. L. Yvin, M. C. Jaffar, M. P. Frenkiel, M. Sourisseau, O. Schwartz, G. Butler-Browne, P. Despres,**

A. Gessain, and P. E. Ceccaldi. 2007. Human muscle satellite cells as targets of Chikungunya virus infection. PLoS ONE **2:**e527.

166. **Palmer, D. R., P. Sun, C. Celluzzi, J. Bisbing, S. Pang, W. Sun, M. A. Marovich, and T. Burgess.** 2005. Differential effects of dengue virus on infected and bystander dendritic cells. J Virol **79:**2432-9.
167. **Pan, G., J. Ni, Y. F. Wei, G. Yu, R. Gentz, and V. M. Dixit.** 1997. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science **277:**815-8.
168. **Pang, T.** 1983. Delayed-type hypersensitivity: probable role in the pathogenesis of dengue hemorrhagic fever/dengue shock syndrome. Rev Infect Dis **5:**346-52.
169. **Pellegrini, S., J. John, M. Shearer, I. M. Kerr, and G. R. Stark.** 1989. Use of a selectable marker regulated by alpha interferon to obtain mutations in the signaling pathway. Mol Cell Biol **9:**4605-12.
170. **Phuong, C. X., N. T. Nhan, R. Kneen, P. T. Thuy, C. van Thien, N. T. Nga, T. T. Thuy, T. Solomon, K. Stepniewska, and B. Wills.** 2004. Clinical diagnosis and assessment of severity of confirmed dengue infections in Vietnamese children: is the world health organization classification system helpful? Am J Trop Med Hyg **70:**172-9.
171. **Pitti, R. M., S. A. Marsters, S. Ruppert, C. J. Donahue, A. Moore, and A. Ashkenazi.** 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem **271:**12687-90.
172. **Potula, R., L. Poluektova, B. Knipe, J. Chrastil, D. Heilman, H. Dou, O. Takikawa, D. H. Munn, H. E. Gendelman, and Y. Persidsky.** 2005. Inhibition of indoleamine 2,3-dioxygenase (IDO) enhances elimination of virus-infected macrophages in an animal model of HIV-1 encephalitis. Blood **106:**2382-90.
173. **Pryor, M. J., S. M. Rawlinson, R. E. Butcher, C. L. Barton, T. A. Waterhouse, S. G. Vasudevan, P. G. Bardin, P. J. Wright, D. A. Jans, and A. D. Davidson.** 2007. Nuclear localization of dengue virus nonstructural protein 5 through its importin alpha/beta-recognized nuclear localization sequences is integral to viral infection. Traffic **8:**795-807.
174. **Qi, X., L. Sun, W. W. Hauswirth, A. S. Lewin, and J. Guy.** 2007. Use of mitochondrial antioxidant defenses for rescue of cells with a Leber hereditary optic neuropathy-causing mutation. Arch Ophthalmol **125:**268-72.
175. **Raftery, M. J., M. Schwab, S. M. Eibert, Y. Samstag, H. Walczak, and G. Schonrich.** 2001. Targeting the function of mature dendritic cells by human cytomegalovirus: a multilayered viral defense strategy. Immunity **15:**997-1009.
176. **Raghupathy, R., U. C. Chaturvedi, H. Al-Sayer, E. A. Elbishbishi, R. Agarwal, R. Nagar, S. Kapoor, A. Misra, A. Mathur, H. Nusrat, F. Azizieh, M. A. Khan, and A. S. Mustafa.** 1998. Elevated levels of IL-8 in dengue hemorrhagic fever. J Med Virol **56:**280-5.
177. **Rajajee, S., S. Ezhilarasi, and K. Rajarajan.** 2005. Benign acute childhood myositis. Indian J Pediatr **72:**399-400.

178. **Reyes-Del Valle, J., S. Chavez-Salinas, F. Medina, and R. M. Del Angel.** 2005. Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol **79:**4557-67.
179. **Rico-Hesse, R.** 1990. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology **174:**479-93.
180. **Robinson, C. M., K. A. Shirey, and J. M. Carlin.** 2003. Synergistic transcriptional activation of indoleamine dioxygenase by IFN-gamma and tumor necrosis factor-alpha. J Interferon Cytokine Res **23:**413-21.
181. **Roehrig, J. T., R. A. Bolin, and R. G. Kelly.** 1998. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. Virology **246:**317-28.
182. **Sariol, C. A., J. L. Munoz-Jordan, K. Abel, L. C. Rosado, P. Pantoja, L. Giavedoni, I. V. Rodriguez, L. J. White, M. Martinez, T. Arana, and E. N. Kraiselburd.** 2007. Transcriptional activation of interferon-stimulated genes but not of cytokine genes after primary infection of rhesus macaques with dengue virus type 1. Clin Vaccine Immunol **14:**756-66.
183. **Sato, K., S. Hida, H. Takayanagi, T. Yokochi, N. Kayagaki, K. Takeda, H. Yagita, K. Okumura, N. Tanaka, T. Taniguchi, and K. Ogasawara.** 2001. Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta. Eur J Immunol **31:**3138-46.
184. **Sato, K., T. Nakaoka, N. Yamashita, H. Yagita, H. Kawasaki, C. Morimoto, M. Baba, and T. Matsuyama.** 2005. TRAIL-transduced dendritic cells protect mice from acute graft-versus-host disease and leukemia relapse. J Immunol **174:**4025-33.
185. **Secchiero, P., F. Corallini, M. G. di Iasio, A. Gonelli, E. Barbarotto, and G. Zauli.** 2005. TRAIL counteracts the proadhesive activity of inflammatory cytokines in endothelial cells by down-modulating CCL8 and CXCL10 chemokine expression and release. Blood **105:**3413-9.
186. **Secchiero, P., A. Gonelli, E. Carnevale, D. Milani, A. Pandolfi, D. Zella, and G. Zauli.** 2003. TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways. Circulation **107:**2250-6.
187. **Secchiero, P., M. Vaccarezza, A. Gonelli, and G. Zauli.** 2004. TNF-related apoptosis-inducing ligand (TRAIL): a potential candidate for combined treatment of hematological malignancies. Curr Pharm Des **10:**3673-81.
188. **Sedger, L. M., D. M. Shows, R. A. Blanton, J. J. Peschon, R. G. Goodwin, D. Cosman, and S. R. Wiley.** 1999. IFN-gamma mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression. J Immunol **163:**920-6.
189. **Severa, M., M. E. Remoli, E. Giacomini, V. Annibali, V. Gafa, R. Lande, M. Tomai, M. Salvetti, and E. M. Coccia.** 2007. Sensitization to TLR7 agonist in IFN-beta-preactivated dendritic cells. J Immunol **178:**6208-16.
190. **Shaheduzzaman, S., V. Krishnan, A. Petrovic, M. Bittner, P. Meltzer, J. Trent, S. Venkatesan, and S. Zeichner.** 2002. Effects of HIV-1 Nef on cellular gene expression profiles. J Biomed Sci **9:**82-96.

191. **Shi, L., S. L. Zhang, K. Li, Y. Hong, Q. Wang, Y. Li, J. Guo, W. H. Fan, L. Zhang, and J. Cheng.** 2008. NS5ATP9, a gene up-regulated by HCV NS5A protein. Cancer Lett **259:**192-7.
192. **Shimpo, M., D. A. Morrow, E. O. Weinberg, M. S. Sabatine, S. A. Murphy, E. M. Antman, and R. T. Lee.** 2004. Serum levels of the interleukin-1 receptor family member ST2 predict mortality and clinical outcome in acute myocardial infarction. Circulation **109:**2186-90.
193. **Shresta, S., J. L. Kyle, P. Robert Beatty, and E. Harris.** 2004. Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice. Virology **319:**262-73.
194. **Shresta, S., J. L. Kyle, H. M. Snider, M. Basavapatna, P. R. Beatty, and E. Harris.** 2004. Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. J Virol **78:**2701-10.
195. **Shresta, S., K. L. Sharar, D. M. Prigozhin, P. R. Beatty, and E. Harris.** 2006. Murine model for dengue virus-induced lethal disease with increased vascular permeability. J Virol **80:**10208-17.
196. **Shresta, S., K. L. Sharar, D. M. Prigozhin, H. M. Snider, P. R. Beatty, and E. Harris.** 2005. Critical roles for both STAT1-dependent and STAT1-independent pathways in the control of primary dengue virus infection in mice. J Immunol **175:**3946-54.
197. **Siegmund, D., S. Klose, D. Zhou, B. Baumann, C. Roder, H. Kalthoff, H. Wajant, and A. Trauzold.** 2007. Role of caspases in CD95L- and TRAIL-induced non-apoptotic signalling in pancreatic tumour cells. Cell Signal **19:**1172-84.
198. **Simmons, C. P., S. Popper, C. Dolocek, T. N. Chau, M. Griffiths, N. T. Dung, T. H. Long, D. M. Hoang, N. V. Chau, T. T. Thao le, T. T. Hien, D. A. Relman, and J. Farrar.** 2007. Patterns of host genome-wide gene transcript abundance in the peripheral blood of patients with acute dengue hemorrhagic fever. J Infect Dis **195:**1097-107.
199. **Song, J. H., H. J. Kim, C. H. Lee, S. J. Kim, S. Y. Hwang, and T. S. Kim.** 2006. Identification of gene expression signatures for molecular classification in human leukemia cells. Int J Oncol **29:**57-64.
200. **Song, K., Y. Chen, R. Goke, A. Wilmen, C. Seidel, A. Goke, and B. Hilliard.** 2000. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression. J Exp Med **191:**1095-104.
201. **Sosothikul, D., P. Seksarn, S. Pongsewalak, U. Thisyakorn, and J. Lusher.** 2007. Activation of endothelial cells, coagulation and fibrinolysis in children with Dengue virus infection. Thromb Haemost **97:**627-34.
202. **Stadler, K., S. L. Allison, J. Schalich, and F. X. Heinz.** 1997. Proteolytic activation of tick-borne encephalitis virus by furin. J Virol **71:**8475-81.
203. **Steinman, R. M.** 2007. Lasker Basic Medical Research Award. Dendritic cells: versatile controllers of the immune system. Nat Med **13:**1155-9.
204. **Stewart, J. D., T. L. Masi, A. E. Cumming, G. M. Molnar, B. M. Wentworth, K. Sampath, J. M. McPherson, and P. C. Yaeger.** 2003.

Characterization of proliferating human skeletal muscle-derived cells in vitro: differential modulation of myoblast markers by TGF-beta2. J Cell Physiol **196:**70-8.

205. **Strater, J., U. Hinz, H. Walczak, G. Mechtersheimer, K. Koretz, C. Herfarth, P. Moller, and T. Lehnert.** 2002. Expression of TRAIL and TRAIL receptors in colon carcinoma: TRAIL-R1 is an independent prognostic parameter. Clin Cancer Res **8:**3734-40.
206. **Sun, J. X., W. G. Zhang, Y. X. Chen, W. H. Zhao, W. Tian, Y. Yang, and S. H. Liu.** 2007. Indoleamine 2, 3-dioxygenase expression in cells of human acute monocyte leukemia (M(5)) and acute lymphocyte leukemia and therapeutic effect of its inhibitor 1-methyl tryptophan. Zhongguo Shi Yan Xue Ye Xue Za Zhi **15:**478-82.
207. **Sweet, M. J., B. P. Leung, D. Kang, M. Sogaard, K. Schulz, V. Trajkovic, C. C. Campbell, D. Xu, and F. Y. Liew.** 2001. A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. J Immunol **166:**6633-9.
208. **Tajima, S., K. Oshikawa, S. Tominaga, and Y. Sugiyama.** 2003. The increase in serum soluble ST2 protein upon acute exacerbation of idiopathic pulmonary fibrosis. Chest **124:**1206-14.
209. **Takezako, N., M. Hayakawa, H. Hayakawa, S. Aoki, K. Yanagisawa, H. Endo, and S. Tominaga.** 2006. ST2 suppresses IL-6 production via the inhibition of IkappaB degradation induced by the LPS signal in THP-1 cells. Biochem Biophys Res Commun **341:**425-32.
210. **Takikawa, O., R. Yoshida, R. Kido, and O. Hayaishi.** 1986. Tryptophan degradation in mice initiated by indoleamine 2,3-dioxygenase. J Biol Chem **261:**3648-53.
211. **Tassaneetrithep, B., T. H. Burgess, A. Granelli-Piperno, C. Trumpfheller, J. Finke, W. Sun, M. A. Eller, K. Pattanapanyasat, S. Sarasombath, D. L. Birx, R. M. Steinman, S. Schlesinger, and M. A. Marovich.** 2003. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J Exp Med **197:**823-9.
212. **Thebault, P., T. Condamine, M. Heslan, M. Hill, I. Bernard, A. Saoudi, R. Josien, I. Anegon, M. C. Cuturi, and E. Chiffoleau.** 2007. Role of IFNgamma in allograft tolerance mediated by CD4+CD25+ regulatory T cells by induction of IDO in endothelial cells. Am J Transplant **7:**2472-82.
213. **Thorburn, J., L. M. Bender, M. J. Morgan, and A. Thorburn.** 2003. Caspase- and serine protease-dependent apoptosis by the death domain of FADD in normal epithelial cells. Mol Biol Cell **14:**67-77.
214. **Tominaga, S.** 1989. A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. FEBS Lett **258:**301-4.
215. **Tominaga, S., K. Kuroiwa, K. Tago, H. Iwahana, K. Yanagisawa, and N. Komatsu.** 1999. Presence and expression of a novel variant form of ST2 gene product in human leukemic cell line UT-7/GM. Biochem Biophys Res Commun **264:**14-8.

216. **Trajkovic, V., M. J. Sweet, and D. Xu.** 2004. T1/ST2--an IL-1 receptor-like modulator of immune responses. Cytokine Growth Factor Rev **15:**87-95.
217. **Uyttenhove, C., L. Pilotte, I. Theate, V. Stroobant, D. Colau, N. Parmentier, T. Boon, and B. J. Van den Eynde.** 2003. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med **9:**1269-74.
218. **Vassina, E., M. Leverkus, S. Yousefi, L. R. Braathen, H. U. Simon, and D. Simon.** 2005. Increased expression and a potential anti-inflammatory role of TRAIL in atopic dermatitis. J Invest Dermatol **125:**746-52.
219. **Vidalain, P. O., O. Azocar, B. Lamouille, A. Astier, C. Rabourdin-Combe, and C. Servet-Delprat.** 2000. Measles virus induces functional TRAIL production by human dendritic cells. J Virol **74:**556-9.
220. **Wajant, H., J. Gerspach, and K. Pfizenmaier.** 2005. Tumor therapeutics by design: targeting and activation of death receptors. Cytokine Growth Factor Rev **16:**55-76.
221. **Wang, J., A. A. Lobito, F. Shen, F. Hornung, A. Winoto, and M. J. Lenardo.** 2000. Inhibition of Fas-mediated apoptosis by the B cell antigen receptor through c-FLIP. Eur J Immunol **30:**155-63.
222. **Wang, L., R. F. Chen, J. W. Liu, H. R. Yu, H. C. Kuo, and K. D. Yang.** 2007. Implications of dynamic changes among tumor necrosis factor-alpha (TNF-alpha), membrane TNF receptor, and soluble TNF receptor levels in regard to the severity of dengue infection. Am J Trop Med Hyg **77:**297-302.
223. **Wang, M., Y. Liu, S. Liu, and D. Zheng.** 2004. 8-Chloro-adenosine sensitizes a human hepatoma cell line to TRAIL-induced apoptosis by caspase-dependent and -independent pathways. Oncol Rep **12:**193-9.
224. **Warke, R. V., K. J. Martin, K. Giaya, S. K. Shaw, A. L. Rothman, and I. Bosch.** 2008. TRAIL is a novel antiviral protein against dengue virus. J Virol **82:**555-64.
225. **Warke, R. V., K. Xhaja, K. J. Martin, M. F. Fournier, S. K. Shaw, N. Brizuela, N. de Bosch, D. Lapointe, F. A. Ennis, A. L. Rothman, and I. Bosch.** 2003. Dengue virus induces novel changes in gene expression of human umbilical vein endothelial cells. J Virol **77:**11822-32.
226. **Weaver SC, F. I.** 2005. Togaviruses. In:, 10th ed. ed. Hodder Arnold., London.
227. **Weinberg, E. O., M. Shimpo, S. Hurwitz, S. Tominaga, J. L. Rouleau, and R. T. Lee.** 2003. Identification of serum soluble ST2 receptor as a novel heart failure biomarker. Circulation **107:**721-6.
228. **Werenskiold, A. K., S. Hoffmann, and R. Klemenz.** 1989. Induction of a mitogen-responsive gene after expression of the Ha-ras oncogene in NIH 3T3 fibroblasts. Mol Cell Biol **9:**5207-14.
229. **Witayathawornwong, P.** 2005. DHF in infants, late infants and older children: a comparative study. Southeast Asian J Trop Med Public Health **36:**896-900.
230. **Wu, S. J., G. Grouard-Vogel, W. Sun, J. R. Mascola, E. Brachtel, R. Putvatana, M. K. Louder, L. Filgueira, M. A. Marovich, H. K. Wong, A. Blauvelt, G. S. Murphy, M. L. Robb, B. L. Innes, D. L. Birx, C. G. Hayes,**

and S. S. Frankel. 2000. Human skin Langerhans cells are targets of dengue virus infection. Nat Med **6:**816-20.

231. **Xu, D., W. L. Chan, B. P. Leung, F. Huang, R. Wheeler, D. Piedrafita, J. H. Robinson, and F. Y. Liew.** 1998. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. J Exp Med **187:**787-94.
232. **Yamamoto, K., T. Sokabe, N. Ohura, H. Nakatsuka, A. Kamiya, and J. Ando.** 2003. Endogenously released ATP mediates shear stress-induced Ca2+ influx into pulmonary artery endothelial cells. Am J Physiol Heart Circ Physiol **285:**H793-803.
233. **Yamanaka, A., S. Kosugi, and E. Konishi.** 2008. Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. J Virol **82:**927-37.
234. **Yanagisawa, K., Y. Naito, K. Kuroiwa, T. Arai, Y. Furukawa, H. Tomizuka, Y. Miura, T. Kasahara, T. Tetsuka, and S. Tominaga.** 1997. The expression of ST2 gene in helper T cells and the binding of ST2 protein to myeloma-derived RPMI8226 cells. J Biochem **121:**95-103.
235. **Yanagisawa, K., T. Takagi, T. Tsukamoto, T. Tetsuka, and S. Tominaga.** 1993. Presence of a novel primary response gene ST2L, encoding a product highly similar to the interleukin 1 receptor type 1. FEBS Lett **318:**83-7.
236. **Yanagisawa, K., T. Tsukamoto, T. Takagi, and S. Tominaga.** 1992. Murine ST2 gene is a member of the primary response gene family induced by growth factors. FEBS Lett **302:**51-3.
237. **Yoshida, R., Y. Urade, M. Tokuda, and O. Hayaishi.** 1979. Induction of indoleamine 2,3-dioxygenase in mouse lung during virus infection. Proc Natl Acad Sci U S A **76:**4084-6.
238. **Zhang, X., R. M. Cheung, R. Komaki, B. Fang, and J. Y. Chang.** 2005. Radiotherapy sensitization by tumor-specific TRAIL gene targeting improves survival of mice bearing human non-small cell lung cancer. Clin Cancer Res **11:**6657-68.
239. **Zheng, J. K., Y. Wang, A. Karandikar, Q. Wang, H. Gai, A. L. Liu, C. Peng, and H. Z. Sheng.** 2006. Skeletal myogenesis by human embryonic stem cells. Cell Res **16:**713-22.