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**DISTINCT BEHAVIORS OF INFECTED AND BYSTANDER DENDRITIC CELLS
FOLLOWING EXPOSURE TO DENGUE VIRUS**

A DISSERTATION PRESENTED
BY

ZACHARY DAVIS NIGHTINGALE

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

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IN

BIOMEDICAL SCIENCES

SEPTEMBER 17, 2007

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ZACHARY DAVIS NIGHTINGALE

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Here I sit, safe and warm, dry and sated. How short a time it has been since my ancestors were huddled in the lee of some massive monolith, huddled for warmth against raging blizzards or driving rain, surrounded by nightmare dangers I will likely never know. It is the inexorable march of humanity...to explore, and to conquer, and to create from mere thoughts the world that surrounds us. That we fight eternally to survive is natural...that we fight to understand, and to use that understanding to aid others in surviving, is very human. Science and medicine, inexorably tied, are the exemplification of this aspect of humanity, and exist only because our predecessors willed them into existence. I wonder now what my will has wrought.

I sit and think that the well must be drained...close my eyes for a moment and realize how much there is to write, still. To even begin to think of all of the people who've impacted my life over the course of this long, often lonely, journey only serves to remind me of how *not* alone I am. I find that I wonder much less about what I will say, and wonder much more if I will somehow be remiss and omit a name, a contribution, a story. It strikes me as shocking, the time and effort spent on producing a tome of brobdignagian proportions, so rarely to be read by others that it defies all sense of rationality. And then I realize, once again, that it is not so much about the destination, but the journey, and who or what one encounters along the way.

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And lastly, for Cathy Watson, and my Aunt Sue...never stop fighting.

ABSTRACT

Dengue viruses (DV) are re-emerging mosquito-borne pathogens for which four distinct lineages, grouped based on serology and referred to as serotypes 1-4 (D1V-D4V), have been described. Epidemiological data imply that re-infection with a “heterologous” serotype, i.e., one other than that to which the individual was originally exposed, enhances the risk for development of severe disease, dengue hemorrhagic fever (DHF). The hallmark of DHF is a transient capillary leakage syndrome of rapid onset, temporally associated with the resolution of fever and viremia. In its most grave form, the vascular permeability phenomenon in DHF may progress to dengue shock syndrome (DSS), which is often fatal in the absence of appropriate medical care.

Despite the fulminant nature of vascular leakage during DHF/DSS, this phenomenon does not appear to be due to direct cytopathic effects of DV. Rather, inappropriate reactivation and/or regulation of dengue-specific memory are the prevailing theorized (immunopathological) etiologies. Traditional vaccine development techniques have proven insufficient for DV, since any vaccine must offer complete protection against all four serotypes to avoid enhanced pathology on natural viral challenge. Understanding the underlying mechanisms that contribute to dengue disease, particularly the development of dengue-specific memory, is therefore of critical importance.

Dengue immunopathology and the specific aspects of immunological memory that determine disease severity are heatedly debated. Previous research in our lab has suggested that T cell responses contribute to the severity of dengue illness. Clinical data indicate enhanced immune activation in more grave cases of DV infection, and serotype

cross-reactive T cells from multiple individuals are present after both primary and secondary dengue infections. However, little is known about the conditions under which T cells are primed and dengue-specific memory is generated.

Dendritic cells (DCs) are bone marrow-derived cells that play a central role in directing activity within the immune system. DCs shape quantitative and qualitative aspects of adaptive immunity, and therefore the intrinsic characteristics of host memory to a pathogen. DCs are essential in generating primary immune responses, due to their particular effectiveness in stimulating naïve T cells. DCs also play important roles in the reactivation of memory to an infectious agent, and as reservoirs for the dissemination of invading microorganisms. Exposure to pathogens or their products initiates a series of phenotypic and functional changes in DCs, termed maturation. DC maturation involves a coordinated response of immunomodulatory surface molecule elaboration and cytokine production, culminating in antigen presentation to, and co-stimulation of, T cells specific for the invading agent. The DC response is ostensibly tailored to facilitate effective elimination by regulating effective downstream interactions of the DC with T cells.

A number of viruses have evolved to infect DCs and alter their functional behavior, facilitating their own survival within the host, and the herd. DV readily infects DCs both in primary cell cultures and *in vivo*. However, reports on the effects of DV infection on DC maturation vary both with regard to some of the cytokines produced, and the phenotypes of infected versus bystander cells. Although DCs appear to be activated following DV exposure, responses on the single-cell level appear to depend on the infection state of the cell, hypothetically driven by intracellular virus-mediated effects. Therefore, downstream responses to these divergent populations—i.e., actively infected

cells versus uninfected bystander cells—are likely to be the consequence of at least two modes of DC behavior. Because DCs play a pivotal role in adaptive immune development, and because the resulting memory response appears to be critical in affecting disease pathology after heterologous DV re-infection, I sought to explore the phenomena of DC maturation in response to dengue exposure, and to begin to answer the question of how active infection alters the functional capabilities of DCs. Notably, primary dengue infection is generally well-controlled with minimal pathology. Therefore, this thesis addresses the hypothesis that DV infection of DCs results in cellular activation and stimulation of antiviral immunity, despite virus-mediated alteration of DC maturation.

In order to address this hypothesis, I examined both DV infection-dependent and -independent effects on DC functional responses including surface molecule regulation, secretory activity, and CD4 T cell allostimulatory priming. DCs derived from human peripheral blood monocytes were readily infected with multiple strains of DV. DV infection of DCs derived from separate donors was dose-dependent, with substantial variability in DC susceptibility to infection. Exposure to live DV activated surface molecule expression in DCs, similar to the effects of defined maturation stimuli, including a combination of TNF- α and IFN- α , or LPS. In addition, UV-inactivated DV induced expression of cell surface molecules, albeit to a lesser extent than did live virus, demonstrating inherent stimulatory properties of DV particles. Using intracellular staining for DV envelope (E) protein, I detected increased surface molecule expression on both infected DCs and uninfected bystander DCs from the same culture, as compared to mock-infected DCs. These data indicate that activation was not prevented in cells

undergoing active viral replication. However, the degree of surface molecule induction depended on the infection state of the cell. Infected DCs had enhanced PD-L2 and MHC II expression relative to uninfected bystander cells, while PD-L1, CD80, CD86, and MHC I expression were suppressed with active infection. Therefore, intracellular DV replication altered the process of cell surface molecule regulation within these cells.

DV infection of DCs also resulted in the secretion of a broad array of cytokines and chemokines. These included the antiviral cytokine IFN- α , inflammatory cytokines TNF- α , IL-6, and IL-1 α , and inflammatory chemokines IP10, MCP-1, MIP-1 α , and RANTES. DV infection did not induce DC production of the IL-12 p70 heterodimer, and secretion of the immunosuppressive cytokine IL-10 was low in most experiments. Similar to the results seen with surface molecule induction, UV inactivation of DV reduced, but did not eliminate, cytokine and chemokine responses. At the single-cell level, TNF- α and IP10 production profiles of infected DCs and uninfected bystander DCs were distinct. DV infection in DCs reduced production of IP10, but stimulated TNF- α , as compared to uninfected bystander cells in the same culture. Blocking experiments demonstrated that IFN- α/β produced by DCs in response to infection actively inhibited viral protein expression and drove IP10, but not TNF- α , production.

DV infection of DCs did not consistently suppress DC stimulation of allogeneic CD4 T cell proliferation. In cases where infection enhanced DC stimulatory function, T cell proliferation was less pronounced than that induced by DCs activated with exogenous TNF- α plus IFN- α . Increasing multiplicity of infection (MOI) of DCs with DV resulted in increasing DC infection rates, but a statistically significant trend at the highest MOIs for decreased T cell alloproliferation, suggesting that direct infection of

DCs reduces their CD4 T cell priming function. MOI-dependent reduction in DC stimulatory function depended on replication-competent virus. Increased MOIs during DV infection of DCs did not cause an elevation in detectable IL-10 in supernatants derived from T-DC co-cultures. In addition, increased DV MOI of DCs was not associated with increased levels of either IL-13 or IFN- γ in supernatants from T-DC co-culture, suggesting that actively infected DC do not skew CD4 T cells towards a specific T_h phenotype. These data demonstrate that DV infection induces functional maturation of DCs that is modified by the presence of virus through both IFN-dependent and independent mechanisms. However, the allostimulatory phenotype of DCs was not universally enhanced, nor was it skewed towards antiviral (T_h1)-type responses.

These data suggest a model whereby dengue infection during primary illness results in controlled immune stimulation through activation of bystander DCs, and the generation of mixed T_h-type responses. Direct DV infection of DCs appears to attenuate activation of, and potentially clearance by, antiviral mechanisms. During secondary infection, reduced IP10 production and enhanced TNF- α secretion by infected cells, coupled with MHC I downregulation and enhanced PD-L2 expression, would subvert both T_h1 CD4 T cell recruitment and result in CD8 T cell suppression and death. Furthermore, DV-specific effects on DCs would allow for continued viral replication in the absence of effective clearance. These DV-mediated effects would modify T cell memory responses to infected DC, and potentially facilitate the expansion of pathologic T cell subsets. Contributing to this pathological cascade, antibody-dependent enhancement of infection in monocytic cells and macrophages would shift antigen presentation and cytokine production paradigms, increasing the risk of DHF.

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LIST OF ABBREVIATIONS

ADE	antibody-dependent enhancement
AIDS	acquired immunodeficiency syndrome
APC	antigen-presenting cell
C	capsid
CEF	chick embryonal fibroblast
CCR7	CC-chemokine receptor-7
DC	dendritic cell
DC-LAMP	dendritic cell-lysosome associated membrane protein
DC-SIGN	dendritic cell-specific ICAM-3-grabbing non-integrin
DHF	dengue hemorrhagic fever
DIC	disseminated intravascular coagulation
DSS	dengue shock syndron
D1V - D4V	dengue virus serotypes
E	envelope
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
F _c	constant fragment
GP	glycoprotein
HBV	hepatitis B virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTNV	hantaan virus
HUVEC	human umbilical vein endothelial cell

ICAM-3	intracellular adhesion molecule
iDC	immature monocyte-derived DC
IFN	interferon
IFNAR2	interferon-alpha receptor subunit 2
IL	interleukin
InfV	influenza virus
IP10	interferon gamma inducible protein-10 (CXCL10)
IRF	interferon regulatory factor
ISRE	interferon-stimulated response element
JEV	Japanese encephalitis virus
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
L-SIGN	liver/lymph node-specific ICAM-3-grabbing non-integrin
M	membrane
MAPK	mitogen-activated protein kinase
MV	measles virus
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MOI	multiplicity of infection
MV	measles virus
NGC	New Guinea C (strain)
NO	nitric oxide
NS	non-structural
NDV	Newcastle disease virus
OAS	2'-5' oligoadenylate synthetase
ORF	open reading frame

PAWB	PBS-azide wash buffer
PBMC	peripheral blood mononuclear cells
PD (-L)	programmed death (-ligand)
prM	pre-membrane
RANTES	regulated on activation, normal T-cell expressed and secreted
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SEB	superantigen staphylococcus enterotoxin B
SI	stimulation index
SNP	single nucleotide polymorphism
sTNFR	soluble TNF- α receptor
TBE	tick-borne encephalitis
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TSST	toxic shock syndrome toxin
VDR	vitamin D receptor
YFV	Yellow fever virus
WHO	World Health Organization
WNV	West Nile virus

CHAPTER I

INTRODUCTION

■ AN OVERVIEW OF VACCINATION AND EMERGING VIRAL DISEASE

The past two centuries have seen remarkable changes in Western medicine; perhaps most notably in the way we combat infectious disease. Just before the turn of the 18th century, Edward Jenner's scientific approach to vaccination against smallpox provided a documented means to combat *variola major*, without the use of variolation—inoculation with the less virulent form of smallpox, *variola minor* (Jenner, 1798). Word of the success of vaccination swept Europe, and the procedure became increasingly customary, rapidly gaining in public acceptance. By the close of the 1800s, advances in public and personal hygiene, combined with the work of the pillars of the scientific community (particularly Ehrlich, Pasteur, Koch, and von Behring), heralded great advances in the ability of humankind to combat infectious agents. However, with the notable exceptions of the viruses rabies and smallpox, vaccines were essentially restricted to controlling bacterial disease (reviewed by Hilleman, 1998).

A few short decades later the H1N1 influenza pandemic of 1918-1920 left in its wake an estimated death toll of 50 million human lives. The panic and devastation that influenza caused in both civilian and military populations arguably ended the First World War and eventually led to a massive overhaul of medical institutions in the U.S (Barry, 2004). Federal intervention in U.S. medical practice included wholesale restructuring (and often elimination) of the vast majority of medical schools, and instigated widespread rethinking of medical education, including far more rigorous selection and training of

candidate physicians (Barry, 2004). The resulting changes facilitated the rise of modern medical science in the United States—specifically, medical practice based on scientific principles, as espoused in Europe (Barry, 2004).

After the end of World War I, viral vaccine technological development accelerated. Sterile culture methods allowed Theiler to generate a remarkably safe and effective vaccine against yellow fever virus (YFV), marking yet another pivotal move in medicine's chess match against viral disease. By the late 1950s, inactivated vaccines included those for Japanese encephalitis, influenza, poliomyelitis, and adenoviruses. In particular, Enders's work with poliomyelitis paved the way for the generation of live attenuated vaccines, which would soon include those for measles (MV), mumps, and rubella in the late 1960s (Hilleman, 1998). By the close of the 20th century, an improved understanding of genetics accompanied by the generation of recombinant viral proteins in cultured vectors (in this case, yeast) provided the means to mass-produce a vaccine against the hepatitis B virus (HBV) in 1984 (McAleer et al., 1984).

Arguably, the halcyon moment of these advances was to come as a return to the starting point. More than two hundred years in the making, and perhaps the single greatest accomplishment in medical history, was the elimination from natural circulation of humankind's deadliest and most feared foe, smallpox (*variola major* and *minor*). The last documented natural infection with smallpox occurred in Somalia, on the 26th of October in 1977. Shortly thereafter (in 1980), culminating an aggressive decades-long vaccination strategy on a global scale, the World Health Organization (WHO) officially declared this ancient scourge—which may have been responsible for the deaths of Egyptian pharaohs and the fall of the Aztec empire in Mexico—eradicated (WHO, 1980).

However, despite many great successes, the flow of new vaccines against viral disease was drying up as the 20th century neared its close.

It wasn't long before the general sense of urgency was joined with the funding needed to rekindle vaccination studies. On June 5, 1981, principal author Michael Gottlieb and collaborators at UCLA and Cedars-Mt. Sinai reported five cases of a typically rare opportunistic infection with the parasite *Pneumocystis carinii*, in otherwise healthy young men (Gottlieb et al., 1981). Today, the virus responsible is known as human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome, AIDS. HIV and its associated constellation of opportunistic and neoplastic diseases became a source of intense public and media focus, and arguably helped drive the burgeoning research and healthcare establishment in the late 20th century.

Lost in the furor over HIV's devastating global impact is the fact that this virus is clearly not alone as an emerging infectious disease with major implications for public health. Also in 1981, another epidemic was occurring in Cuba. In the most severe cases, patients experienced abnormal bleeding, hepatomegaly, and a fulminant capillary leakage syndrome resulting in circulatory failure, shock and sometimes death (Guzman et al., 1990). The causative agent was dengue virus (DV), a small RNA virus closely related to the virus that causes yellow fever. This and previous dengue epidemics, particularly in Southeast Asia, represented a cautionary message to the world: a viral threat, largely ignored, was in the midst of a dramatic resurgence. Unlike yellow fever, dengue had not been so amenable to vaccine development, despite more than five decades of scientific

progress. Today, several vaccines are in development but none is yet commercially available, although this may change given growing public demand.

■ A HISTORY OF DENGUE ILLNESS

Putative dengue disease had been described in the Americas as early as the late 1700s. In 1780, Philadelphia physician Benjamin Rush reported treating patients who suffered from a dengue-like illness in his home city (Rush, 1789). Because of profound differences in the understanding of disease etiology at that time, a definitive clinical history of the emergence of DV is limited and largely speculative. However, it has been suggested based on an analysis of nucleotide substitution rates in multiple dengue viruses that the most likely timeframe for dengue viral emergence from sylvatic (i.e., with monkeys being the primate host) to sustained human transmission occurred between 280 and 380 years ago, with accelerated genetic divergence appearing in the last century (Twiddy et al., 2003).

During World War II, Japanese scientists first isolated DV (Kimura, 1944), and American scientists confirmed their findings just a few years later. Interest in dengue as a disease had been piqued by the toll it exacted on both American and Japanese troops during the war in the Pacific theater. By 1952, American scientists had isolated and described another distinct dengue virus (or serotype, as this distinction is based on serology) and were gaining an understanding of the relation of DV serology to disease (Sabin, 1952). Today, it is understood that there are four dengue virus serotypes (D1V-D4V), which may circulate simultaneously within a given region, a state termed “hyperendemicity.” The interrelated roles that antigenic variability and co-circulation of

multiple DV serotypes play in disease will be discussed in greater detail in coming sections.

In the years following World War II and up until the present day, major dengue outbreaks have occurred in tropical regions around the globe and appear to be doing so with increasing frequency and severity. Today, the global incidence of dengue infection is estimated at over 50 million cases per year (reviewed by Gubler, 2002), with the WHO reporting 200 thousand to 500 thousand cases of severe disease, dengue hemorrhagic fever (DHF) (WHO, 2002).

■ ECOLOGY OF DENGUE VIRUSES AND THEIR VECTORS

DVs are members of the genus *Flavivirus*, in the family of viruses *Flaviviridae*. Yellow fever virus is the prototype, and the agent from which the nomenclature is derived (*flavus* designating yellow in Latin). The flavivirus genus contains over 70 viruses, of which several are the cause of emergent or re-emergent diseases. Phylogenetic analyses clearly indicate that flaviviruses that belong to the mosquito-borne clades represent a distinct evolutionary lineage from the other vector (tick)-borne flaviviruses (Kuno et al., 1998; Billoir et al., 2000). More than half of the flaviviruses are transmitted by mosquito vectors, as is the case for YFV, DV, and two additional important human pathogens such as Japanese encephalitis virus (JEV) and West Nile virus (WNV) (reviewed by Mackenzie et al., 2004). *Aedes aegypti* and *Aedes albopictus* mosquitoes are the primary vectors for dengue transmission; the former also transmits YFV, particularly in Africa. In the life cycle of both viruses primates are the vertebrate host, although unlike YFV,

DV does not require a sylvatic reservoir, a characteristic that may have evolved in concert with the feeding habits of its major vector, *Aedes aegypti*.

Aedes aegypti is a “domesticated” species, having adapted efficiently to thrive in areas affected by human habitation, where it reproduces in standing water collected in old tires, barrels, and similar refuse, or in stagnant pools in cleared areas. *Aedes aegypti* is active throughout the day, and thus adapted to human diurnal behavior. Commonly found in urban environments, mosquitoes of this species are fastidious feeders, and a single mosquito with access to multiple individuals is likely to bite often, and repeatedly, especially when disturbed (reviewed by Gubler, 1998). This particular characteristic may be exaggerated by a host of neurological effects that DV infection has on the mosquito, increasing the time needed for feeding and hypothetically increasing the chance for interruption and movement to another host (Platt et al., 1997).

During the first half of the 20th century in the Americas, efforts to reduce yellow fever through mosquito control, which included spraying and the elimination of reservoirs for standing water, were effective in limiting the range of endemic dengue (Gubler, 1998). However, relaxation of these control measures has resulted in re-establishment of endemic dengue in many historically affected areas. International travel has exacerbated the problem by introducing variant—and potentially more virulent—strains into new environs (Gubler, 1998). In effect, human mobility combined with lax vector control, reduced public education, and significant genetic diversity among DVs all appear to be contributing to the broadening range and apparent increase in severity of dengue disease.

■ THE BIOLOGY OF DENGUE VIRUSES

Flaviviruses are single-stranded RNA viruses of (+) polarity, with an approximately 11-kb genome surrounded by capsid (C) protein. The viral capsid is enclosed in a lipid envelope that is derived from host cellular membranes and contains the two other dengue structural proteins, membrane (M) and envelope (E). The structural proteins are encoded at the 5' end of the dengue genome, in the order C, pre-membrane (prM), and E. The E protein mediates binding to the host cell membrane, at which point the virus is internalized through receptor-mediated endocytosis. The seven non-structural proteins follow, in sequence order, 5' to 3': NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. 5' and 3' untranslated regions flank the coding portion of the genome, which is translated from a single open reading frame (ORF) to yield a single polypeptide (reviewed by Lindenbach, 2001).

A number of molecules have been proposed as the host receptors for dengue virus, including heat shock proteins (Reyes-Del Valle et al., 2005), CD14-associated molecules (Chen et al., 1999) and glycosaminoglycans such as heparan sulfate (Chen et al., 1997), which may be involved in concentrating the virus at the host cell surface.

Several groups have also demonstrated that the C-type lectin, dendritic cell (DC)-specific intracellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN), mediates infection of dendritic cells, which naturally express DC-SIGN at high levels prior to activation (Tassaneetrithep et al., 2003; Lozach et al., 2005; Kwan et al., 2005).

Immortalized cell lines that are normally inefficiently infected by DV become highly susceptible to infection following ectopic expression of DC-SIGN or its homologue, liver/lymph node-specific ICAM-3-grabbing non-integrin (L-SIGN). L-SIGN is expressed on specialized endothelial cells in the liver and lymph nodes, suggesting that

high-level dengue infection may target multiple tissues *in vivo*. An antibody that cross-reacts with both DC-SIGN and L-SIGN blocks infection in either ectopic expression system; this suggests that infection requires binding to a shared epitope on the two molecules (Tassaneeritthep et al., 2003). Surprisingly, in two reports the internalization sequence of DC-SIGN was dispensable for dengue infectivity, suggesting that additional molecules are involved in virus internalization (Lozach et al., 2005; Tassaneeritthep et al., 2003). The broader context of these findings will be discussed later in this Introduction (see “Dengue virus infection of dendritic cells”).

Studies with the flavivirus tick-borne encephalitis (TBE) virus show that the E protein undergoes a conformational change upon endosomal acidification, which induces an irreversible change from a packed dimeric structure to a more open trimeric structure (reviewed by Mukhopadhyay et al., 2005) and reveals the active site for membrane fusion (Allison et al., 2001). The nucleocapsid is released into the cytoplasm where dissociation of the viral RNA permits the initiation of translation. Translation occurs at the surface of the endoplasmic reticulum (ER), with cleavage of the polypeptide mediated by host signalases and the viral serine protease. The capsid protein mediates translocation of prM into the lumen of the ER, which in turn mediates similar translocation of the E protein. Following multiple cleavage events, the prM and E proteins form stable heterodimers in the ER lumen, while C remains associated with the ER at the cytoplasmic surface. The RNA genome is then packaged and the immature virion moves through the trans-Golgi system, undergoing pH-dependent structural rearrangement. This change appears to allow for the cleavage of a peptide fragment from prM by host furin, which facilitates switching from a spiked particle surface composed of prM-E heterodimers to a smooth particle surface comprised of E homodimers, with the mature M protein essentially

obscured. At this point, the virus is infectious, and is exocytosed from the cell (Mukhopadhyay et al., 2005).

During the process of infection, the multiple dengue non-structural proteins serve a variety of both indispensable and accessory functions, many of which researchers are discovering and defining. NS1 has been implicated in cell signaling through a glycerophosphatidylinositol (GPI)-linked membrane-bound form which, when expressed in HeLa cells, induced protein phosphorylation following NS1-specific antibody treatment (Jacobs et al., 2000). NS1-driven signaling also occurs through interaction with signal transducer and activator of transcription (STAT)-3 β , resulting in the production of the inflammatory cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-6 following transfection into murine DCs (Chua et al., 2005). NS2B is a cofactor required for proper function of NS3, which has multiple functions including serine protease activity, nucleoside triphosphatase and helicase functions required for viral RNA synthesis, and 5'-triphosphatase activity involved in RNA capping (reviewed by Clyde et al., 2006). NS5 serves as the viral RNA-dependent RNA polymerase and has methyltransferase function (Clyde et al., 2006). NS5 has also been reported to activate gene transcription resulting in the secretion of the chemokine IL-8 (Medin et al., 2005). IL-8 has been demonstrated to interfere with antiviral IFN- α function by inhibiting 2'-5' oligoadenylate synthetase (OAS) activity (Khabar et al., 1997).

It has been demonstrated that the non-structural proteins NS2A, NS4A, and NS4B directly suppress the effects of interferon (IFN)-mediated signaling. Expression of these proteins from plasmids transfected into chick embryonal fibroblast (CEF) and human lung epithelial carcinoma A549 cell lines facilitated the replication of IFN-sensitive

Newcastle disease virus (NDV) (Munoz-Jordan et al., 2003). In the same study (using 293T cells), NS2A, NS4A, and NS4B inhibited activity from an IFN-stimulated response element (ISRE)-54 reporter construct in response to IFN- β stimulation, but not Sendai virus infection. This indicates that the noted effect was secondary to inhibition of IFN- β signaling, as opposed to effects on interferon regulatory factor (IRF)-3; IRF-3 is critical in initiating IFN responses to viral infection by itself eliciting IFN- β production (Sato et al., 2000; Fitzgerald et al., 2003).

The effects of the aforementioned dengue NS proteins were synergistic, but NS4B by itself was sufficient to inhibit STAT-1 phosphorylation and nuclear localization in response to either exogenous IFN- β or IFN- γ (Munoz-Jordan et al., 2003). Additional studies implicated amino acids 77-125 as the NS4B inhibitory active site, with a separate region responsible for ER localization required for IFN-inhibitory function (Munoz-Jordan et al., 2005). As previously noted, dengue genome replication occurs at the ER surface, suggesting a potential role for NS4B in shielding the viral RNA from intracellular sensory mechanisms during replication. IFN inhibition appears to be of critical importance for dengue survival and replication in the host since IFN- α is readily detected in the plasma of children with dengue infection (Kurane et al., 1993), and a murine model of primary dengue infection demonstrated a requirement for the combination of IFN- α and γ to clear the virus (Johnson and Roehrig, 1999).

■ CLINICAL PRESENTATION OF DENGUE INFECTION

Dengue illness is manifested in a spectrum of symptoms and disease severity. Many dengue infections are mild, either asymptomatic or presenting as an undifferentiated

febrile illness. Undifferentiated dengue fever (DF) is particularly common in infants and very young children, who may also exhibit a maculopapular rash. Such cases represent most infections in endemic regions, with two separate studies in Thailand reporting from 50% (Endy et al., 2002) to 90% (Burke et al., 1988) of infections as being sub-clinical. In older children and adults dengue disease may manifest with more severe symptoms, or “classical” dengue fever. The classical presentation is characterized by high, sometimes biphasic fever, accompanied by constitutional symptoms that include retro-orbital pain and headache, and the severe muscle pain (myalgia) for which dengue garnered the moniker “break-bone fever” (Rigau-Perez, 1998). Anorexia, vomiting, and abdominal pain or tenderness are not uncommon. There may also be signs of mild coagulation dysfunction, with mucosal injection, micro hemorrhages and petechiae, and occasionally occult GI bleeding, although the latter is rare. Patients suffering from DF typically display leucopenia and may also present with thrombocytopenia. Recovery from DF is generally complete with few lasting sequelae although fatigue and depression may persist for weeks to months after the resolution of fever (WHO, 1997).

The most severe cases of dengue infection are termed dengue hemorrhagic fever (DHF). DHF tends to be seen most often in young children and infants, but is not limited to this age range (WHO, 1997). Beyond high fever ($>39^{\circ}\text{C}$) and constitutional symptoms, the hallmarks of DHF are increased vascular permeability, hemorrhagic phenomena, and hepatomegaly. The clinical case definition of DHF as described by the World Health Organization includes the presence of all of the following: high fever, bleeding tendency (including but not limited to a positive tourniquet test, petechiae and signs of micro hemorrhage, mucosal bleeding, hematemesis and/or melena), severe

thrombocytopenia ($<100,000/\text{mm}^3$), and evidence of plasma leakage, including elevated hematocrit or signs of pleural effusion (WHO, 1997).

The clinical severity of DHF is graded according to WHO guidelines, with grade 1 being defined by the minimal criteria, where the only evidence of hemorrhage is a positive tourniquet test or bruising tendency. Grade 2 includes the additional criterion of spontaneous bleeding. DHF grades 3 and 4 are cases where dengue shock syndrome (DSS) is present. DSS is diagnosed when the patient presents with signs of circulatory failure due to functional hypovolemia. Such signs range from a weak, rapid pulse and cold, clammy skin accompanied by restlessness (grade 3) to profound shock, with undetectable pulse and blood pressure (grade 4). DSS is a critical condition, often being fatal if untreated, as poor organ perfusion leads to multi organ system failure. However, the plasma leakage syndrome, which is roughly concurrent with defervescence, is rapid and transient. Appropriate medical care, particularly fluid resuscitation and monitoring for occult bleeding, results in full recovery from DHF (Dung et al., 1999).

■ IMMUNOPATHOLOGY OF SEVERE DENGUE ILLNESS

The etiology of enhanced vascular permeability that is seen during some cases of dengue infection is ill-defined, although there has been considerable research done in identifying risk factors for the development of DHF/DSS. The most prominent of these is heterologous secondary⁴ infection, that is, infection with a dengue serotype other than the one to which the individual was first exposed (Nisalak et al., 2003; Guzman et al., 1990; Burke et al., 1988). It is generally believed that heterologous re-infection activates inappropriate humoral and cellular immune responses, resulting in disease through failed

immune regulation and inappropriate cytokine cascades, as opposed to direct viral cytopathic effects. The dysregulation of secondary immune responses appears to involve multiple immune system components of diverse nature, with contributions from factors intrinsic to both host and virus. The following sections will discuss these issues, and introduce the roles that host and viral factors play in target cell infection.

As previously noted, the majority of DHF cases are associated with heterologous dengue re-infection. In some instances DHF has been noted in infants experiencing primary infections. It has been hypothesized that reduction in the levels of passively transferred maternal antibody results in infants becoming susceptible specifically when antibody levels become sub-neutralizing and may in fact enhance infection (Kliks et al., 1988). A study from Bangkok, Thailand reported that the serotypes most commonly isolated during primary infection in infants (D2V and D3V) were the same serotypes most commonly seen in secondary infection in older children, suggesting common pathologic mechanisms (Nisalak et al., 2003). Cross-reactive sub-neutralizing antibodies are responsible for the *in vitro* phenomenon of antibody-dependent enhancement (ADE), where antibody-coated virus enters phagocytic cells, especially monocytes and macrophages, via F_c-gamma receptors (F_cγR). FcγR on the cell surface recognize the constant fragment (F_c) of virus-coating IgG antibody, and mediate uptake of infectious immune complexes (reviewed by Tirado and Yoon, 2003).

ADE has been demonstrated *in vitro* for several viruses, including DV and HIV (Littau et al., 1990; Morens and Halstead, 1990; Jolly and Weiss, 2000). This process not only increases cellular infection, it can also induce distinct signaling patterns in infected cells when compared to naked virus, including IL-10 production and suppression

of antiviral responses (Mahalingam and Lidbury, 2002). Further evidence for the role of an *in vivo* ADE-type phenomenon in severe dengue disease is supported by the observation of a protective effect on the risk of developing DHF for a histidine to arginine mutation at position 133 in the FcγRIIA allele (Loke et al., 2001). The arginine mutation was shown to be responsible for reducing uptake of IgG1 but not IgG2b when compared with the common histidine variant (Clark et al., 1991).

Mathematical modeling suggests that evolutionary pressure on dengue viruses confers a selective advantage on strains that develop the capacity for ADE, thus increasing their virulence (Cummings et al., 2005). However, that model also predicted that as multiple serotypes developed greater ADE capability, the system would become destabilized and chaotic. There would be large oscillations in the overall incidence of infection, risking viral extinction through exhaustion of secondary infection-susceptible hosts. Despite the aforementioned findings, there is some dispute regarding the role that pre-existing cross-reactive antibodies play in determining the severity of dengue illness. Studies performed on clinical samples to correlate levels of enhancing-antibody with an outcome of increased disease severity have not proven definitive, with both supporting (Kliks et al., 1989) and refuting (Laoprasopwattana et al., 2005) evidence in the literature.

An *in vivo* ADE-type phenomenon is not the only theory to espouse a link between humoral immune responses and severe dengue disease. During acute dengue disease, the viral NS1 protein has been measured in plasma at high levels in a soluble form that may also be involved in dengue pathogenesis (Young et al., 2000; Libraty et al., 2002). In one study, polyclonal antibodies generated against dengue NS1 in mice recognized epitopes on human targets, particularly clotting factors, platelets, and

endothelial cells (Falconar, 1997). Monoclonal antibodies developed in the same study reacted with multiple human proteins, including ICAM-1, fibrinogen, factors VII and IX, and prothrombin. Work performed by a separate group found that binding of antibody specific for NS1 to endothelial cells induced caspase-dependent death; this effect was enhanced in the presence of complement (Lin et al., 2003). Anti-NS1 antibodies may also induce endothelial cell nitric oxide (NO) production, resulting in apoptosis (Lin et al., 2002) and potentially blood vessel dilatation. Thus, dengue NS1 appears to contribute both to vascular leakage and platelet and coagulation factor consumption, risking disseminated intravascular coagulation (DIC), hemorrhage, and shock.

Humoral immunity represents only one of the two arms of adaptive immune responses. It is also theorized that cell-mediated immunity contributes to the immunopathological mechanisms in DHF (Rothman and Ennis, 1999). Early studies found evidence of increased T cell activation during dengue disease, and suggested that these effects were exacerbated in cases with greater pathology (Kurane et al., 1991; Green et al., 1999a). Subsequent work has identified the presence of serotype cross-reactive T cells following both primary (Green et al., 1997; Kurane et al., 1998; Bashyam et al., 2006) and secondary infections (Mathew et al., 1998; Mongkolsapaya et al., 2003). MHC Class I HLA polymorphisms have also been correlated with disease risk as well, further suggesting that certain T cell responses may be either protective or pathogenic, and may have a strong host genetic factor (Loke et al., 2001).

Work specifically addressing the role of CD8 T cells in secondary dengue infection reported expansion of CD8 T cells with higher affinity for epitopes from heterologous serotypes, rather than the subsequent infecting serotype, an effect termed

“original antigenic sin” (Mongkolsapaya et al., 2003). That same study also reported that the CD8 T cells responding during acute secondary infection were apoptotic. Separate studies on whole peripheral blood mononuclear cells (PBMC) obtained from individuals following experimental dengue vaccination found that CD8 T cells were responsive to peptide epitopes from the homologous strain as well as variant peptides derived from heterologous strains (Bashyam et al., 2006). In that study, there were also clear differences between vaccinated donors regarding quantitative and qualitative aspects of the dengue-specific CD8 T cell memory pool. Cytokine production profiles varied both between donors for a given epitope and within a given donor for variant epitopes, revealing donor- and epitope-dependent variation in function of dengue-specific CD8 T cells following reactivation.

Beyond CD8 T cell responses, CD4 T cells have also been examined in regards to the pathogenesis of dengue infection. In a prospective study of dengue illness in Thai schoolchildren, dengue antigen stimulation of pre-secondary illness PBMC demonstrated poor responses against the secondary infecting serotype (D3V). However, there was a broad serotype-cross-reactive IFN- γ secretory response, particularly in non-hospitalized subjects, while TNF- α secretory responses were found only in subjects who were hospitalized during secondary infection (Mangada et al., 2002). Further studies using intracellular cytokine staining of PBMC from dengue-vaccinated individuals found a trend for elevated TNF- α^{\dagger} /IFN- γ^{\dagger} CD4 T cell ratios following heterologous antigen stimulation, again suggesting a role for TNF- α in pathologic immune reactivation (Mangada and Rothman, 2005).

These results demonstrate that functionally diverse T cell repertoires are present following infection with DV, and that specific T cell subsets as well as some functions of the cells comprising these subsets may be associated with immunopathology or immunoprotection. When reviewing these studies, interesting questions emerge regarding the priming history of the cells being studied. Elucidation of the potential range of functional activities (including secretion of IL-10, IL-13, or granulocyte-macrophage colony stimulating factor (GM-CSF), which all affect APC function and/or T cell function) in these cells may reveal additional insight into the mechanisms of severe disease. The qualitative and quantitative aspects of T cell priming induced by APCs that have been exposed to or infected with DV is thus an important avenue of investigation.

One consistent theme of the multiple hypotheses regarding DHF pathogenesis is the influence of soluble mediator cascades. A number of studies with clinical samples have identified cytokines and chemokines associated with increased risk of severe disease. Several studies have demonstrated that elevated plasma levels of IL-10, which has immunosuppressive properties, are independently correlated with risk of severe disease or thrombocytopenia (Perez et al., 2004; Green et al., 1999b; Libraty et al., 2002). Elevated levels of plasma IL-10 also correlate with poor disease outcome in Ebola virus infection of humans, which results in a fulminant hemorrhagic fever associated with high mortality (Baize et al., 2002; Villinger et al., 1999). These findings suggest that induction of immunosuppression may enhance virulence for some RNA viruses, although it may alternatively be postulated that elevated IL-10 production associated with severe disease is a function of enhanced counter-regulatory mechanisms induced following uncontrolled initial activation. Interestingly, IFN- γ concentrations in plasma do not correlate with

disease severity in dengue infection—in agreement with findings that suggest a protective role for this cytokine in dengue illness (Mangada et al., 2002). Because of the antagonistic role that IL-10 plays in IFN- γ signaling, these findings suggest that elevated IL-10 may exert pathogenic effects by blocking protective IFN- γ activity early in illness when antiviral activity may be important in suppressing viral load and immune activation.

Another cytokine associated with severe dengue disease is TNF- α . Serum levels of TNF- α were found to be elevated in the sera of patients with dengue, as compared to controls, and this effect correlated with hemorrhagic manifestations (Azeredo et al., 2001). In a study of children and adults with DHF, the highest serum TNF- α levels were found in patients with DHF grades III and IV (Hober et al., 1993). One study investigating the role of TNF- α in Thai children with dengue found that 80-kilodalton soluble TNF- α receptor (sTNFR80) concentrations in plasma were higher in patients with DHF than with DF or other febrile illnesses and correlated with the pleural effusion index, a measure of capillary leakage. That same study also detected TNF- α in plasma more frequently in cases of DHF than DF (Green et al., 1999a). TNF- α is thought to regulate changes in vascular endothelium, and acute sera from DF patients has been demonstrated to induce microvascular endothelial cell activation that was inhibited by addition of TNF- α blocking antibodies; the same sera induced apoptosis in these cells as well (Cardier et al., 2005).

The chemokines IL-8 and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) have also been investigated in studies of dengue disease severity. In a report from India, serum IL-8 levels were demonstrated to be highest in

patients experiencing DSS, with statistically significant differences in DHF grade IV patients as compared to grades I and II, while IL-8 was not detected in the sera of healthy controls (Raghupathy et al., 1998). A separate study from Indonesia reported similar results, detecting IL-8 most frequently and at the highest median levels in DHF grade IV patients as compared to other DHF grades, patients with DF, or healthy controls. That study also found elevation in plasma elastase levels with severe disease, which the authors proposed might have been due to IL-8-mediated neutrophil activation (Juffrie et al., 2000). Plasma levels of RANTES, on the other hand, were shown to be lower in DHF patients later during the course of disease than were levels in patients with DF, or in healthy controls (Perez et al., 2004).

Taken as a whole, these data suggest that specific cytokine responses (i.e., high-level IL-10, TNF- α , and IL-8 production, or weak RANTES induction) may predispose an individual to development of pathology during dengue infection. This provides further evidence for the importance of immunopathogenesis in dengue illness and indicates that both host- and virus-associated factors that influence cytokine and chemokine cascades may dictate disease outcome.

Epidemiological evidence is compelling for a role of virus-associated factors in determining the severity of dengue illness. The occurrence of DHF in the Western Hemisphere has been specifically linked to the arrival of Asian genotypes of D2V, despite the presence of circulating American D2V genotypes (Rico-Hesse et al., 1997). For example, during an outbreak of American genotype D2V in Iquitos, Peru, in a population previously exposed to D1V, severe dengue illness in the form of DHF/DSS was conspicuously absent (Watts et al., 1999). In a longitudinal study from Bangkok,

Thailand, although multiple serotypes were associated with epidemic dengue, D2V was the most frequent serotype isolated from secondary infection, yet it was the only serotype that did not independently contribute to the presence of DHF during an epidemic (Nisalak et al., 2003). The most severe epidemics (based on incidence) were associated with dengue 3 serotypes, and D3V was the most common isolate from primary DHF (Nisalak et al., 2003). Lastly, the frequency of isolating D1V correlated with the yearly proportion of primary disease cases, suggesting that D1V strains in this region had particular virulence in children who lacked prior dengue priming (Nisalak et al., 2003). Together, these studies highlight the complex role that viral characteristics play in determining disease pathology. *In vitro* studies aimed at determining virulence factors examined regions in the 5' and 3' UTRs of the dengue genome and those resulting in changes in the non-structural genes, which might also be important in determining disease severity (Mangada and Igarashi, 1997; Mangada and Igarashi, 1998).

In dengue research, it is particularly important to combine clinical and epidemiological data with *in vitro* studies because of the prohibitive ethics of studying the priming process *in vivo* in the natural human host. Although the murine system is arguably ideal for studying basic immunological principles, mouse models are inherently problematic when studying dengue infection. Major issues include the drastic gene manipulation, non-physiologic infection routes, and/or specific virus strains required to generate dengue disease in susceptible murine infection models (Johnson and Roehrig, 1999; Shrestha et al., 2006; Kuruvilla et al., 2007). Also, disease pathology in mice does not effectively recapitulate that seen in humans. Fundamental differences in the human and murine immune systems might also be considered prohibitive, particularly given the

species-specific adaptation of dengue to human hosts. Therefore, a great deal of effort has been directed at investigating the effects of DV infection on the responses of human target cells *in vitro*.

■ CELLULAR TARGETS OF INFECTION

Several reports indicate a range of susceptible *in vivo* targets of dengue infection, including leukocytes, endothelial cells, and hepatocytes (Jessie et al., 2004; Rosen et al., 1989; Couvelard et al., 1999). However, these data are far from definitive and rely on small sample numbers, with low detection rates. Obtaining human tissue samples for analysis is difficult since the potential research benefit does not justify the danger to patients inherent in biopsy of internal organs. Therefore, the use of *in vitro* model systems for dengue infection has served as a surrogate for *in vivo* work.

Due to the ease of manipulation and maintenance, researchers have directed a great deal of effort towards the use of immortalized cell lines in the study of DV. The potential involvement of the liver *in vivo* has lead several groups to address infection using the hepatoma cell line HepG2. Dengue infection of HepG2 cells was found to induce apoptosis through NF κ B activation (Marianneau et al., 1997), as well as induction of the chemokines IL-8, RANTES, and macrophage inflammatory protein (MIP)-1 α , and MIP-1 β (Medin et al., 2005), suggesting that hepatocyte infection *in vivo* might be associated with pathology via direct mechanisms as well as damage through the recruitment of cellular immune effectors.

Attempts to understand the vascular permeability phenomenon that occurs during severe dengue infection have led some researchers to explore the role of histamine, which

is secreted by mast cells and basophils and has potent vasodilatory functions. Histamine has been implicated in disease for both human patients (Tuchinda et al., 1977) and in a murine dengue infection model (Chaturvedi et al., 1991). The human mast cell line KU812 could be infected with DV through an ADE-mediated mechanism, releasing inflammatory cytokines IL-1 β and IL-6 (King et al., 2000). In a comparison of ADE of infection of KU812 cells and the monocytic cell line U937, KU812 cells alone induced production of the chemokines RANTES, MIP-1 α and MIP-1 β by 72 hours post-infection (King et al., 2002). Surprisingly, however, neither study implicated DV as a stimulus for mast cell degranulation and histamine release, suggesting that a role for histamine may involve more complex mechanisms than cellular infection alone. One possibility is that in some individuals, DV may stimulate IL-13 production by T cells during priming, with elevation of DV-specific IgE production (and mast cell degranulation on secondary exposure).

As noted previously, *ex vivo* analyses have not provided definitive evidence for lymphocyte infection, although one study implicated B cells specifically as being the predominantly infected cell in PBMC (King et al., 1999). DV infection of both lymphocytes and monocytic cell lines has been accomplished, with the authors reporting the establishment of persistently infected cell lines, indicating the susceptibility of multiple cell lineages to dengue infection, at least *in vitro* in immortalized cells (Kurane et al., 1990). Most notably, monocytic cell lines were of particular interest in early *in vitro* studies given the putative role of ADE in severe dengue etiology. Investigators identified roles for Fc γ RI and Fc γ RII in mediating ADE in infection of U937 and K562 cell lines, respectively (Littau et al., 1990). IFN- γ enhanced this process by increasing

Fc γ R expression (Kontny et al., 1988). Unlike much of the evidence regarding infection of other cell lines, infection of monocytic cells has translated rather consistently into investigations of primary cell culture, although infection rates are generally low.

Similar to research on cell lines, early studies of dengue infection in primary cells focused predominantly on cells of myeloid/monocytic lineage (Morens and Halstead, 1990; Kliks et al., 1989). More recent literature reports of monocytic/macrophage cell infection, including liver sinusoid-resident Kupffer cells, suggest that these cells are activated during infection and produce inflammatory cytokines, although infection of Kupffer cells was not productive (Chen and Wang, 2002; Marianneau et al., 1999). Infection of monocytic cells and macrophages was a prominent topic of study for some time, although recent work has expanded to include other primary cell types as well. DV infection of primary human umbilical vein endothelial cells (HUVEC) results in virion production and multiple inflammatory events, including the secretion of IL-6 and IL-8, and platelet adhesion (Huang et al., 2000; Huang et al., 2002). Despite these findings of primary cell activation following DV exposure, infection rates are low. This is true even for monocytes and macrophages, particularly in the absence of enhancing antibody. DCs, however, are a notable exception to the typically poor susceptibility to infection that is seen with other primary cells.

As previously noted, DV binds to the C-type lectin DC-SIGN, a molecule expressed at high levels specifically on DCs. These findings followed the seminal discovery that DV infects human DCs, both *in vivo* and *in vitro* (Wu et al., 2000). In that study, the authors first reported the infection of blood monocyte-derived DCs following exposure to the prototype D2V strain New Guinea C. Infected DCs failed to induce

dendritic cell-lysosome associated membrane protein (DC-LAMP) compared with uninfected bystander cells, suggesting that dengue infection interferes with normal processes associated with DC activation. DC infection occurred *in situ* in skin explants derived from cadavers, with dengue protein expression in 60-80% of emigrant HLA DR-expressing cells following tissue inoculation. Although the authors also reported Langerhans cell infection, this was proposed based on CD1a expression (a marker also expressed by monocyte-derived DCs), and the emigration of the infected cells following superficial skin inoculation, therefore leaving this conclusion in question. The authors also identified cells co-staining for dengue antigens and the DC marker CD1a in skin biopsies from a dengue-vaccinated donor. By directly demonstrating efficient infection of human skin-derived cells both during acute infection and in tissue explants and correlating these findings with an established *in vitro* model, this study opened an intriguing avenue into investigating the pathogenesis of dengue infection. Definitive evidence for an *in vivo* cell target highly susceptible to infection was identified, and the particular cell type, the DC, was known to be intimately involved in the generation of immune responses. What follows in the remainder of this introduction is the story of DCs—their origins, functions, and role in generating adaptive immunity. The growing body of work on DV infection of DCs, and the results of similar studies with other viruses, will also be addressed.

■ THE BIOLOGY OF DENDRITIC CELLS

DCs are a vital component of the innate immune system and are critical in the detection of and response to microbial challenge. DCs are derived from bone marrow precursors,

and include cells of both plasmacytoid and conventional lineages (reviewed by Villadangos and Schnorrer, 2007). Conventional (i.e., myeloid) DCs migrate via the blood to final sites of residence, where their differentiation is arrested in an “immature,” or pre-activated state. Immature DCs are present in the epidermis (as Langerhans cells), vascularized interstitial tissues of the dermis and solid organs, and mucosal surfaces such as those of the gastrointestinal, and respiratory tracts (Banchereau and Steinman, 1998). Immature DCs constitutively sample their surrounding environment and employ a vast array of intracellular and cell-surface-bound receptors. Many of these receptors act as sensory mechanisms to detect both endogenous and exogenous antigens (Villadangos and Schnorrer, 2007). The range of antigen moieties recognized is strikingly diverse: DCs express receptors for each of the major classes of biomolecules, including proteins, nucleic acids, sugars, and lipids. In many cases, binding of pathogen-derived products to specific receptors on the DC induces signal transduction cascades that culminate in transcriptional, functional, and morphological changes to the DC. A microbial “danger signal” (Matzinger, 1994) thus initiates a program of DC “maturation” (Banchereau and Steinman, 1998).

Functionally, DC maturation includes a transient burst of antigen uptake followed by a switch to enhanced antigen processing and presentation, the secretion of cytokines and chemokines, and homing to secondary lymphoid tissues to stimulate T cell responses (Banchereau and Steinman, 1998; Reis e Sousa, 2006). The maturation process evolves as the DC encounters additional signals, i.e., intracellular viral double-stranded RNA (dsRNA) (Cella et al., 1999), or the binding of CD40 ligand (CD40L) (O'Sullivan and Thomas, 2002), which is expressed on T cells during co-stimulation. Plasticity in DC

activity ostensibly allows for priming of the most appropriate adaptive response for pathogen clearance.

DC co-stimulatory molecule expression influences quantitative aspects of T cell responses to DCs. A major aspect of DC maturation is the regulation of surface molecules involved in DC-T cell interactions. Early studies of DCs indicated that inflammatory mediators such as TNF- α or IFN- α stimulated increased expression of surface molecules that are involved in initiating T cell proliferation (Sallusto and Lanzavecchia, 1994; Luft et al., 1998). Some of the earliest DC-expressed molecules studied included the B7-family co-stimulators CD80 and CD86, which bind both activating and deactivating receptors on responding T cells during T cell-dendritic cell co-regulation. The more recently discovered programmed death-1 ligands (PD-L), PD-L1 and PD-L2, are members of the same family of molecules as CD80 and CD86 (Freeman et al., 2000; Latchman et al., 2001). However, these proteins appear to act as T cell suppressor molecules through binding to programmed death-1 (PD-1) expressed on activated T cells (Cai et al., 2004; Freeman et al., 2000; Latchman et al., 2001). The phenotypic changes DCs underwent after exposure to activating stimuli also demonstrated the induction of CD83, a marker considered specific for mature DCs (Zhou and Tedder, 1995; Zhou and Tedder, 1996). The DC surface-bound TNF-family co-stimulator CD40 binds CD40 ligand (CD40L) expressed on activated T cells, and is crucial for both effective T cell "help" and memory generation and for antibody isotype switching (reviewed by Quezada et al., 2004). DCs present endogenous and/or exogenous peptides in the context of class I and II Major Histocompatibility Complex proteins, which in turn signal to antigen-specific CD8 and CD4 T cells, respectively.

DCs are effective cross-presenters as well, i.e., they are able to load peptides normally destined for MHC II presentation into the context of MHC I (Villadangos and Schnorrer, 2007). Therefore, expression of DC surface molecules serves to initiate and control T cell responses, and differential expression of specific molecules allows DCs to finely tune the responses of antigen-specific T cells.

DC co-stimulatory molecule expression also influences qualitative aspects of T cell responses to DCs. In a study using the experimental allergic encephalitis model in mice, blocking CD80 signaling reduced disease incidence, while blocking CD86 enhanced disease severity (Kuchroo et al., 1995). These effects were related not to the magnitude of T cell responses, but to selective induction of a transferable, protective T_h2 -type response when CD80 was blocked during immunization. Since the co-administration of anti-IL-4 antibody abolished the effect, the authors concluded that blocking CD80 enabled endogenous IL-4 production in responding T cells, facilitating the protective T_h2 response (Kuchroo et al., 1995). *In vitro*, while PD-L1 suppresses both T_h1 and T_h2 -type responses, PD-L2 appears to selectively suppress T_h1 responses only (Latchman et al., 2001). CD40 expression on DCs can aid in T_h1 skewing during interaction with CD40L, through the stimulation of DC IL-12 secretion and induction of T cell IFN- γ production (Cella et al., 1996).

IL-12 represents the most well-defined DC-derived cytokine responsible for the induction of T_h1 -type immune responses (reviewed by Moser and Murphy, 2000). However, human DCs are further able to initiate and direct additional immune responses through the production of a wide variety of cytokines and chemokines (Banchereau and Steinman, 1998). DCs secrete IFN- α , which in humans can direct T_h1 development (i.e.,

IFN- γ production) through phosphorylation of STAT4, which does not occur in mice (Moser and Murphy, 2000). This effect is in addition to the well-described antiviral functions of type I IFNs, the downstream activities of which include mRNA translation inhibition, RNA degradation, RNA editing, nitric oxide (NO) production, and the induction of MHC molecules involved in the presentation of viral antigens to T cells (reviewed by Samuel, 2001). DCs also produce inflammatory cytokines such as TNF- α , IL-1, and IL-6. TNF- α in particular can affect T cell responses through killing of actively proliferating T cells (Zheng et al., 1995; reviewed by Lenardo et al., 1999). IL-10 production by DCs can affect a variety of cells, including the DC itself. IL-10 inhibits cytokine signaling including that by IFNs, regulates T cell proliferation and cytokine release, and facilitates B cell development (Pestka et al., 2004). DCs also produce a vast array of chemokines, a function exemplified by a report examining virus infection of human DCs (Piqueras et al., 2006). In that report, chemokine secretion by DCs occurred in three temporal waves, each associated with recruitment of distinct immune effector populations. Chemokines such as IP10 (CXCL10), ITAC (CXCL11), and Mig (CXCL9) are associated with the recruitment of T_h1 T cells, enhancing T_h1-type responses, while others, such as MDC (macrophage-derived chemokine, CCL22) attract T_h2 cells. The range of secreted products induced following DC activation is incredibly diverse, highlighting the central role of these cells in the stimulation and orchestration of immune responses.

DCs are kinetic beasts. The response of a DC to stimulation, even with a purified, non-living agent, is not a snapshot—the adoption of a fixed functional phenotype—but rather a dynamic continuum of complex signaling. Two factors that modify T cell

responses to DCs are 1) the temporal relationship to stimulus exposure and 2) the antigen dose presented to the T cell in the context of the DC. During co-culture with autologous naïve CD4 T cells and toxic shock syndrome toxin (TSST), T_h1 priming was enhanced by reducing the time after lipopolysaccharide (LPS) stimulation of DCs, increasing the antigen (i.e., TSST) dose, and addition of exogenous IL-12 (Langenkamp et al., 2000). Conversely, in the same study, increasing the time post-stimulation of DCs, low antigen dose, and IL-4 supplementation all enhanced T_h2 priming. The same conditions associated with T_h2 priming also preferentially induced non-polarized CC-chemokine receptor-7 (CCR7)-expressing “central memory” T cells which secreted the T cell growth factor IL-2, but not T_h effector molecules IFN- γ or IL-4, at levels comparable to CCR7 “effector memory” T cells (Langenkamp et al., 2000). Thus, DCs have intrinsic properties for priming T_h and memory phenotypes of naïve CD4 T cells, which depend on multiple facets of DC function at any given point in time.

The strength and duration of the signal that T cells receive during priming are also critical in the development of T cell memory, particularly the T cell’s “fitness.” T cells that are weakly stimulated can proliferate in response to IL-2, but they fail to adopt characteristics that allow them to persist in a homeostatically controlled environment, where survival depends on successfully competing for space and growth factors such as IL-7 and IL-15 (Gett et al., 2003). As a result, when T cells respond to virus-infected DCs, weak stimulation and activation may limit their viability during homeostatic expansion and contraction. Their relative numbers in the memory pool would thus decline, making cell-mediated immunity upon re-exposure to the pathogen less effective.

■ VIRAL INFECTION OF HUMAN DCs: A BRIEF OVERVIEW

The infection of DCs by viruses can be instrumental in viral dissemination (Wang et al., 2007; MacDonald and Johnston, 2000). This infection process is a common phenomenon, having been demonstrated for multiple RNA and DNA viruses responsible for a broad range of diseases. Conventional DCs are targets for a variety of RNA viruses, including measles virus (MV) (Grosjean et al., 1997), respiratory syncytial virus (RSV) (de Graaff et al., 2005), HIV (Granelli-Piperno et al., 1996; Canque et al., 1996), hantaan virus (HTNV) (Raftery et al., 2002), YFV 17D (Barba-Spaeth et al., 2005), and influenza viruses (InfV) (Cella et al., 1999). The observation that many viruses infect DCs may indicate that viruses have evolved a common “targeting” mechanism designed to subvert or exploit DC immune functions. Viruses can thus facilitate their own survival by infecting DCs and altering the interface of innate and adaptive immunity in the process.

Although literature reports often use DC phenotypic changes to denote “maturation,” this connotation does not account for functional activity. Phenotypic changes and cytokine production have been traditionally associated with enhanced DC function during co-culture with T cells. However, these activation events often belie broad diversity in DC APC function that is based on stimulus, time, and the products generated by (and in response to) infectious agents (reviewed by Reis e Sousa, 2006). With regards to infection, diverse function often includes agent-specific modulations that reveal themselves only upon discrimination between infected and uninfected cells or further perturbation of the culture system, such as the addition of T cells. An example of this is measles virus (MV) infection of DCs, where T cell-associated signals such as CD40L enhance viral replication and elicit IL-10 production that is not seen in uninfected

cells (Servet-Delprat et al., 2000). The following sections address the effects of viral infection of DCs, with specific attention to virus-mediated modulation of DC functions.

■ VIRUSES ASSOCIATED WITH *IN VITRO* SUPPRESSIVE RESPONSES

Multiple early studies *in vitro* using MV to infect DCs indicated that proliferative responses of both syngeneic, activated whole T cells and allogeneic resting CD4 T cells to MV-infected DCs were profoundly reduced when compared to responses initiated by uninfected DCs (Fugier-Vivier et al., 1997; Schnorr et al., 1997; Grosjean et al., 1997). These findings contrasted starkly to the observation that DCs infected with measles “matured,” by increasing their surface expression levels of a wide number of immunoregulatory proteins, including co-stimulatory molecules CD40, CD80, and CD86, the DC activation marker CD83, and MHC molecules (Schnorr et al., 1997; Servet-Delprat et al., 2000). It was also demonstrated that enhanced expression of CD80 and CD86 was a function of the autocrine activity of IFN- α/β released in response to MV infection, indicating that the DCs had preserved IFN responsiveness (Dubois et al., 2001).

Determining which mechanisms were involved in suppressing T cell proliferative responses to MV-infected DCs began with the finding that CD40-CD40L interactions—normally associated with facilitating immune responses—triggered both potent DC down-regulation of surface molecule expression and an increase in MV replication (Servet-Delprat et al., 2000; Fugier-Vivier et al., 1997). Massive cell death was observed during T cell-DC co-culture, with apoptosis of the latter cells (but not the former) depending on Fas-mediated signals, which also facilitated virion release through DC lysis (Servet-Delprat et al., 2000). Additional studies suggested that DCs were inducing death

signals themselves through expression of TNF-related apoptosis-inducing ligand (TRAIL), which was found to be sequestered intracellularly (Vidalain et al., 2000). The effects of infection on DC function during co-culture depended on viral replication, since UV-inactivated MV treatment of DCs did not have the profound consequences that infection with live virus did (Fugier-Vivier et al., 1997; Grosjean et al., 1997). Also, the observed effects were not the result of T cell trans-infection, since treatment of infected DCs with paraformaldehyde fixation or UV irradiation to eliminate infectious virus did not restore T cell proliferation, and T cell expression of measles antigen during co-culture was rare (Grosjean et al., 1997; Schnorr et al., 1997; Fugier-Vivier et al., 1997).

MV replication in DCs also appeared to modify intracellular cytokine induction pathways, since CD40-CD40L interactions specifically facilitated IL-10 production in MV-infected DCs, yet did not appear to prevent IL-12 production (Fugier-Vivier et al., 1997; Servet-Delprat et al., 2000; Dubois et al., 2001). IL-12 production was also preserved following stimulation of MV-infected DCs with LPS (Schnorr et al., 1997). However, IL-10 has multiple immunosuppressive activities, as evidenced by the accelerated apoptosis of, and suppressed surface expression of immunostimulatory molecules by, epidermal DCs (Langerhans cells) in response to this cytokine (Ludewig et al., 1995). IL-10 also opposes the signaling induced by IFN- γ and IFN- α (Ito et al., 1999).

The message of these findings is that MV utilizes a number of strategies to prevent or exploit immune responses initiated by DCs after infection. These include the induction of T cell and DC death, the utilization of T-DC interactions (specifically, CD40-CD40L binding) to increase virion production and reduce co-stimulatory molecule

expression, the death of DCs to facilitate virion release, and the induction of IL-10 to inhibit antiviral responses and suppress DC and T cell functions. Expression of the MV glycoprotein itself on the surface of infected DCs may be crucial for some of these observed effects, particularly suppression of T cell proliferation (Dubois et al., 2001). Thus, MV appears to facilitate multiple APC-opposing feedback loops, in which the prototypical antiviral agents IFN- α and IFN- γ are functionally marginalized due to DC IL-10 production, whilst at the same time eliminating substantial MV-specific immunity through the deletion of DCs and T cells. *In vivo*, these T-DC interactions would be dependent on cognate recognition, suggesting that MV is able to ablate MV-specific adaptive immune responses induced by infected DCs. The mechanisms described here are clearly advantageous to the virus for its survival and dissemination, increasing viral replication while likely retarding viral clearance, and permitting sufficient time for transfer to another susceptible host.

Respiratory syncytial viral (RSV) is similar to MV in that they are both typically diseases of childhood, yet they differ significantly in terms of the generation of immunity. While MV results in transient profound immunosuppression followed by lasting immunity, RSV appears to have particularly poor immunogenicity. In the absence of vaccination (no effective vaccine being available), individuals are exposed to RSV throughout life, may be repeatedly infected, and are subject to the risk of more severe disease with age-related immune senescence and underlying disease (reviewed by Schmidt et al., 2004). Clinically, RSV-specific antibody production is not necessarily correlated with protection, and may even be involved in the pathogenic manifestations of disease (Chin, 1969).

Reminiscent of findings with MV, *in vitro* studies with primary human cells showed that despite elevated surface molecule expression on DCs after RSV exposure, infected DCs essentially ablated autologous naive CD4 T proliferative responses in response to either the superantigen staphylococcus enterotoxin B (SEB), or anti-CD3 antibodies, during co-culture. T cells derived from these same co-cultures demonstrated weak cytokine production (including IFN- γ , IL-4, IL-13, and TNF- α) as compared to cells co-cultured with DC infected with influenza virus, or treated with synthetic dsRNA (in the form of poly I:C, a toll-like receptor (TLR)-3 agonist), or IL-1 β plus TNF- α (de Graaff et al., 2005). Although suppression of proliferation appeared to be due to a soluble mediator, neither IL-10 nor transforming growth factor (TGF)- β was responsible for the reduced proliferation seen in response to RSV-infected DCs (de Graaff et al., 2005). These data suggest that RSV infection of DC induces a functionally defective state, in which the DC is unable to facilitate T cell priming, reminiscent of the poor immunogenicity of RSV *in vivo*.

The results highlighted above reflect earlier *in vitro* work with a similar system examining T cell responses to autologous RSV-infected DCs, with cells being derived from children at birth, age 1, and age 4. Sorted naive CD4 T cells in co-culture with DCs and the superantigen toxic shock syndrome toxin (TSST) revealed deficient IFN- γ production by intracellular staining in response to RSV-infected DCs, an effect that was stable over time for a given individual. In the same report, the authors performed an analysis on children at the age of 1 who were sorted based on RSV disease history, using the same culture system. In this comparison, lower frequencies of IFN- γ producing T cells were found in co-cultures with cells derived from individuals with a history of

bronchiolitis rather than a benign disease course (Schauer et al., 2004). Previous work by the same group had demonstrated that the reduced IFN- γ production by T cells in this co-culture system was dependent on replication-competent RSV infection of DCs, was associated with selective DC CD86 induction, and could be overcome by stimulation of the DCs with poly I:C, but not by CD40L stimulation (Bartz et al., 2003). Because of the role of dsRNA in IFN- α/β induction and activation of DCs (Cella et al., 1999), this suggests that RSV may have evolved to avoid stimulating DC functions specifically associated with type I IFNs. Indeed, a clinical isolate of RSV was associated with ablation of typically potent IFN- α induction in plasmacytoid DCs (Schlender et al., 2005), and RSV encodes IFN-antagonizing proteins (Spann et al., 2004). Taken together, these data indicate that DC functional maturation following RSV infection is defective, that at least some of the defects are associated with poor type I IFN responses, and that these defects are influenced by viral and host genetic components.

In RSV infection, the T_h type-skew induced during primary exposure may have consequences for disease pathology during repeated challenges, as evidenced by increased incidence in severe RSV disease noted following use of a formalin-inactivated RSV vaccine (Chin, 1969; Fulginitti 1969). This vaccine is thought to have biased RSV-specific responses towards a T_h2 -type response due to the nature of the adjuvant (alum precipitation was used in vaccine preparation) included with the inactivated virus, and the pulmonary eosinophilia noted following post-vaccination natural infection (Chin, 1969). There is also evidence, derived from experiments in a murine model of RSV infection, that additional factors may predispose the host to development of pathogenic T cell subsets. This argument is based on the observation that an oligoclonal, potentially

expanded subset of V β 14 CD4 T cells specific for the RSV G protein are associated with RSV-mediated pathology, with elimination of this subset providing protection against severe disease upon RSV challenge (Varga et al., 2001). However, the role of DC in generation of these pathogenic T cells (i.e., their priming history) is not yet defined. In the vast majority of human RSV cases, the individual recovers effectively from infection; this may occur serially until adaptive RSV-specific responses are sufficient to make re-exposure asymptomatic.

Viral infection of DCs associated with immune suppression is also implicated in the development of indolent infection and chronic disease. Studies in humans and mice suggest that, like acute immunosuppression, chronicity may be achieved through utilization of IL-10 to attenuate host antiviral responses. This has been proposed to be the case in humans for HCV (Hofer et al., 2005), and has been elegantly demonstrated for the arenavirus, lymphocytic choriomeningitis virus (LCMV) clone 13 infection of mice (Brooks et al., 2006). In the latter case, LCMV clone 13 is a derivative of LCMV strain Armstrong. Although infection with the Armstrong strain is acute in nature, clone 13 establishes persistent infection in adult mice. A single amino acid change in the LCMV glycoprotein (GP) is known to enhance binding to DCs (Oldstone, 2002). In the experiments by Brooks et al., clone 13, but not Armstrong, was associated with DC IL-10 production, and persistent infection induced by clone 13 infection was absent in IL-10 knockout mice.

Taken as a whole, these data serve to illustrate multiple pathways by which viruses can subvert DC function to facilitate their own survival and replication. In particular, several of the aforementioned systems highlight the induction of IL-10 and

suppression of IFN (particularly IFN- α/β) as convergent evolutionary tactics utilized by viruses to modulate host immunity. Paradoxical T cell suppression in the context of phenotypic activation is not uncommon, and in some cases appears to reflect DC functions that are elicited by reverse signaling (i.e., T cell signals to the DC) during priming and co-stimulation events.

■ VIRAL INFECTION ASSOCIATED WITH DENDRITIC CELL ACTIVATION AND T CELL STIMULATION

Not all cases of DC infection with viruses result in immunosuppressive outcomes. In a study examining infection of DCs with the attenuated yellow fever virus, vaccine strain 17D, despite active replication in DCs, these cells did not express CD83, which is considered a prototypical marker of myeloid DC maturation. However, infected DCs were able to stimulate YFV-specific autologous CD4 T cell and CD8 T cell responses in a YF-immune donor, as well as responses in a flavivirus-naïve donor when an influenza peptide was inserted into the NS2B-3 junction of the 17D virus used for DC infection (Barba-Spaeth et al., 2005).

Additional studies on YFV indicated that infection of human monocyte-derived DCs induced TNF- α , IL-6, and, to a lesser extent, IL-10 production, in addition to the chemokines IP10 and MCP-1. While IP10 and IL-6 induction were abrogated when the virus was either UV-inactivated or heat inactivated, TNF- α was absent only with heat inactivation. Together, these data suggest a multi-tiered response to YFV 17D infection in DC, with alternate pathways activated by distinct viral components or processes. The authors reported only very modest DC CD83 induction with YFV infection, similar to the

previous report (Barba-Spaeth et al., 2005), and only at the highest MOI of 1. However, YFV induced both CD80 and CD86, demonstrating a disconnection between the traditional DC maturation marker, CD83, and two of the B-7 family co-stimulatory molecules often associated with phenotypic maturation. The same study, using murine DCs *in vitro*, revealed TLR-2, 7, 8, and 9- dependent activation of CD11c⁺ DCs by YFV in the absence of DC infection. The authors also reported DC activation *in vivo* in 17D-challenged mice, demonstrating YFV-specific expansion of CD8 T cells. Notably, the data suggested a mixed T_h1/T_h2 response to the virus, with T_h1/T_c1 responses appearing to be inhibited through TLR2 activation (Querec et al., 2006).

Taken together, these two studies suggest that YFV 17D activates DCs and facilitates their T cell priming capabilities. One could speculate further that, given the superior effectiveness of the 17D vaccine, ideal vaccination strategies target multiple DC signaling modalities and thus promote a balanced T_h1/T_h2 response in addition to cytotoxic CD8 T cell activity.

It has also been demonstrated that Hantaan virus (HTNV), a bunyavirus that causes hemorrhagic fever with renal syndrome, infects both blood- and culture-derived DCs (Raftery et al., 2002). Infection neither induced apoptosis, nor inhibited HLA-DR-specific antibody-induced apoptotic signaling. The authors reported production of both TNF- α and IFN- α in response to infection; following exposure to HTNV, the DCs increased expression of CD40, CD80, CD86 and HLA molecules to a level similar to that of cells treated with TNF- α . In co-culture with whole allogeneic T cells, the response of infected DCs again proved similar to TNF- α treated cells, as HTNV infection of DCs improved their capacity to induce T cell proliferation (Raftery et al., 2002).

These reports demonstrate that, for some viruses, infection results in a DC phenotype associated with functional activation, including cytokine production and, importantly, the ability to prime T cell responses. This observation is in contrast to the immunosuppressive effects of viruses described in the previous section, and serves to highlight the broad diversity in DC responses induced by a range of viral pathogens. Lastly, beyond simply regulation of DC function, viruses may utilize DCs as reservoirs for transmission to other cell types and/or dissemination of infection.

■ VIRAL DISSEMINATION AND THE PECULIAR CASE OF HIV

In the case of HIV, there is considerable evidence that DCs are not a primary target of infection in the classical sense, in that there is little replication of the virus in the infected cell. The “infection” process appears to occur through binding of the HIV virion to DC-SIGN, an event that preserves the infectivity of the virus and results in transfer to, and infection of, resting T cells (Geijtenbeek et al., 2000a). In particular, DC-SIGN expression on the DC induces T cell clustering through binding of T-cell expressed ICAM-3, allowing HIV to enrich its microenvironment with available targets. In this case, the DC acts as a “Trojan horse”, internalizing and sequestering the virus and maintaining it in a non-lysosomal low pH compartment, and presenting it to CD4 T cells, which are then infected in trans, an effect that has been observed in both *in vitro* and *in vivo* (Kwon et al., 2002; Cameron et al., 1992). Therefore, HIV is able to utilize DCs for temporary survival in the host while accumulating target cells, a strategy that fosters rapid replication and dissemination and eventually establishes a state of chronic disease. To the point, these observations demonstrate that DCs need not serve as an active source

of virion production to facilitate viral replication. Rather, viruses may use DCs as a temporary "safe house," enabling the virus to avoid detection whilst manipulating DC functions to further their own ends.

■ DENGUE INFECTION OF DENDRITIC CELLS

As noted previously, DCs were originally shown to be infected by DV both *ex vivo* and *in vitro* by Wu et al (2000). Shortly thereafter, subsequent work demonstrated that DV induces DC phenotypic changes and cytokine production in DCs generated *in vitro* from adherent blood monocytes (Libraty et al., 2001; Ho et al., 2001). In one study, these included increases in co-stimulatory molecules CD80, CD86, and HLA DR, induction of CD83, and secretion of TNF- α and IFN- α , but not IL-12 or IL-6. In the absence of feeding cytokines, infected DCs showed slightly higher levels of apoptosis before 22 hours, but had greater survival by 46 hours (Ho et al., 2001). Similar results were found in a second study, in which following infection DCs induced CD40, CD83, CD80, CD86, and both MHC class I and II molecules at 48 hours (Libraty et al., 2001). In that study, intracellular staining for dengue antigen revealed that infected DCs had significantly reduced expression of CD83, CD80, CD86, and MHC Class I when compared to uninfected bystanders. Consistent with the report by Ho et al (2001), the authors reported that in response to infection DCs secreted TNF- α and IFN- α without a significant IL-12 p70 response, and DV infection did not induce IL-10. Libraty et al. (2001) also noted that IFN- γ treatment at the time of infection reduced virus output, restored surface molecule expression in infected cells, and increased IL-12 p70 secretion, suggesting that

early IFN- γ induction *in vivo* during secondary infection might enhance DC activation and adaptive immune stimulation.

A later *in vitro* study addressed the issue of virus strain on DC infection. The investigators generated chimeric infectious clones by replacing specific regions of the SE Asian D2V strain 16681 with sequences from another SE Asian D2V strain or an American D2V strain. When monocyte-derived DCs were exposed to these viruses, the infectious clone generated from 16681 and the 16681 parent virus infected DCs more efficiently than either an infectious clone with three American genotype structure inserts (and a SE Asian backbone) or the parent American genotype virus (Cologna and Rico-Hesse, 2003). In that report, of the three American structures introduced, the E protein mutation was most critical for reduced virion output.

The importance of the E protein becomes more apparent when viewed in light of the role that this protein serves in binding to the DC receptor, DC-SIGN, as discussed in the section on dengue virus biology. Strengthening the hypothesis that DC infection is critically involved in dengue pathogenesis, a multi-site study performed in Thailand found that the more rare "G" polymorphism in the promoter region of DC-SIGN (denoted DCSIGN1-336 by the authors) had a significantly lower frequency in individuals diagnosed with dengue fever, with an incidence in DF cases of 4.7%. The frequency of individuals carrying the G allele among DHF patients (22.4%) was similar to that in the control population (19.5%). These results suggest that the G form of the allele is associated with protection from DF but not DHF, potentially by affecting an Sp1 binding site and reducing transcriptional activity from the DC-SIGN locus (Sakuntabhai et al., 2005). In particular, this may reduce susceptibility of DCs to infection by DV in

individuals carrying the G allele. Additional evidence for host genetics affecting DC function in dengue infection may be implied from the observation that a single nucleotide polymorphism (SNP) in the vitamin D receptor (VDR) is associated with reduced risk of DSS (Loke et al., 2002). Signaling through the VDR has been shown to attenuate the activation and function of DCs (Griffin et al., 2001).

During preparation of this thesis, additional reports emerged on the effects of DV on DCs, including suppression of T cell stimulation. The first of these indicated that although dengue infection activated bystander cells, actively infected DCs failed to increase surface molecule expression, with CD40 and HLA DR reduced even in comparison to mock infected cells (Palmer et al., 2005). In accordance with earlier studies (Libraty et al., 2001; Ho et al., 2001), Palmer et al. (2005) reported TNF- α and IFN- α secretion with minimal detection of IL-12 p70. In contrast to the study by Libraty et al. (2001), Palmer et al. (2005) also reported IL-10 production by DC in response to dengue infection, and elevated levels of IL-10 when dengue-infected as opposed to mock-infected DC were co-cultured with allogeneic T cells. Thus, the authors hypothesized that IL-10 was responsible for the observation of lower T cell proliferative responses to DCs infected at higher MOIs (Palmer et al., 2005).

Additional work by the same group found that CD40 ligation on DCs following DV infection increased both the percentage of infected DCs and virion output in the exposed cultures, similar to what had been observed for MV a number of years prior (Sun et al., 2006). In that study, TNF- α and IL-12 p70 secretion were enhanced, while IFN- α secretion appeared to be suppressed by CD40L stimulation (Sun et al., 2006). Also in that study, although DC apoptosis following dengue infection was lower than that seen

with mock infection, the addition of CD40L reduced cell death in both populations, in contrast to observations in the MV system. Lastly, CD40L stimulation of infected cultures restored CD80, CD86, and CD83 expression on infected DCs when compared to bystanders and enhanced IFN- γ production in co-culture with allogeneic CD4 T cells, but did not reverse the decrease in T cell proliferative responses to DCs seen with increasing MOIs (Sun et al., 2006).

A later study compared DC responses on the single-cell level following infection with either parent (strain 16681) or attenuated (PDK53) D2V strains. Regardless of the infection state of the DC, both strains increased CD80, CD83, and CD86 expression compared to control treatment while MHC class I and II expression were elevated following 16681 exposure alone (Sanchez et al., 2006). That study also used intracellular staining for cytokines and dengue protein, demonstrating that after PDK53 infection, TNF- α production occurred primarily in infected cells, while both infected and bystander DCs produced TNF- α following 16681 infection; only 16681 induced IL-6, which was detected in both infected and bystander DCs (Sanchez et al., 2006). Therefore, active replication and virus genetics both contribute to the surface expression and secretory phenotype of DCs following DV infection. IL-12 p70 production and secretion were minimal, and although the authors reported some low-level IL-10 secretion, intracellular cytokine staining did not reveal IL-10 production and thus the infection state of the responsible cells (Sanchez et al., 2006). This may have been a failure in either the quality of the staining antibody or a function of low IL-10 production levels, resulting in inadequate signal strength for flow cytometry (Sanchez et al., 2006).

Consistent with reports of IFN-interference by DV in cell lines, DV has also been shown to alter IFN-dependent signaling in primary human DCs. Work by Ho et al (2005) demonstrated that, similar to findings in multiple cell lines (Diamond et al., 2000), IFN- α and IFN- γ could inhibit DV infection and replication in DCs, although IFN- α required pretreatment to be effective. DV induced STAT1, 2, and 3 phosphorylation, with STAT3 showing early (3hr) activation and DNA binding, while slower kinetics (24hr) were observed for STAT1. Infected DC were resistant to exogenous IFN- α mediated activation of STAT1 and 3, but responded to IFN- γ (Ho et al., 2005), reminiscent of IFN- γ effects on DC phenotype in work by Libraty et al (2001). The phosphorylation of STAT1, but not STAT3, depended on endogenous IFN- α production, and active infection reduced IFN- α mediated Tyk2 phosphorylation (Ho et al., 2005):

As a whole, the studies highlighted here demonstrating DV infection of DC begin to elucidate some of the mechanisms by which the virus is able to modulate host immune responses. Blockade of IFN- α/β , elicitation of IL-10, and interference with the expression of DC surface molecules all represent manipulations of DC function that can have profound consequences for the development of dengue-specific immunity *in vivo*.

■ THESIS OBJECTIVES

Primary dengue infection is associated with the generation of serotype-specific protective immune responses, with complete clearance of the virus and, in the vast majority of cases, minimal pathology. Memory to dengue appears to play a critical role in the etiology of the most severe manifestation of disease, DHF. Dengue infection of dendritic cells likely plays a crucial role in determining downstream immune responses, serving as

a source of virus production, initiating immune activation, and driving T cell differentiation and functional behavior. The presence of replicating virus in DCs, resulting in production of dengue proteins and RNA, means that infected DCs are influenced in a manner that differentiates them from uninfected, bystander cells that are exposed to an otherwise identical microenvironment. Therefore, the functions of these two distinct populations are expected to differ, with consequences for the development of dengue-specific immunity.

My hypothesis was therefore that dengue virus infection of dendritic cells results in the activation of functions consistent with immune regulation and antiviral immunity, with intracellular replication inhibiting activity critical to viral clearance.

The experimental work of this thesis is presented in three sections:

Chapter III: INFECTION AND PHENOTYPIC MATURATION OF DENDRITIC CELLS FOLLOWING DENGUE VIRUS EXPOSURE

Enhanced expression of surface molecules on DCs prior to, and during, antigen presentation has contact-dependent consequences for the nature of T cell priming. Both quantitative and qualitative regulation of T cell function can be affected, determining the effectiveness of downstream immune responses. Therefore, I sought to determine if DV exposure was a stimulus for the increased expression of DC surface molecules involved in T cell regulation. In addition, exposure to viral particles affects DC phenotypic change through multiple pathways, including the direct effects of binding, internalization and replication, and the indirect effects of subsequent soluble mediator release. Thus, I also sought to elucidate the contributions of each aforementioned pathway to changes in DC phenotype. These aims were addressed as follows:

- a) Generation of monocyte-derived DCs and infection with DV
- b) Whole culture phenotypic maturation responses of DCs following DV infection and comparison to defined stimuli and killed virus
- c) Comparison of phenotypic maturation responses of actively infected DCs and uninfected bystanders in the same culture

Chapter IV: INDUCTION AND REGULATION OF DENDRITIC CELL CYTOKINE AND CHEMOKINE RESPONSES BY DENGUE VIRUS

Soluble mediators released following exposure of DCs to virus have autocrine and paracrine effects on DCs. These effects include induction of an infection-resistant state, changes in cell surface phenotype, and secondary release of further soluble mediators. Soluble factors further influence DC function by modulating their interactions with downstream cellular responses. This can include the selective recruitment of specific sets of cellular effectors, as well as regulation of the activity of these effectors, based on the signaling induced in responding cells by DC-released molecules. Therefore, I sought to determine the effects of DV on DC cytokine and chemokine production, by again addressing both direct (particle- and replication-dependent) and indirect (secondary secretory response) effects. I addressed these issues by:

- a) Whole culture secretory responses of DCs to infection with DV and comparison to LPS or killed virus
- b) Dose-responsiveness of whole culture secretory responses of DCs to infection with DV
- c) Comparison of TNF- α and IP10 response profiles in infected DCs as compared to uninfected bystanders
- d) Effects of IFN- α/β blockade on single-cell TNF- α and IP10 production

- e) Differences in single-cell DC IP10 production profiles following infection with variant D2V strains

Chapter V: EFFECTS OF DENGUE INFECTION ON THE ABILITY OF DENDRITIC CELLS TO PRIME ALLOGENEIC RESTING CD4 T CELLS

Arguably the most critical function of DCs following exposure to pathogens such as viruses is the induction of T cell –mediated immune responses. Proper regulation of T cell responses is crucial in determining both the appropriate magnitude of the response, as well as the direction of that response with regards to effector actions of T cells. A properly regulated T cell response will facilitate effective elimination of the invading pathogen. T cell expansion is particularly important, given the relative paucity of pathogen-specific T cells in a naïve individual. While T_h1 -type responses characterized by $IFN-\gamma$ production are considered critical for elimination of intracellular pathogens such as viruses, T_h2 -type responses characterized by IL-4 and -13 are thought to be important in effective antibody production by B cells and elimination of extracellular agents. IL-10 is important in the attenuation of the magnitude of T cell responses, as well as inhibition of $IFN-\gamma$ function. I therefore sought to determine if DV infection of DCs promoted proliferation and T_h -skewing in responding CD4 T cells.

- a) Comparison of resting CD4 T cell proliferative responses to DV-infected DCs versus mock-infected or cytokine-matured DCs
- b) Effects of increasing DV MOI of DC on their CD4 T cell priming function
- c) Effects of infectious immune-complexed virus on DC priming function

CHAPTER II

MATERIALS AND METHODS

■ MONOCYTE ISOLATION AND CULTURE

We generated monocyte-derived DCs following the methods of Sallusto and Lanzavecchia (1994) with minor modifications. Briefly, peripheral blood mononuclear cells (PBMC) were obtained from the heparinized blood of healthy adult volunteers in a dengue non-endemic region using Ficoll-hypaque (Amersham/GE Healthcare Bio-Sciences AB, Uppsala Sweden), under a protocol approved by the University of Massachusetts Medical School Institutional Review Board. Monocytes were isolated from PBMC using MACS™ (Miltenyi) CD14 positive selection, according to the manufacturer's protocol, and were separated on an LS column (Miltenyi) using a MidiMACS™ magnet (Miltenyi). The CD14⁺ fraction was collected and used for T cell isolation (see below). CD14⁺ cells were washed and re-suspended at $1.4-1.8 \times 10^6$ cells/mL in 24-well plates, in RPMI 1640 containing penicillin/streptomycin (1 mg/mL each), and 10% heat-inactivated fetal calf serum (FCS; Hyclone) supplemented with 800 U/mL recombinant human (rh) GM-CSF and 500 U/mL rhIL-4 (Peprotech). At day 3-4, 0.5 mL of medium was removed and replaced with fresh medium containing 1600 U/mL rhGM-CSF and 1000 U/mL rhIL-4. On day 6-7, vigorous pipetting and gentle scraping collected DCs. These cells were CD3⁻, CD8⁻, CD14⁺, CD19⁻, and CD56⁻, but were MHC class II⁺, MHC class I^{low}, and expressed the myeloid DC marker CD1a⁺. Cells had an immature DC phenotype (as shown in Figure 1) by low staining for CD83.

■ T CELL ISOLATION

CD45RA⁺ CD45RO⁻ CD4 T cells were isolated by negative selection using a naïve T cell isolation kit (StemSep; StemCell Technologies) according to the manufacturer's protocol. Briefly, the CD14⁻ fraction of cells generated by positive selection of monocytes was washed and re-suspended with MACS™ buffer at a final concentration of 5×10^7 cells per mL. Biotinylated anti-CD45RO was added and incubated for 30 minutes at 4° C. Anti-lineage/anti-biotin antibodies were added and the mixture was incubated for 30 minutes at 4° C. Cells were washed two times with 5 mL of MACS buffer and separated using a MidiMACS™ magnet and LS column (Miltenyi). The cells were washed, counted, and re-suspended in RPMI 1640 supplemented with penicillin/streptomycin and 10% heat-inactivated human AB serum (Gemini BioProducts) (RPMI AB10) at 1.0×10^6 cells/mL. Human AB serum was used in lieu of FCS at all steps during isolation and culture of T cells due to an observed tendency of FCS to induce non-specific proliferation. T cells isolated in the manner described were typically 90-95% CD3⁺, and >95% CD4⁺. The CD3⁺CD4⁺ cells were routinely >98% CD45RA⁺CD45RO⁻ (not shown). T cells were added to wells in a 96-well plate at 50,000 T cells in 50 μ L for co-culture experiments.

■ DENGUE VIRUS INFECTION OF DENDRITIC CELLS

D2V New Guinea C (NGC) is a laboratory-adapted prototype Southeast Asian D2V strain obtained from ATCC. D2V 16681 and D3V CH53489 are prototype strains originally obtained from the Walter Reed Army Institute of Research. D4V 814669 is a prototype strain originally obtained from the National Institutes of Health. D2V INH and VEN are American genotype viruses originally obtained from Dr. Rebeca Rico-Hesse,

Southwest Foundation for Biomedical Research. Dengue 2 strain C0166/96 is a low-passage clinical isolate from a Thai DF patient (Vaughn et al., 1997). Viruses were propagated in the mosquito cell line C6/36. Virus titers were determined by plaque assay on Vero cells. Inactivated, non-replicative virus was prepared by placing an aliquot of virus stock 5-6 cm under a germicidal lamp ($2300 \mu\text{W}/\text{cm}^2$ UVA irradiation at 254 nm) and incubating for 30 minutes on ice.

For infection, DC pellets in 15 mL polypropylene tubes were incubated at 37°C 5% CO_2 for 90 minutes with supernatant from C6/36 cultures (mock infection), UV-inactivated virus, or live virus. Tubes were left loosely capped and the pellets were disrupted using vortex mixing every 30 minutes. Following adsorption, the cells were washed and plated in 24-well plates (1.0 mL/well) or 48-well plates (0.50 mL/well) at 1.0×10^6 cells/mL and maintained at 37°C , 5% CO_2 . TNF- α at 50 ng/mL (R&D systems; Peprotech) and IFN- α 1000 U/mL (5 ng/mL) (Peprotech), or LPS (50 ng/mL, from *E. coli*; Sigma) was added where noted. For DC infection using a range of MOIs, virus stock was serially diluted 5-fold in medium as needed, and DCs were infected in a fixed volume as described above.

■ INFECTION OF DENDRITIC CELLS WITH IMMUNE-COMPLEXED

DENGUE 2 VIRUS

Uninfected C6/36 mosquito cell supernatant or live D2V NGC was combined with either control plasma or plasma from a dengue 3 immune donor at a dilution of 1:20, which was previously determined to enhance DV infection of K562 cells (Laoprasopwattana et al., 2005). The virus-plasma mixtures were incubated on ice for 30 minutes prior to addition

to DCs at an MOI=1. Following adsorption, DCs were washed and re-cultured for 24 hours as previously described.

■ MULTIPLEX ANALYSIS OF CYTOKINES AND CHEMOKINES

Analysis of cell culture supernatants for cytokines and chemokines utilized BeadLyte cytokine assay kits (Upstate) as per the manufacturer's protocol; analyses were performed at the Baylor Institute for Immunological Research. Cytokine concentrations were calculated using Bio-Plex Manager 3.0 software with a 5-parameter curve-fitting algorithm applied for standard curve calculations. Assay specifics: In Figure 7, the lower limit of detection threshold was 10 pg/mL for all analytes, with the exception of IL-12 p40 (40 pg/mL), IFN- γ (13 pg/mL), Eotaxin (69 pg/mL) and MIP-1 α (86 pg/mL). In this assay, upper limits of detection were greater than 10,000 pg/mL with the exception of RANTES (8750 pg/mL). Samples were diluted 2.5-fold for analysis. For the purposes of graphical analysis, samples outside the limits of detection were plotted at the limit. In this assay, IP10 levels in DV-infected DC supernatants were not quantifiable according to the analysis software. Samples from which the data in Figures 8 and 9 were derived were diluted 7.5-fold for analysis. Lower limits of detection were 10 pg/mL. Samples from which the data in Figure 10 were derived were diluted 2-fold for analysis. Some data for IP10 in Figure 10 represent extrapolated values beyond the high value in the five-point standard curve based on the linearity of the high end of the curve. These values were not determined to be out of range by the analysis software and represent quantifiable values (John Connolly, Baylor Luminex Core Facility, personal communication). Lower limits

of detection were 10 pg/mL. Samples from which the data presented in Figure 18 were derived were diluted 2-fold for analysis; lower limits of detection were 10 pg/mL.

■ ANTIBODIES FOR FLOW CYTOMETRIC ANALYSIS

The following monoclonal antibodies to human targets were purchased from BD/Pharmingen: FITC anti-CD3, FITC anti-CD45RA, FITC IgG_{2a}, FITC IgG_{1k}, PE anti-HLA A, B, C, PE anti-CD40, PE anti-IP10, PE anti-CD45RO, PE anti-CD56, PE anti-CD1a, PE-anti PD-L1, PE anti-CD80, PE IgG_{2a}, PE IgG_{1k}, PerCP anti-CD4, PerCP IgG_{1k}, APC anti-CD3, APC anti-CD19, APC anti-CD56, APC anti-CD83, APC anti-CD1a, APC anti-HLA DR, APC anti-PD-L2, APC anti-CD86, APC anti-TNF- α , and APC IgG_{1k}. Purified mouse IgG_{1k} was purchased from BD and used as a blocking antibody in some experiments, Monoclonal cross-reactive anti-dengue complex antibody (clone M8051125, IgG_{2a}) was purchased from Fitzgerald Industries (Concord, MA) and was either custom-conjugated to FITC or labeled with Zenon™ Pacific Blue (Molecular Probes/Invitrogen) reagent according to the manufacturer's protocol. In some experiments, viral envelope staining was performed using FITC goat anti-mouse IgG (Sigma) as a secondary antibody.

■ IMMUNOCYTOCHEMISTRY AND FLOW CYTOMETRIC ANALYSIS

For staining of cell surface markers, DCs were washed twice with DPBS (Gibco) 2% FCS, 0.5% sodium azide, (PBS-azide wash buffer, PAWB). In some experiments, DCs were incubated for 15 minutes with 500 ng mouse IgG_{1k} to reduce non-specific staining. Cell-marker specific antibodies were added at 50 ng per antibody for 30 minutes at 4°C.

Cells were washed three times and fixed using Cytofix (BD) or Cytofix/Cytoperm (BD) according to the manufacturer's protocol. Cells were assayed for dengue E protein expression by permeabilization and staining for 30 minutes with anti-dengue (1 μ g) antibody. For intracellular cytokine staining, 1 μ g of brefeldin A (Golgiplug, BD) per million cells was added 6-8 hours prior to harvesting and fixation. DCs were washed 2 times with Perm/Wash (BD), and then anti-IP10 (0.5 μ g) and/or anti-TNF- α (0.5 μ g), and anti-dengue (1 μ g) antibody were added for 30 minutes, after which the cells were washed three times with Perm/Wash buffer and re-suspended in PAWB for analysis. Flow cytometry was performed on FACSCalibur, FACS Aria, or LSRII flow cytometers (BD Immunocytometry Systems, San Jose, CA) with data collection utilizing CellQuest 3.1.3 or FACSDiva 5.x software. Data were analyzed using FlowJo™ ver.6 and up software (Treestar).

■ INTERFERON ALPHA/BETA BLOCKING EXPERIMENTS

DCs were harvested and washed as previously described. The cells were then incubated with medium alone, or with medium plus IgG_{2a} or anti-CD118 (interferon-alpha receptor subunit 2; IFNAR2) antibody (PBL laboratories) at 50 μ g/mL for 30 minutes. The addition of virus resulted in dilution of antibody to 10 μ g/mL. Viral adsorption, washing, and DC culture were carried out as previously described, in the presence of antibody where specified. Upon re-suspension, the appropriate antibody (IgG_{2a} or anti-IFNAR2) was added at 10 μ g/mL for the duration of culture as required.

■ DENDRITIC CELL-T CELL CO-CULTURE

For co-cultures examining a range of T: DC ratios, D2V-infected, mock-infected, or cytokine-treated DCs were harvested, washed, and re-suspended in RPMI AB10 at 10^5 cells/mL, then serially diluted as needed. DCs were added to T cells (5×10^4 cells in 50 μ L) in 100 μ L aliquots in quintuplicate wells for each condition to a final volume of 150 μ L/well in 96-well plates. In experiments utilizing a range of MOIs, DCs were washed and re-suspended as described above, with a final count of 5×10^4 cells/mL, and added to 100 μ L of T cell suspension (at 5×10^5 T cells/mL) in aliquots of 50 μ L (2500 DC, T: DC ratio of 20:1) in quintuplicate wells in 96-well plates. For experiments with immune-complex treated DCs, DCs were washed and re-suspended as previously described, at 5×10^4 cells/mL, then serially diluted as needed and 50 μ L of the appropriate dilution added to 100 μ L of T cells (at 5×10^5 T cells/mL) in quintuplicate wells in 96-well plates (T: DC ratio of 20:1). T cells incubated with 1 μ g/mL phorbol myristoyl acetate (PMA) plus 0.1 μ g/mL ionomycin (Sigma) served as positive controls. Negative control wells were T cells receiving medium alone.

■ TRITIATED THYMIDINE INCORPORATION

Following 3-4 days of co-culture, tritiated thymidine (Perkin-Elmer) was added to each well at a concentration of 1 μ Ci in 50 μ L of RPMI AB10, 18-20 hours before harvesting. Cells were harvested onto glass fiber filter mats and the incorporation of radioactive thymidine measured using a Betaplate liquid scintillation β -counter. PMA/ionomycin stimulated cells routinely produced counts in the range of 50,000 to greater than 100,000 cpm; negative controls were routinely less than 100 cpm (not shown).

■ STATISTICS

All statistical analyses utilized the nonparametric Wilcoxon signed-rank test. For cell surface phenotyping, the ratio of mean fluorescence intensities (MFI) was calculated for each comparison and compared to a hypothetical value of 1. Statistical analyses of co-culture experiments were performed between groups on mean raw cpm values derived from replicate (quintuplicate) wells. For all analyses, P values of <0.05 were considered statistically significant.

CHAPTER III

INFECTION AND PHENOTYPIC MATURATION OF DENDRITIC CELLS FOLLOWING DENGUE VIRUS EXPOSURE

■ GENERATION OF IMMATURE MONOCYTE-DERIVED DENDRITIC CELLS AND THEIR SUSCEPTIBILITY TO INFECTION WITH DENGUE VIRUS

Immature monocyte-derived DCs (iDC) are the *in vitro* counterparts of *in vivo* myeloid DCs, including dermal interstitial DCs (Banchereau and Steinman, 1998). Early studies demonstrated dengue virus infection of *in vitro*-cultured DCs (Wu et al., 2000; Ho et al., 2001; Libraty et al., 2001). Therefore, in order to study the effects that dengue virus has on the functional activities of target cells, we generated myeloid DC in culture and infected them with the laboratory-adapted D2V New Guinea C strain (NGC). Positive selection of monocytes from the PBMC of anonymous whole blood donors with MACS™ allowed us to isolate highly purified populations of CD14-expressing monocytes. Following supplementation with GM-CSF and IL-4, monocyte-derived DCs exhibited uniform forward- and side-scatter morphology, large proportions (typically 85% or more) of CD1a-expressing cells, and low expression of CD83 (*Figure 1A*). Cells generated in this manner also expressed high levels of HLA DR, intermediate levels of HLA A, B, C, and lost CD14 expression (not shown), characterizing them as immature myeloid DCs.

Following generation of these cells, we first sought to determine the period of maximal viral protein production following infection with D2V. A previous report

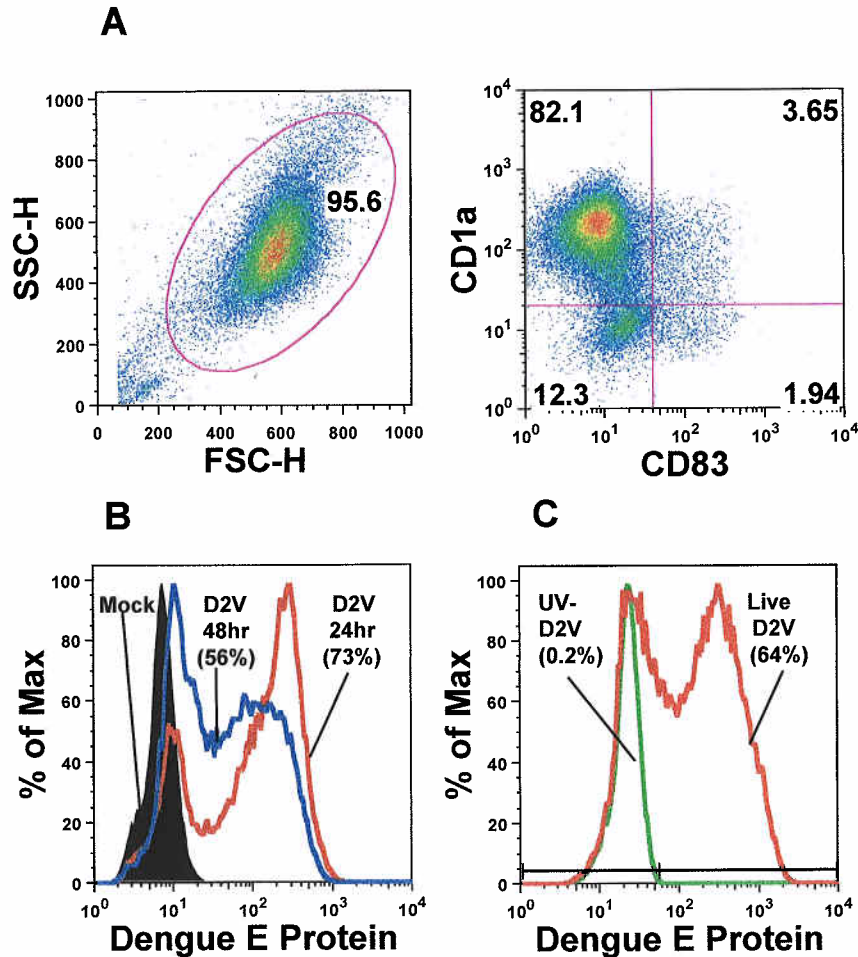


Figure 1 Monocyte-derived dendritic cells (DCs) are highly susceptible to infection with dengue virus.

A) Flow cytometric analysis of immature DCs derived from CD14-positively selected monocytes. Results are representative of at least 10 experiments. DCs were CD14⁺ HLA A, B, C^{mid} HLA DR⁺ (not shown). **B)** DCs were mock infected (black fill) or infected for 24 (red line) or 48 (blue line) hours with live dengue 2 virus New Guinea C strain (D2V) at a multiplicity of infection (MOI) of 2. Infection was performed by adsorbing with virus for 90 minutes, washing, and returning the DCs to culture. At the specified time, DCs were intracellularly stained for dengue E protein using a FITC monoclonal anti-dengue complex antibody. The data shown are representative of three similar experiments. **C)** DCs were infected with live virus (red line) as in (B), or with virus pre-treated for 30 minutes on ice using short-wave (254nm) UV irradiation (green line). Gating based on dengue-specific staining of mock-infected cells is represented by the black line at the base of the figure. The data shown are representative of more than five experiments.

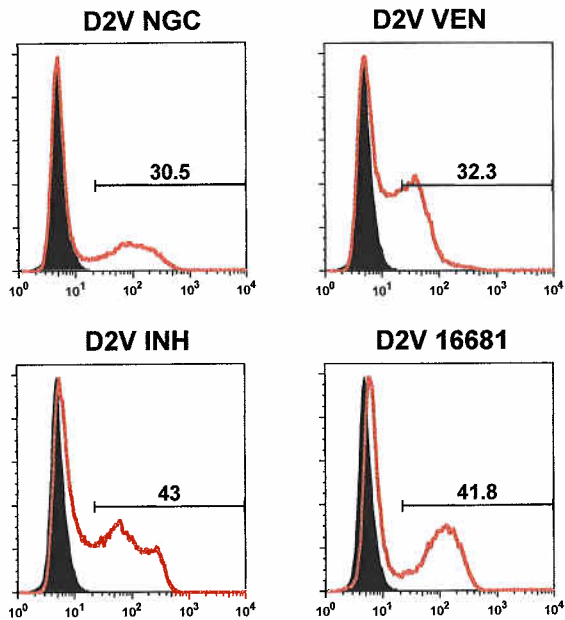
indicated that D2V NGC infection of DCs resulted in the appearance of viral protein at 12 hours and peak production between 24 and 48 hours (Wu et al., 2000). Therefore, after 6-8 days in culture, we harvested and infected DCs with D2V NGC, then returned them to culture for 24 or 48 hours. After the respective time period, we harvested the DCs and fixed them, then performed intracellular staining for dengue viral E protein using a monoclonal anti-dengue E protein antibody. Following infection with D2V NGC, we found maximal E protein production before 48 hours (*Figure 1B*).

One of the principal functions of DCs is antigen uptake and processing. To ensure that dengue E protein staining was indicative of *de novo* viral protein synthesis and was not simply unprocessed viral protein taken up via endocytosis, we compared staining for E protein after infection with live or UV-inactivated D2V NGC. At 24 hours, only DCs treated with live D2V, and not cells exposed to UV-irradiated virus, expressed detectable dengue E protein (*Figure 1C*). These data demonstrate that D2V readily infects DCs, and that dengue E protein was synthesized *de novo* in infected cells.

■ DENDRITIC CELL INFECTION WITH MULTIPLE DENGUE VIRUS STRAINS

Since D2V New Guinea C is a laboratory-adapted virus, we sought to determine if other dengue viruses were able to infect DCs. We included prototype strains of dengue serotypes 2 (strain 16681), 3 (strain CH53489), and 4 (strain 814669) and low-passaged dengue 2 strains isolated from patients (C0166/96, VEN, INH) at an MOI=2.

Intracellular staining at 24 hours post-infection detected the presence of DV E protein in DCs infected with all of the virus strains tested (*Figure 2*). These results demonstrate

A

Relative Cell #

Dengue E Protein

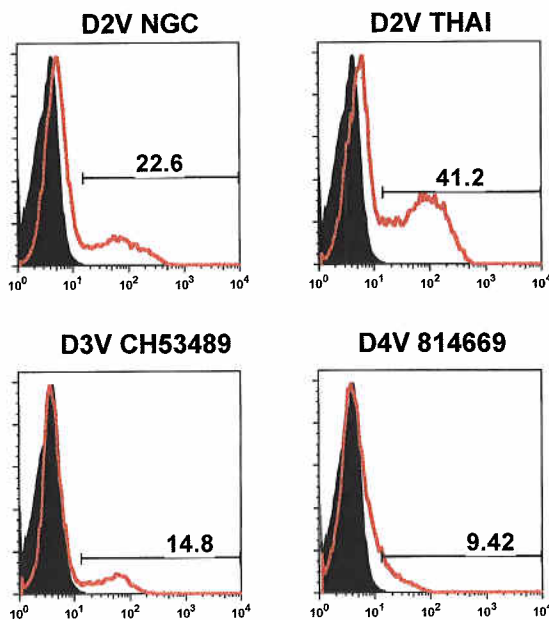
B

Figure 2 Wild type dengue viruses infect DCs.

DCs were infected for 24 hours as previously described, using a range of dengue viruses, then stained for intracellular dengue E protein. Black fill: Mock infection. Red traces: DV infection. **A)** Dengue intracellular E protein staining in DCs from a single donor infected with laboratory-adapted D2V New Guinea C strain, low-passage American D2V strains INH and VEN, and the D2V prototype strain 16681. **B)** Dengue intracellular E protein staining in DCs from a single donor infected with New Guinea C, low-passage DF patient isolate C0166/96 (D2V Thai), D3V prototype strain CH53489, and D4V prototype strain 814669. NGC, Thai, and 16681 strains were tested in at least 5 independent experiments; INH and VEN strains were tested in four donors. D3V and D4V are from a single experiment.

that multiple dengue viruses, including low-passage clinical isolates, are able to efficiently infect immature DCs.

■ DOSE-DEPENDENT INFECTION OF DENDRITIC CELLS WITH DENGUE 2 VIRUS STRAIN NEW GUINEA C

Previous reports using other strains of dengue to infect dendritic cells have utilized varying input levels of virus (Ho et al., 2001; Libraty et al., 2001; Palmer et al., 2005; Sanchez et al., 2006). In order to determine dose-dependence of infection, and the amount of virus required for reproducible, quantifiable infection in DC, we infected cells from multiple (N=7) donors, each with the same range of virus concentrations. To do so, we performed 5-fold serial dilutions of D2V NGC in medium and adsorbed the virus to DCs at MOIs of 0.04, 0.2, 1, and 5. Following adsorption, the cells were washed and returned to culture for 24 hours, after which they were harvested, fixed, and stained for intracellular dengue E protein (*Figure 3*). Non-specific staining of uninfected DC with the anti-dengue antibody was 0.75% or lower (mean±SD of 0.45±0.17%).

Infection was evident in some instances even with the lowest amount of input virus (MOI=0.04), with three of seven donors' DCs demonstrating antigen expression of greater than 2% (range, 2.1-7.8%). Although substantial infection (greater than 20%) occurred in five of seven donors at an MOI=1 (range, 24-50%), dengue E protein was essentially undetectable in donor 2 and was low (2%) in donor 1. We found consistently high levels of dengue infection at an MOI of 5 (mean±SD, 53.5±19.1%, range, 20%-73%). There was substantial variability in infection rates, which was not wholly dependent on the virus preparation used or the day of assay, since donors 1 to 5 were infected using a single virus stock, and donors 1, 2, and 3 were infected simultaneously in

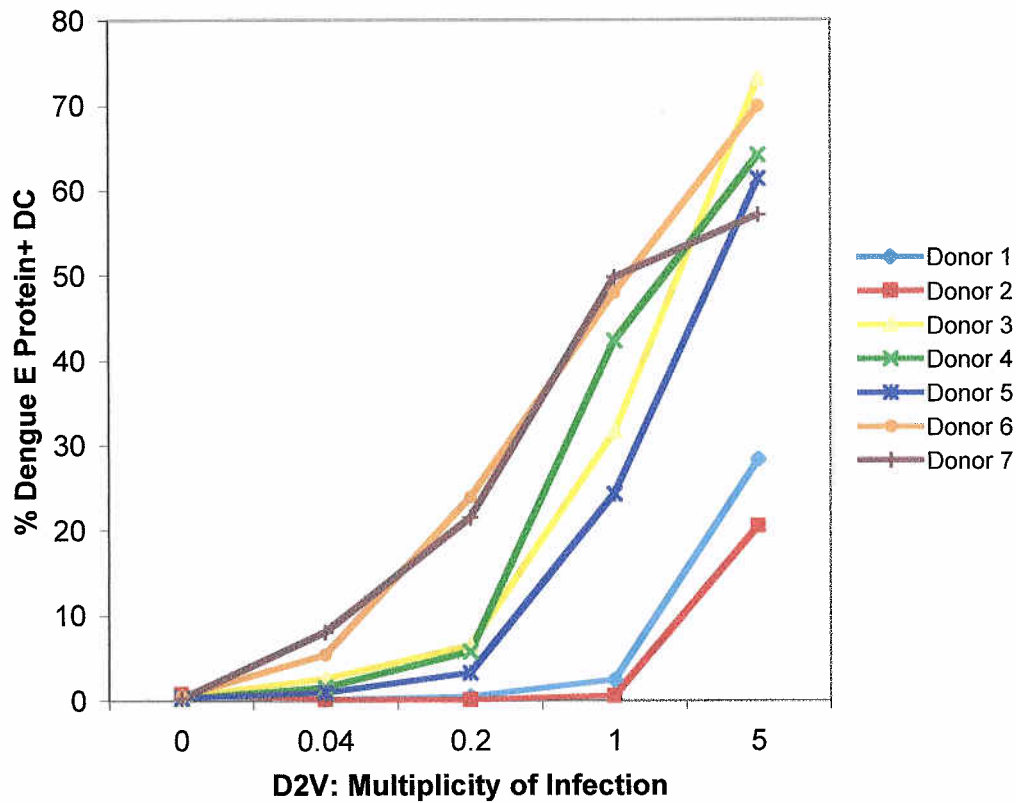


Figure 3. Dose responsiveness of multiple donors' DCs to infection with D2V New Guinea C strain.

DCs from seven donors were infected with D2V NGC at varying MOIs, using five-fold serial dilutions in three independent experiments (experiment 1, donors 1-3; experiment 2, donors 4 and 5; experiment 3, donors 6 and 7). Virus adsorption, washing, and DC re-culture were performed as previously described. At 24 hours, DCs were harvested and intracellularly stained for dengue E protein.

a single experiment. Together, the data indicate that infection of immature DC with dengue virus is dose-dependent, and suggest donor-dependent variability in the permissiveness of DC to infection.

■ DENDRITIC CELL IMMUNOMODULATORY SURFACE MOLECULE EXPRESSION CHANGES AFTER INFECTION WITH DENGUE 2 VIRUS

Cytokines produced in response to viral infection, including IFN- α and TNF- α , are implicated in the process of DC activation and maturation (Luft et al., 1998; Honda et al., 2003; Sallusto and Lanzavecchia, 1994). One aspect of DC maturation is the induction of surface molecules involved in the regulation of adaptive immune responses. To determine if D2V activates DCs and induces expression of these immunoregulatory proteins, we used flow cytometry to compare DC surface phenotypes following infection with live dengue virus, treatment with TNF- α /IFN- α or LPS, or mock infection.

When compared to isotype control staining, mock-infected cells expressed low levels of CD40 and PD-L2 and intermediate levels of MHC class I and PD-L1. CD86, CD80, and HLA DR were expressed by approximately 50%, 90%, and 95%, respectively, of DC following mock infection (*Figure 6*). CD83 expression was similar to isotype control staining, although in most experiments a small population (less than 15%) of DCs were CD83⁺ (*refer to Figure 6*). In order to quantify changes related to treating these DCs with additional stimuli, we calculated fold-expression by normalizing the MFI for each condition relative to mock infection for the same donor's DCs. Both D2V infection and TNF- α /IFN- α treatment induced similar patterns of surface molecule expression

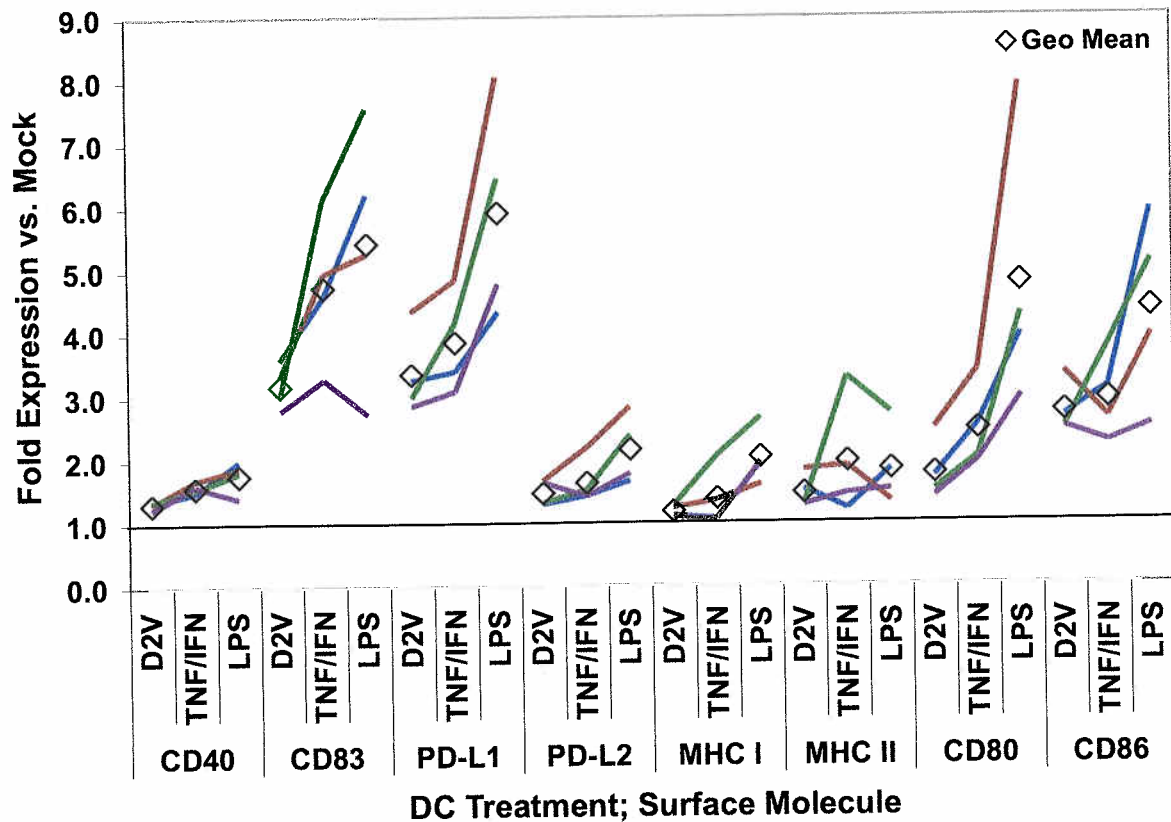


Figure 4: Live dengue virus activates DC surface molecule expression. DCs were adsorbed for 90 minutes with control medium (mock) or D2V New Guinea C, MOI=2, then washed and returned to culture for 24 hours. For positive control comparisons, immediately following mock adsorption, some cells received treatment with TNF- α /IFN- α (50ng/mL and 1000 U/mL=5 ng/mL, respectively) or LPS (from *E. coli*, 50 ng/mL). At 24 hours cells were harvested and surface stained for the specified protein markers. Values are represented as a fold-expression versus mock treatment alone, calculated as a ratio of geometric mean fluorescence intensities: $MFI(\text{treatment})/MFI(\text{mock})$. Each line/color represents a single DC donor. White diamonds represent mean values for each condition from the four experiments. The black line at 1 represents no change in expression from mock treatment (fold expression=1).

(Figure 4). Relative expression to mock infection of CD83 (mean±SEM, 4.73±0.50 versus 3.18±0.15) and CD80 (2.48±0.29 versus 1.77±0.21) was clearly higher in cytokine-matured cells as compared to infected cells, while differences in CD40 (1.58±0.04 versus 1.32±0.02) and HLA DR (1.96±0.41 versus 1.47±0.11) induction were less pronounced. Expression levels of CD86, PD-L1, PD-L2, and HLA A,B,C showed similarly small differences between D2V-infected and cytokine-treated DCs, although cytokine-matured cells had higher mean expression in all cases.

As expected, LPS (50 ng/mL) was a potent stimulus for DC activation, inducing the greatest increases in expression for all markers tested. CD83, CD80, CD86, and PD-L1 showed the greatest induction of expression in response to LPS, with mean geometric MFI values 4- to 6-fold those seen with mock infection (5.44±0.88, 4.80±0.94, 4.37±0.64 and 5.91±0.91, respectively). CD40, PD-L2, HLA A, B, C, and HLA DR showed less induction than the aforementioned products when compared with mock infection, ranging from 1.77±0.11 for CD40 to 2.17±0.24 for PD-L2. These data indicate that live D2V exposure stimulates expression of immunomodulatory surface molecules on DC. The degree of stimulation following infection with D2V was slightly lower than that of TNF- α /IFN- α treatment and clearly less pronounced than seen with high-dose LPS.

■ EFFECTS OF UV LIGHT INACTIVATION OF DENGUE VIRUS ON DENDRITIC CELL SURFACE MOLECULE EXPRESSION

In order to determine if D2V replicative capability was necessary for the induction of surface immunomodulatory molecules, we compared mock-infected DCs to DCs exposed to either UV-inactivated or live D2V (Figure 5). We found that exposure to UV-inactivated virus significantly enhanced expression of the B7-family molecules CD80

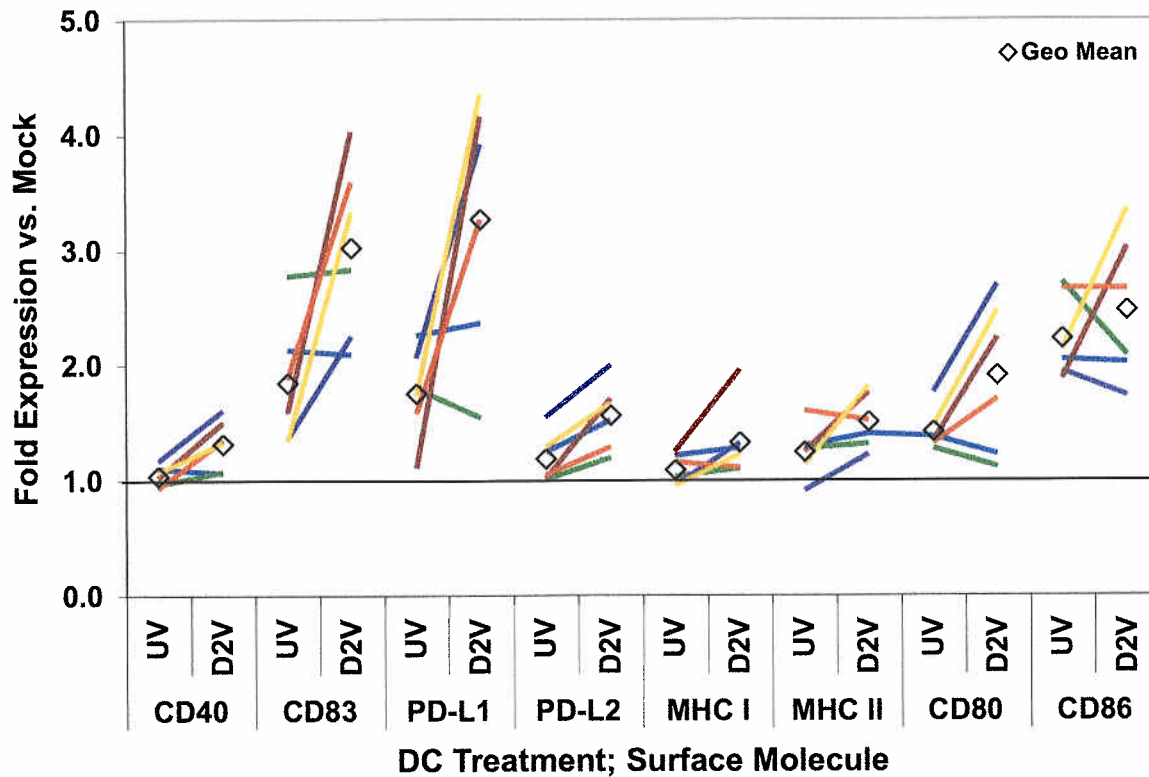


Figure 5: Replication enhances surface molecule expression changes induced following D2V exposure: DC were mock infected, infected with live D2V New Guinea C strain at an MOI=2, or infected with the same virus, inactivated with UV light as previously described (UV-D2V). DCs were harvested and surface stained as previously described, then analyzed by flow cytometry to determine mean fluorescence intensity for each surface marker. Values are represented as a fold-expression versus mock treatment alone, calculated as MFI (treatment)/MFI(mock). Each line/color represents a single DC donor. White diamonds represent mean values for each condition from the six experiments. The black line at 1 represents no change in expression versus mock treatment (fold expression=1).

(mean±SEM, geometric MFI ratio to mock infection, 1.42 ± 0.07 , $p=0.03$), CD86 (2.23 ± 0.14 , $p=0.03$), PD-L1 (1.76 ± 0.15 , $p=0.03$), and CD83 (1.84 ± 0.20). PD-L2 expression following exposure to UV-inactivated virus was also consistently higher than expression following mock infection, although the changes were modest (1.26 ± 0.22 , $p=0.03$). Treatment of DCs with UV-inactivated virus also enhanced MHCII expression, although this effect did not reach statistical significance (1.24 ± 0.08 , $p=0.06$). DC expression of HLA A, B, C (1.09 ± 0.05 , $p=0.29$) and CD40 (1.04 ± 0.03 , $p=0.31$) demonstrated minimal changes in response to UV-inactivated virus, which were not statistically significant. Infection with live virus resulted in statistically significant increases in expression for all molecules tested when compared to mock infection ($p=0.03$), with the greatest increases in PD-L1 (3.27 ± 0.41) and CD83 (3.02 ± 0.28), smaller increases in expression level for CD86 (2.48 ± 0.24) and CD80 (1.91 ± 0.25). Viral induction of CD40 (1.32 ± 0.08), PD-L2 (1.57 ± 0.11), HLA A, B, C (1.33 ± 0.12) and HLA DR (1.51 ± 0.09) was less pronounced. Despite more clear activation in response to live virus than UV-inactivated virus when comparing each treatment to mock infection, only PD-L2 expression was statistically significantly greater in a comparison of live virus to inactivated virus treatment ($p=0.03$); CD40, CD83, and MHC-I showed a trend toward greater expression following live virus infection that did not reach statistical significance ($p=0.06$). Weaker trends were seen for PD-L1 and MHC II ($p=0.09$) and CD80 ($p=0.14$), while CD86 expression was essentially identical ($p=1.0$). These experiments demonstrate that both live and inactivated D2V stimulate increases in the expression of DC surface molecules, and demonstrate that viral replication further enhances DC activation.

■ DIFFERENTIAL EXPRESSION OF DENDRITIC CELL SURFACE MARKERS ON INFECTED VERSUS BYSTANDER CELLS

Previous reports have identified several dengue proteins involved in modulating intracellular signaling (Munoz-Jordan et al., 2003; Munoz-Jordan et al., 2005; Jones et al., 2005; Chua et al., 2005). Additionally, there are conflicting reports regarding the expression of co-stimulatory molecules on dengue infected DCs versus uninfected bystander cells within the same culture (Libraty et al., 2001; Sanchez et al., 2006; Palmer et al., 2005). Therefore, we expanded our studies of cell-surface immunomodulatory proteins by infecting DC cultures and combining surface antibody staining for phenotypic markers with intracellular staining for dengue E protein, to discriminate between infected and bystander DCs (*Figure 6*). Again, we quantitated differences in the expression of surface markers by normalizing geometric MFI values for each molecule to mock infection. Both infected and bystander cells increased surface expression of all molecules tested when compared to mock infection ($p < 0.02$), with the exception of HLA A, B, C on infected DCs ($p = 1.0$). However, we noted reduced expression of the B-7 family molecules CD86 (geometric MFI ratio to mock-infected cells \pm SEM for eight donors, 1.86 ± 0.14 versus 2.61 ± 0.17), PD-L1 (2.62 ± 0.26 versus 3.33 ± 0.32), and CD80 (1.63 ± 0.19 versus 1.82 ± 0.20), as well as MHC class I molecules HLA A, B, C (1.05 ± 0.08 versus 1.36 ± 0.10) ($p < 0.02$ for all comparisons) on infected DCs, in comparison to bystander cells. CD83 was also lower on infected DCs (2.72 ± 0.21 versus 3.10 ± 0.21) when compared to bystanders, although this difference was not statistically significant ($p = 0.055$). Conversely, infected DCs had significantly greater expression than did bystander cells of PD-L2 (geometric MFI relative to mock-infected cells \pm SEM,

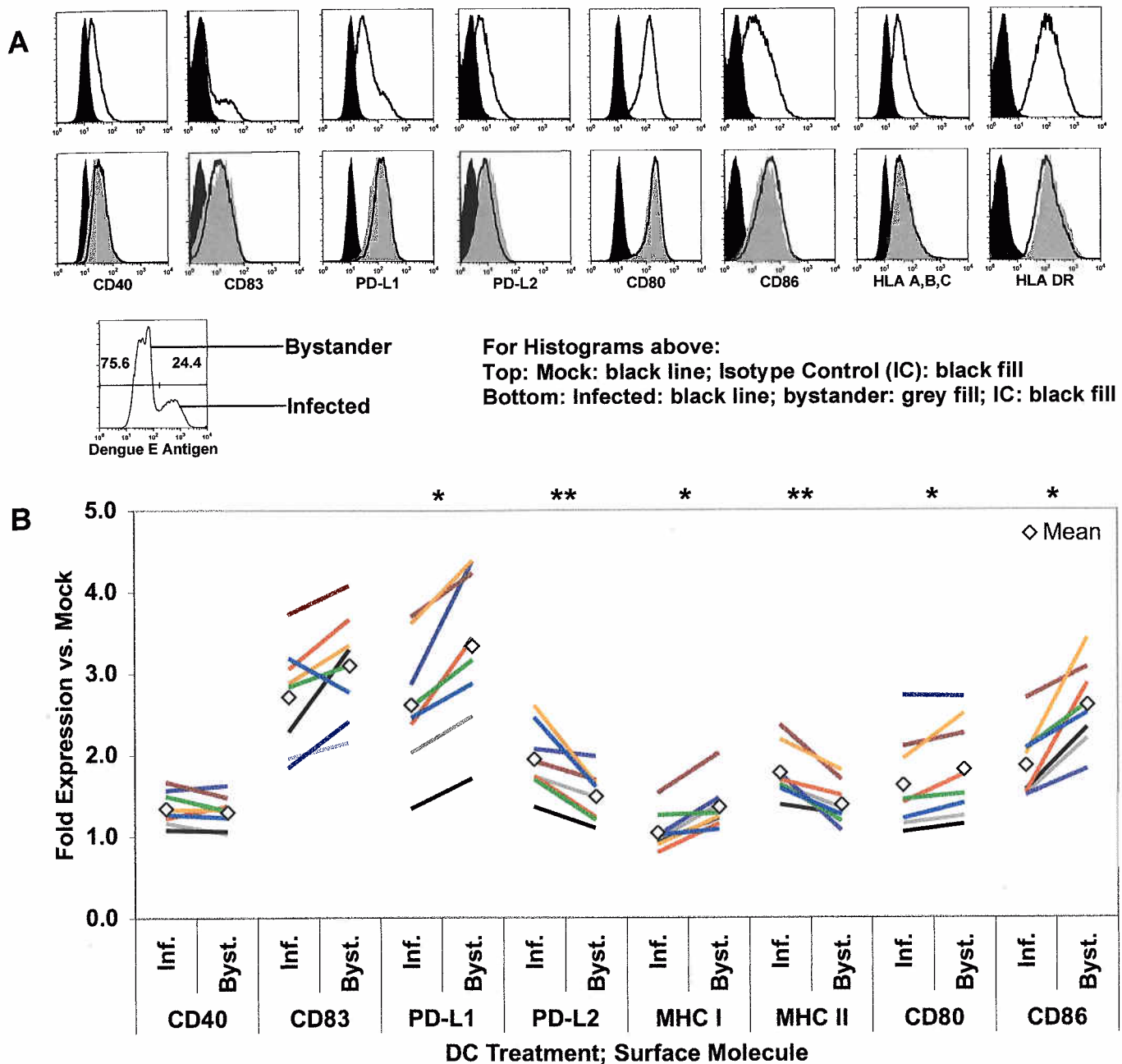


Figure 6: Actively infected cells have altered surface molecule expression when compared to bystanders: DC infected with dengue virus 2 New Guinea C strain MOI=2 were harvested at 24 hours and surface stained for the specified proteins prior to fixation and intracellular staining for dengue E protein. Cells were gated by flow cytometry based on dengue E protein expression, detected with a monoclonal IgG2a anti-dengue E antibody bound to a Pacific Blue™-conjugated anti-IgG2a F_{ab} fragment. **A)** Representative histograms for each surface molecule on DCs from one donor. Top: Mock infection versus isotype control staining. Bottom: Surface molecule expression in infected versus bystander DC. **B)** Expression of each surface molecule on infected and bystander DC populations was quantitated by geometric mean fluorescence intensity and normalized to mock infection for each donor, as previously described. Each line/color represents a single DC donor. White diamonds represent mean values for the eight individual donors. Asterisks above graph (*): P<0.05 for infected<bystander; **: P<0.05 for infected>bystander, both by two-tailed Wilcoxon ranked signs test.

1.95±0.14 versus 1.49±0.10) and HLA DR (1.78±0.11 versus 1.39±0.09) ($p < 0.02$ for both comparisons). CD40 (1.35±0.07 vs. 1.30±0.07) was unaffected by the presence of viral antigen ($p = 0.31$). These data demonstrate that D2V exposure activates DCs through both direct and indirect mechanisms. The differences in protein expression could not be explained solely by viral suppression of IFN- α/β signaling, since relative differences were bi-directional. Active DV replication both positively and negatively modifies DC phenotype, depending on the surface molecule in question.

■ CHAPTER SUMMARY

In this chapter, we demonstrated that DCs could be efficiently generated from PBMC following CD14-positive selection of monocytes and culture with GM-CSF and IL-4. Monocyte-derived DCs were permissive for infection with multiple serotypes and strains of DV, including both prototype viruses and low-passage clinical isolates. Dengue E protein staining was highest at 24 hours post-infection with D2V NGC strain and declined at 48 hours post-infection. While infection with D2V NGC was dose-dependent, there was substantial variability in the susceptibility of DC preparations from different donors.

D2V infection activated DC to express increased levels of surface molecules involved in immunoregulation. The changes seen in response to D2V were similar to, but slightly lower than, those seen with TNF- α /IFN- α , and were clearly lower than those seen following LPS treatment. The enhancement of surface molecule expression did not require replication-competent virus, although the magnitude of the changes seen with live virus were greater than those seen with UV-inactivated virus. While both infected and

bystander DCs had enhanced surface molecule expression, these two populations demonstrated statistically significant differences in phenotype. Specifically, actively infected DCs had reduced expression of CD80, CD86, PD-L1, CD83, and MHC Class I molecules but increased expression of PD-L2 and MHC Class II molecules when compared to bystander DCs in the same culture.

CHAPTER IV

INDUCTION AND REGULATION OF DENDRITIC CELL CYTOKINE AND CHEMOKINE RESPONSES BY DENGUE VIRUS

■ DENDRITIC CELL INFLAMMATORY MEDIATOR SECRETION PATTERNS

Previous *in vitro* studies with other viruses indicated that monocyte-derived DCs were capable of production of a wide range of secreted products (Zilliox et al., 2006; Piqueras et al., 2006). D2V infection of DCs has been reported to cause the production of inflammatory cytokines such as IFN- α and TNF- α (Libraty et al., 2001; Ho et al., 2001), and chemokines including IL-8 and RANTES (Medin et al., 2005). We hypothesized that dengue infection of DCs would induce a largely donor-independent, characteristic secretory response. Using a multiplex cytokine bead assay, we compared the secretory responses in 24-hour supernatants of mock-infected and D2V-infected DCs. Virus infection was a potent stimulus for the secretion of chemokines (*Figure 7*). IP10, RANTES, MIP-1 α , and MCP-1 were all found at substantially higher levels in supernatants from infected cultures when compared to those from mock-infected DCs. Of these, IP10 reached the highest concentrations, uniformly greater than the maximum threshold for quantitation in this assay, at 18750 pg/mL. Concentrations of IL-8 and Eotaxin also increased with virus infection, although the effect of dengue exposure was less pronounced, as basal levels with mock infection were generally higher than those seen for IP10, RANTES, MIP1- α , and MCP-1.

Inflammatory cytokines including TNF- α , IL-6, and IL-1 α were increased in culture supernatants when compared with mock-treated cells. Of these, IL-1 α reached

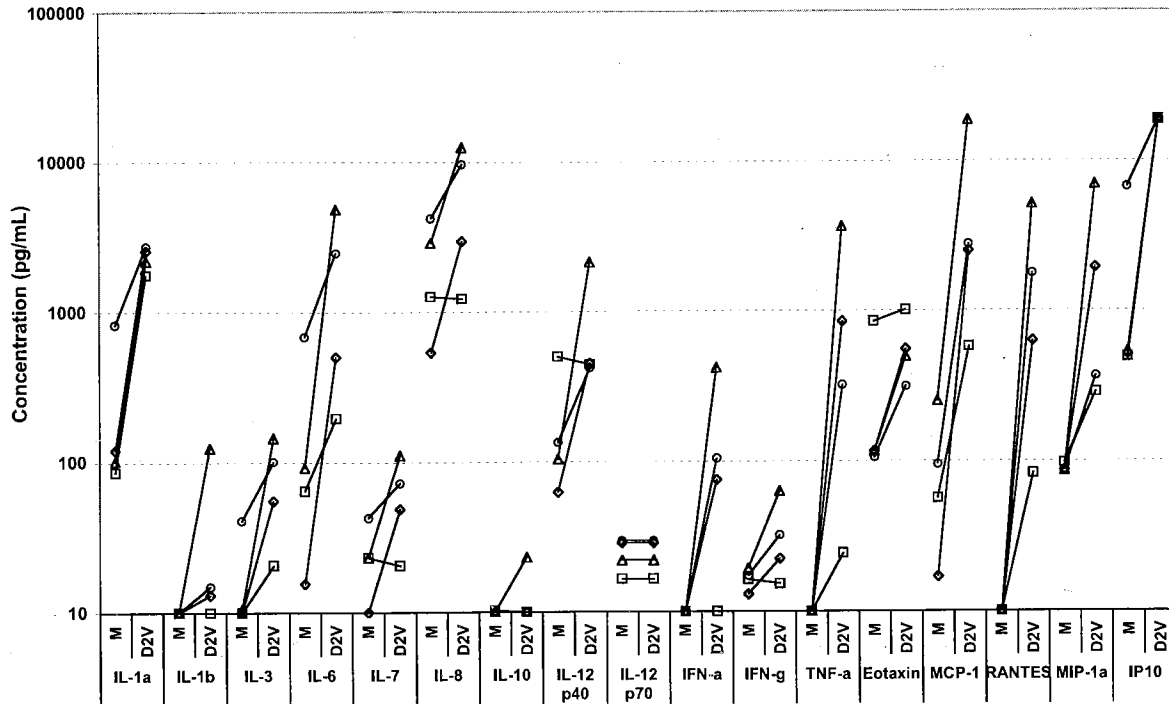


Figure 7 D2V infection of DC cultures induces a broad inflammatory cytokine and chemokine secretory response.

DCs were mock-infected (M) or infected with D2V NGC strain (D2V) as previously described, at an MOI=2. Cells were washed and re-suspended at 10^6 DCs per mL. Supernatants were collected at 24 hours and analyzed using Luminex™ technology for a panel of immune mediators. Shown are the results from four independent experiments. Samples for which the values fell below the limit of detection are plotted at the detection limit. Samples for which the values exceeded the maximum threshold are plotted at the upper limit of quantitation, as determined by the highest value utilized in preparation of the standard curve (See Materials and Methods).

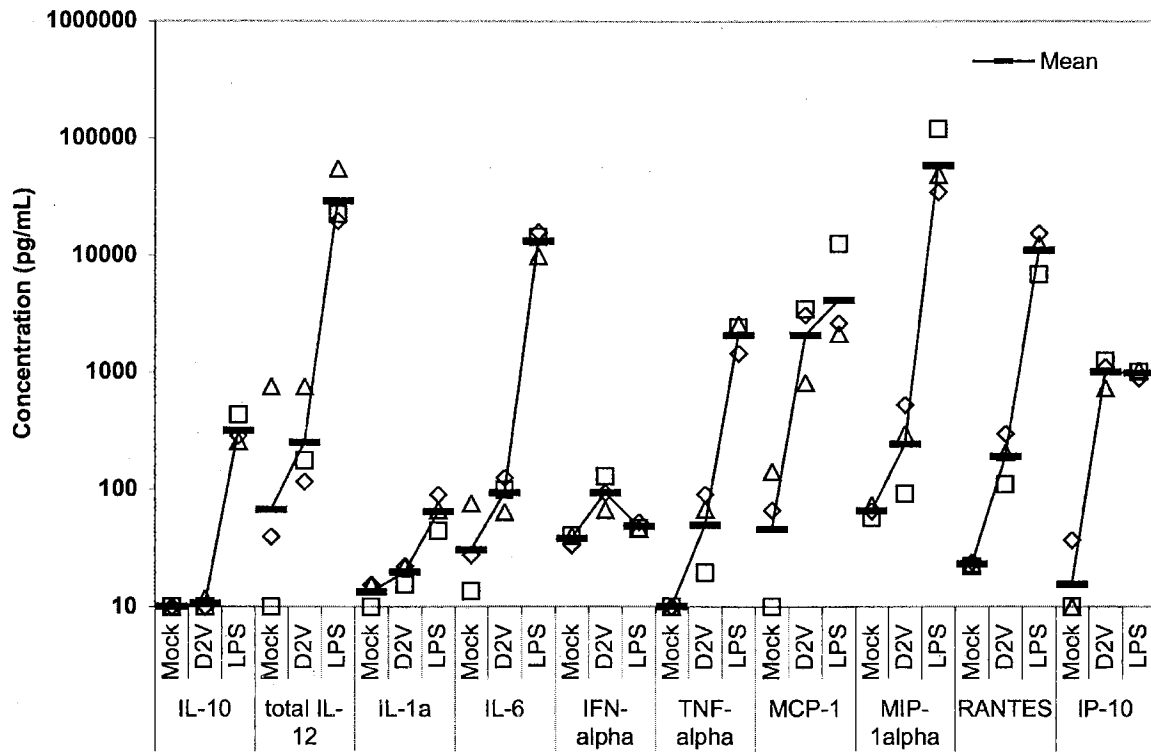


Figure 8 DC responses to LPS and D2V differ both qualitatively and quantitatively. Immature DC from three donors were mock-infected or infected with live D2V NGC at an MOI=2 as previously described. Following the virus adsorption period and return to culture at 1.0×10^6 cells/mL, some mock-treated cells were activated with LPS at 50 ng/mL. Supernatants were collected after 24 hours and stored at -70°C for analysis. Samples were analyzed using a multiplex cytokine assay. Symbols represent three individual donors; black bars and lines represent geometric mean values.

the highest levels (mean, 2316 pg/mL; range, 1764-2734 pg/mL). TNF- α appeared to be the most inducible, being undetectable in all mock-treated cultures and reaching a mean concentration of 1216 pg/mL (range, 24-3673 pg/mL) in supernatants from infected DCs. We also detected elevated levels of the T cell growth factor IL-7 and the PDC growth factor IL-3 following dengue viral infection of DCs. IFN- α secretion was undetectable in mock-infected cells, but became elevated in three of four donors in response to D2V infection (mean, 200 pg/mL; range 75-420 pg/mL). IFN- α was undetectable in supernatants following either treatment in one donor (experiment 2, *see Figure 7*). This donor notably appeared to have a blunted overall response. This was evident in IL-12 p40 production as well, where three of four experiments showed 3- to 20-fold increases following infection, while this donor showed essentially no change, despite an infection rate of 35% of DCs in the culture (not shown). In all experiments the levels of IFN- γ -inducing IL-12 p70 heterodimer were 1-2 orders of magnitude lower than those of the subunit IL-12 p40, and there was no clear virus-specific response for this cytokine. IL-10 production was detected slightly above the assay threshold in one of four mock-infected samples (10 pg/mL) and one of four D2V-infected samples (23 pg/mL). Separate experiments demonstrated no differences in transforming growth factor beta (TGF- β)-1, -2, or -3 isoform secretion between mock- and D2V-infected cultures (data not shown).

To confirm that DCs were capable of IL-10 and high-level IL-12 production, we compared secretory responses in mock infected DC cultures to those treated with live D2V or the known DC activator LPS. This set of experiments compared 24-hour culture supernatants from three donors' DCs after mock-infection, infection with live virus, or stimulation with LPS at 50 ng/mL (*Figure 8*). LPS treatment induced the secretion of

IL-10 (mean, 327 pg/mL; range, 259-433 pg/mL, N=3), while IL-10 was undetectable in mock-infected cultures and was detectable (12 pg/mL) in only one D2V-infected culture supernatant. Secreted levels of IP10 with LPS stimulation were similar to those seen with dengue infection, while IFN- α secretion was slightly lower. Levels of TNF- α , MCP-1, IL-12 p40, MIP-1 α , RANTES, and IL-6 were much greater with LPS treatment than with dengue virus infection. These data demonstrate that iDCs produce and secrete a broad array of immune mediators in response to D2V infection that is clearly distinct from LPS stimulation, with low relative IL-10 production.

■ EFFECTS OF UV LIGHT INACTIVATION OF DENGUE VIRUS ON SECRETORY RESPONSES TO INFECTION

The putative DC receptor for dengue, DC-SIGN, is a C-type lectin capable of intracellular signaling (Caparros et al., 2006; Hodges et al., 2007). Viral replication is associated with the activation of intracellular signaling pathways as well, most notably through dsRNA-sensing pathways such as RIG-I and mitochondrially-associated MAVS (Yoneyama et al., 2004; Hiscott et al., 2006) or endosomal TLR3 (Lee et al., 2006) (Alexopoulou et al., 2001). In order to distinguish cytokine responses to virion binding from those requiring viral replication, we compared cytokine and chemokine levels in supernatants from mock-infected, UV-inactivated virus-treated, or live-virus infected DC (*Figure 9*). In experiments with four separate donors, both UV-inactivated virus and live virus induced the secretion of several cytokines and chemokines, most convincingly

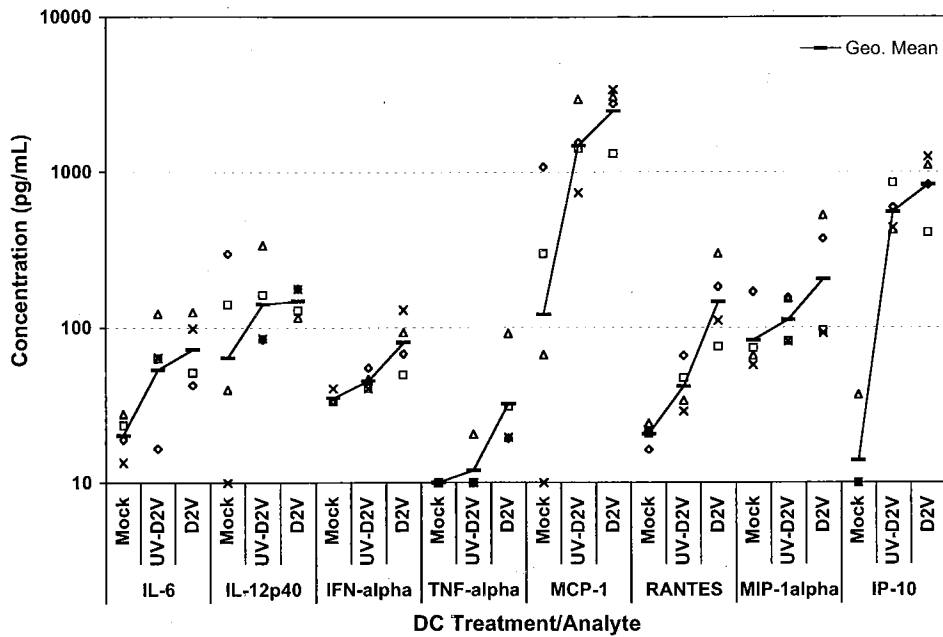


Figure 9 Viral replication enhances DC secretory responses to DV exposure.

DCs were mock-infected, or infected with UV-killed or live dengue 2 virus New Guinea C strain at an MOI=2 as previously described. Following adsorption, cells were washed and re-suspended at 10^6 DC per mL. Supernatants were harvested at 24 hours and analyzed using Luminex™ technology. Each symbol represents a separate DC donor. Lines with bars represent the geometric means of the four independent values for each analyte under each condition.

MCP-1 and IP-10. Levels of MIP-1 α , RANTES, IFN- α , and TNF- α were lower in UV-treated DC supernatants than in those from virus-infected DCs. Neither treatment with inactivated virus nor live virus induced secretion of IL-12 p40 or IL-1 α in these experiments. These results demonstrate that binding of D2V is sufficient for the induction of some secretory responses, but that viral replication augments DC cytokine secretion.

■ DOSE-RESPONSIVENESS OF DENDRITIC CELL SECRETORY RESPONSES TO INFECTION WITH DENGUE VIRUS

In order to determine if the secretion of cytokines and chemokines by DCs following D2V exposure was related to the virus dose and the percentage of infected cells, we measured TNF- α , RANTES, MIP-1 α , IFN- α , IP-10, and IL-10 in culture supernatants from seven donors' DCs following infection with D2V over a range of MOIs (*Figure 10*). In this series of experiments, we noted distinct profiles between these products. Levels of TNF- α , RANTES, and MIP-1 α in the culture supernatants increased with increasing MOI, although DCs from donor 2 were the exception for each cytokine. For example, DCs from donor 2 showed potent TNF- α secretion at lower MOIs (peaking at >700 pg/mL at an MOI=0.2), despite absent detectable dengue E protein, but unlike the remaining six donors, detection of TNF- α decreased at the highest MOIs, when infection became evident.

A similar pattern was detected with both RANTES and MIP-1 α production, as these chemokines were produced at progressively higher levels with increasing MOI for all donors' DCs save donor 2. Both of these chemokines were potently induced at low

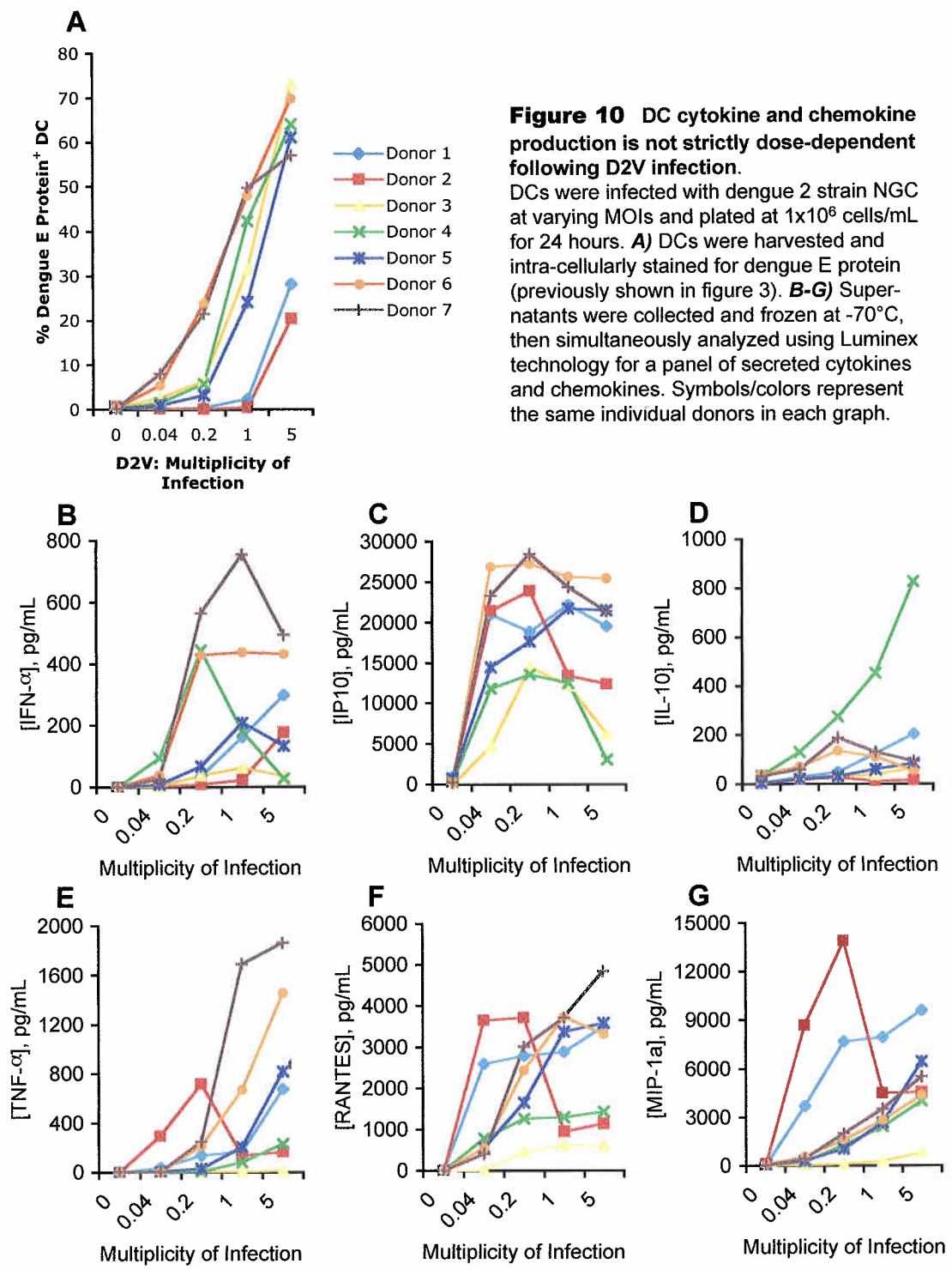


Figure 10 DC cytokine and chemokine production is not strictly dose-dependent following D2V infection.

DCs were infected with dengue 2 strain NGC at varying MOIs and plated at 1×10^6 cells/mL for 24 hours. **A)** DCs were harvested and intra-cellularly stained for dengue E protein (previously shown in figure 3). **B-G)** Supernatants were collected and frozen at -70°C , then simultaneously analyzed using Luminex technology for a panel of secreted cytokines and chemokines. Symbols/colors represent the same individual donors in each graph.

MOIs by DCs from both donor 2 and, to a lesser extent, donor 1, despite neither donors' DCs demonstrating substantial infection except at an MOI of 5. Dose-responsiveness of IFN- α production was highly variable between DCs from different donors. For example, DCs from donor 3 showed minimal IFN- α production at all MOIs studied. On the other hand, supernatants from donors' 4, 6, and 7 DCs showed high IFN- α levels at an MOI=0.2, with detected levels showing a plateau in DC supernatants from donor 6, a rapid drop in donor 4, and a milder drop in donor 7 at higher MOIs. IP10, on the other hand, was secreted at high levels in all donors, even with minimal infection (at an MOI=0.04). IP10 levels reached a plateau in the midrange of MOIs, and in four of seven donors, dropped as infection rates increased.

IL-10 was detected in culture supernatants from DCs derived from all donors in this series of experiments. IL-10 levels showed progressive dose-dependent increases and were highest at an MOI=5 for supernatants from four of the seven donors (1, 3, 4, and 5); supernatants from the remaining donors' DCs demonstrated peak levels at an MOI=0.2. High-level (>800 pg/ml) IL-10 secretion was detected in supernatants from the DCs of donor 4, although infection rate response curves for this donor's DCs were similar to those in DCs from donors 3 and 5, which had much lower (63 pg/mL and 85 pg/mL, respectively) IL-10 production.

These results demonstrate that TNF- α , MIP-1 α , and RANTES production by DCs in response to D2V infection increase progressively as the D2V dose increases, while IFN- α and IL-10 production show similar effects in only some donors, and decrease at higher MOIs in others. High-level IP10 secretion was not dose-dependent.

■ DIFFERENTIAL PRODUCTION OF TNF-ALPHA AND IP10 BY INFECTED VERSUS BYSTANDER CELLS FOLLOWING DENGUE EXPOSURE

Because dengue proteins are implicated in interferon inhibition (Munoz-Jordan et al., 2005; Munoz-Jordan et al., 2003), we hypothesized that a consequence of differential interferon signaling would be distinct cytokine production profiles in actively infected versus bystander DCs. To answer this question, we first mock-infected DCs, or infected them with either UV-inactivated or live D2V, and used brefeldin A treatment coupled with intracellular cytokine staining to measure TNF- α and IP10 production in the 16-24 hour time period, while using anti-dengue staining to distinguish infected from uninfected cells. We chose these analytes based on high levels of secretion suggesting adequate detectability, and to reflect two different types of response, specifically, inflammation versus chemoattraction. In addition, these products showed differing patterns of dose-dependence. Mock-infected cells produced neither TNF- α nor IP10, and UV-inactivated virus induced low levels of IP10, without causing TNF- α production during this timeframe (*Figure 11*). However, in DCs infected with live D2V, we noted marked production of both proteins (*Figures 11, 12*). In addition, we found a clear disparity in production of these proteins on the single-cell level, depending on the presence of detectable viral E protein (*Figure 12A*). IP10 induction occurred primarily in bystander cells, while TNF- α was predominantly associated with infected cells (*Figure 12C*).

Analysis of multiple experiments demonstrated that the frequency of cells staining positive for IP10 was consistently lower in infected cells than in bystanders (mean \pm SD, 0.252 \pm 0.160 vs. 0.455 \pm 0.216), $p=0.001$) (*Figure 12B*). Bystander DCs demonstrated a range of IP10 staining, including IP10-high populations, whereas infected DCs had

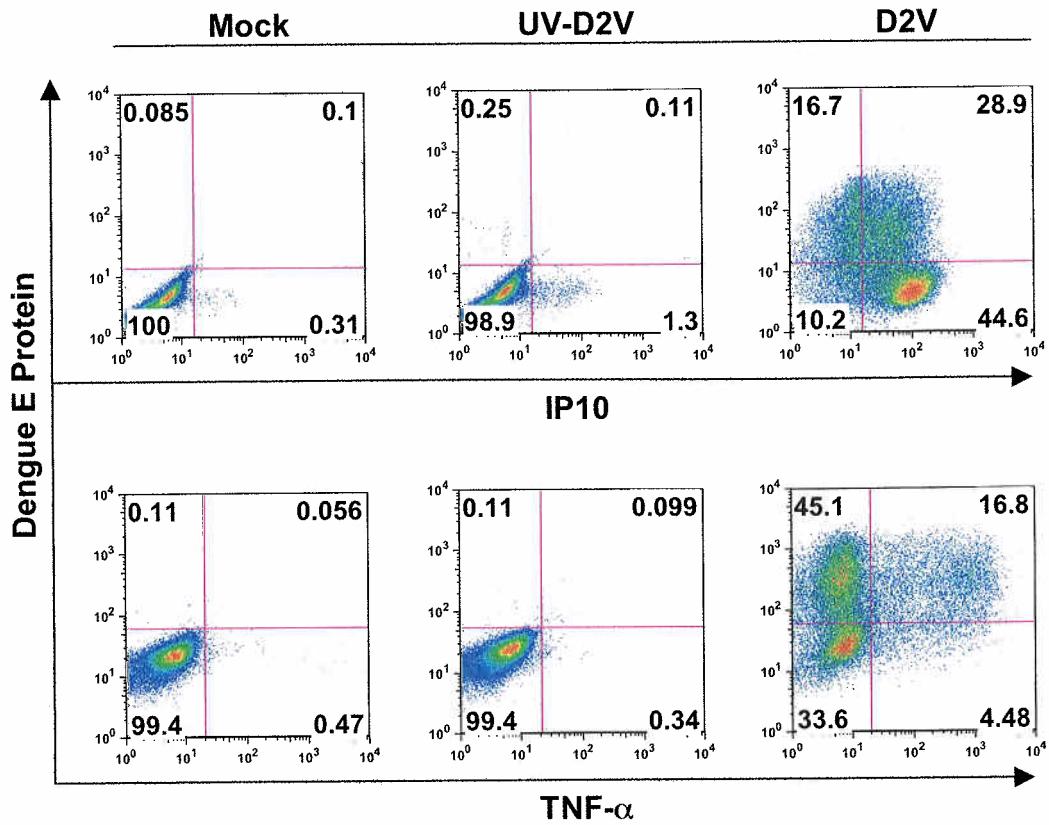


Figure 11: Replication-competent virus is required for detection of intracellular production of TNF- α and IP10. DCs were mock-infected or infected with UV-inactivated D2V NGC at an MOI=2 (UV-D2V), or live D2V NGC at an MOI=2 (D2V) as previously described. Brefeldin A was added at 18 hr., and the cells harvested and fixed at 24 hr. Cells were stained using an anti-dengue E protein monoclonal antibody and monoclonal antibodies for IP10 (top), or TNF- α (bottom). Results are representative of at least 3 independent experiments for each cytokine/chemokine under each specified condition.

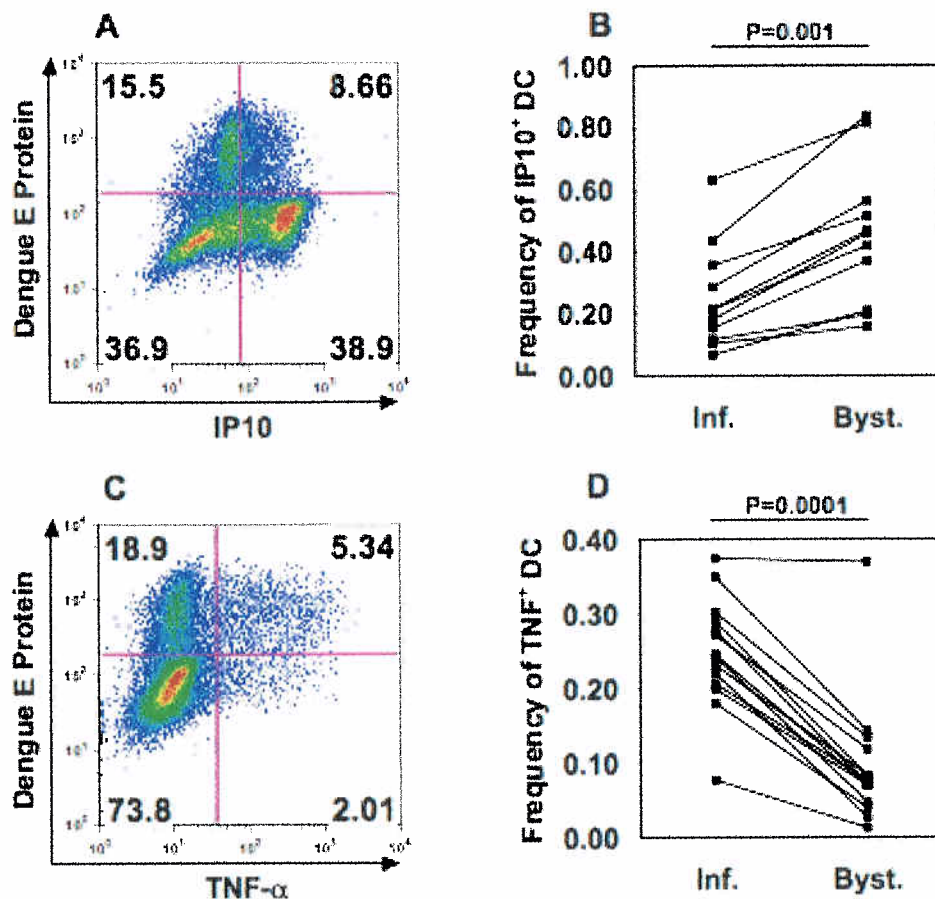


Figure 12: Infected DC are deficient in IP10 production but more effectively induce TNF- α when compared to bystanders:

DCs were infected with D2V NGC at an MOI=2 as previously described and brefeldin A was added after 16 or 18 hr in culture, followed by fixation at 24 hr. Cells were stained using fluorochrome-conjugated anti-dengue E protein monoclonal antibody and monoclonal antibodies directed against IP10 and/or TNF- α . **A)** Representative flow cytometry plot of DCs showing dengue E protein staining versus intracellular IP10. **B)** Analysis of multiple experiments, showing the frequency of IP10-expressing cells in infected or bystander DC populations, as assessed by dengue E protein staining. **C)** Flow cytometric plot from the same donor as shown in A, demonstrating dengue E protein staining compared with TNF- α production. **D)** Analysis of multiple experiments, as described in (B), for TNF- α production.

uniformly low to negative IP10 staining. On the other hand, TNF- α positive cells were 2.6 times more abundant in the infected DC population than in uninfected bystander cells (mean \pm SD frequency, 0.248 \pm 0.072 vs. 0.096 \pm 0.084, $p=0.0001$) (*Figure 12 D*). These findings demonstrate that on a single-cell level, D2V infection of DCs differentially regulates IP10 and TNF- α , inhibiting IP10 production while enhancing that of TNF- α .

■ ENDOGENOUS DENDRITIC CELL-DERIVED IFN-ALPHA/BETA

INHIBITION OF DENGUE VIRUS INFECTION

Dengue susceptibility to the antiviral effects of interferons has been described in immortalized cells, but required pretreatment to be effective (Diamond et al., 2000; Diamond and Harris, 2001). Although monocyte-derived DCs produce substantially less IFN- α than plasmacytoid DCs in response to viruses (Izaguirre et al., 2003; Colonna et al., 2002), we hypothesized that the endogenous IFN- α/β induced following D2V exposure might inhibit active infection. We utilized the anti-CD118 (IFN- α receptor subunit 2; IFNAR2) monoclonal antibody to block IFN- α/β signaling in DCs during virus adsorption and culture. DCs infected in the presence of blocking antibody exhibited a higher percentage of dengue antigen-positive cells than DCs infected in the absence of antibody or the presence of a control antibody (*Figure 13A*). In five experiments, the percentage of cells infected at 24 hours was similar for D2V alone (mean \pm SD, 28.0 \pm 8.2%) and D2V plus control IgG_{2a} antibody (26.2 \pm 5.1%), but increased with blocking antibody (38.8 \pm 7.5%, $p=0.06$) treatment (*Figure 13B*). These results demonstrate that IFN- α/β produced by monocyte-derived DCs inhibits ongoing D2V infection.

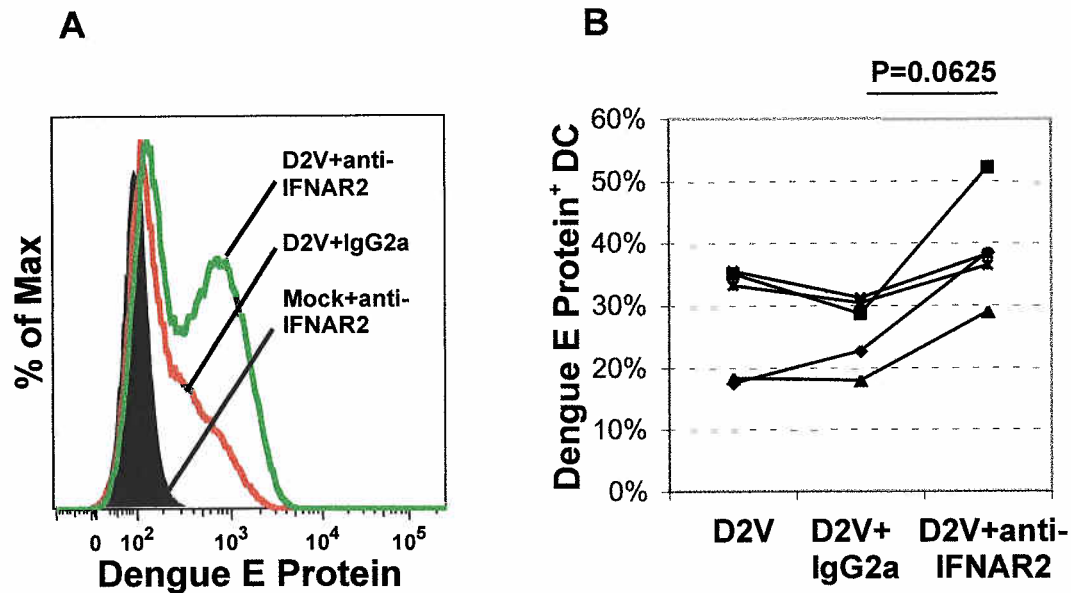


Figure 13 Endogenous type I interferon inhibits dengue infection of DCs: DCs were preincubated for 30 min. with medium, control IgG2a monoclonal antibody, or anti-interferon alpha receptor subunit 2 (IFNAR2, CD118) at 50 μ g/mL. DEN was then adsorbed to DC for 90 minutes (resulting in antibody dilution to 10 μ g/mL) and the cells cultured for 24 hr without Ab, or with IgG2a or anti-IFNAR2 antibody at 10 μ g/mL. Cells were harvested and stained for intracellular DV antigen using a monoclonal anti-dengue envelope antibody. **A)** Representative flow cytometric analysis of dengue antigen staining. Black fill: Mock-infected cells treated with IFNAR2 blocking antibody; Red: Infected cells treated with IgG2a; Green: Infected cells treated with IFNAR2 blocking antibody. **B)** Percent of DC staining positive for dengue E protein in experiments with five separate donors.

■ EFFECTS OF IFN-ALPHA/BETA SIGNALING ON DENDRITIC CELL IP10 AND TNF-ALPHA PRODUCTION AFTER INFECTION WITH DENGUE VIRUS

Viral antigen expression was positively associated with TNF- α production and negatively associated with IP10 production. Potent IP10 production in bystander cells suggested that a soluble factor was responsible, and IFN- α is capable of inducing IP10 in monocyte-derived DCs (Padovan et al., 2002). We therefore hypothesized that blocking IFN- α/β would suppress DC IP10 production in response to infection with D2V, but would not affect the expression of TNF- α . For these experiments, we pre-treated DCs with anti-CD118 (a blocking antibody for the IFN- α receptor subunit 2), isotype control antibody, or medium alone, followed by adsorption of NGC at an MOI of 2, washing, and re-culture in the presence of the original antibody treatment. In control experiments, we used a similar approach to pre-block CD118, followed by mock infection, washing, re-culture, and addition of IFN- α at 1000 U (>5.5 ng) per milliliter, or roughly 5- to 10-fold greater concentrations than the peak levels we observed in supernatants from infected cultures (*refer to Figures 7, 8, 9, and 10*).

In this series of experiments, we had three cultures where we measured greater than 20% of all cells staining positive for IP10 after infection with dengue 2 virus NGC (*Figure 14A*). IP10 production in DCs infected with live virus and treated with anti-CD118 blocking antibody was reduced (mean \pm SD frequency of all IP10⁺ DC, 0.156 \pm 0.041) compared with isotype control antibody (0.414 \pm 0.124) or no antibody (0.381 \pm 0.100) (*Figure 14B*). IP10 inhibition by IFN- α/β block occurred in both the infected and bystander cell populations. In control experiments, we noted total inhibition of IP10 production in response to exogenous IFN- α using this concentration of blocking

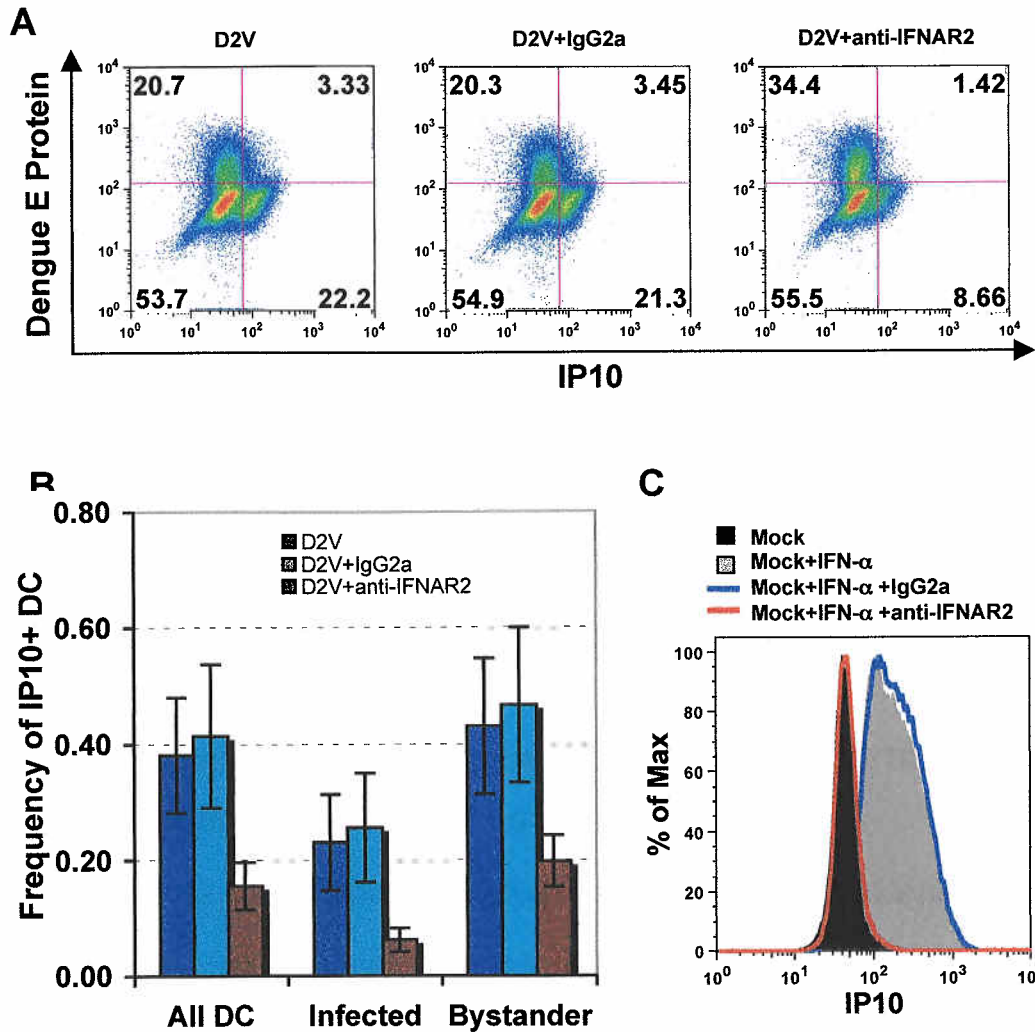


Figure 14 Blocking endogenous type I interferon signaling inhibits DC IP10 production in response to dengue infection.

A) Addition of IFNAR2 blocking antibody, but not isotype control antibody, reduces IP10 production in both infected (upper right quadrant) and bystander (lower right quadrant) DCs. Representative flow cytometry from one of 3 experiments. **B)** The frequency of IP10 expressing cells by flow cytometry was calculated for each cell population specified. Shown are the means \pm SD for three experiments, demonstrating that IP10 production is inhibited in both infected and bystander cells by blocking type I interferon signaling. **C)** Mock-treated cells (black fill) produce IP10 in response to IFN- α treatment at >5 ng/mL (grey fill). Addition of blocking antibody (red line) but not isotype control antibody (blue line) completely abrogates IP10 production in response to IFN- α . Representative of 3 experiments.

antibody (*Figure 14 C*). IFN- α/β block had minimal effects on the overall frequency of TNF- α^+ cells (*Figure 15A and B*). In five experiments, the mean \pm SD frequency of all TNF- α^+ DC with virus treatment alone (0.14 ± 0.018) was similar to cultures treated with the addition of isotype control IgG_{2a} (0.131 ± 0.026), and anti-CD118 antibody (0.117 ± 0.025) treatment had no effect on the overall expression of TNF- α (*Figure 15 B*). These results demonstrate that endogenous IFN- α/β produced by DCs in response to DV infection acts in an autocrine/paracrine manner to induce IP10 production. The lack of complete blocking, in light of the fact that high-dose exogenous IFN- α treatment could be fully inhibited in our system, suggests that IP10 induction was not solely IFN- α/β dependent.

■ STRAIN-DEPENDENT DIFFERENCES IN DENDRITIC CELL IP10 INDUCTION FOLLOWING INFECTION WITH DENGUE VIRUS

Previous studies using intracellular cytokine staining have indicated that there are differences between attenuated and wild type dengue strains in the viral antigen-dependence of dendritic cell cytokine responses, including TNF- α (Sanchez et al., 2006). We therefore postulated that dengue viral strains might also differ in the patterns of induction of IP10. In four experiments, we infected DCs from the same donor using control C6/36 supernatant (mock infection), live D2V strain NGC, or live D2V strain C0166/96, a low-passage clinical isolate from a DF patient in Thailand (Vaughn et al., 1997). Both viruses were tested at an MOI of 2. Using intracellular staining for dengue E protein and IP10 as previously described, we quantitated IP10-producing DCs and

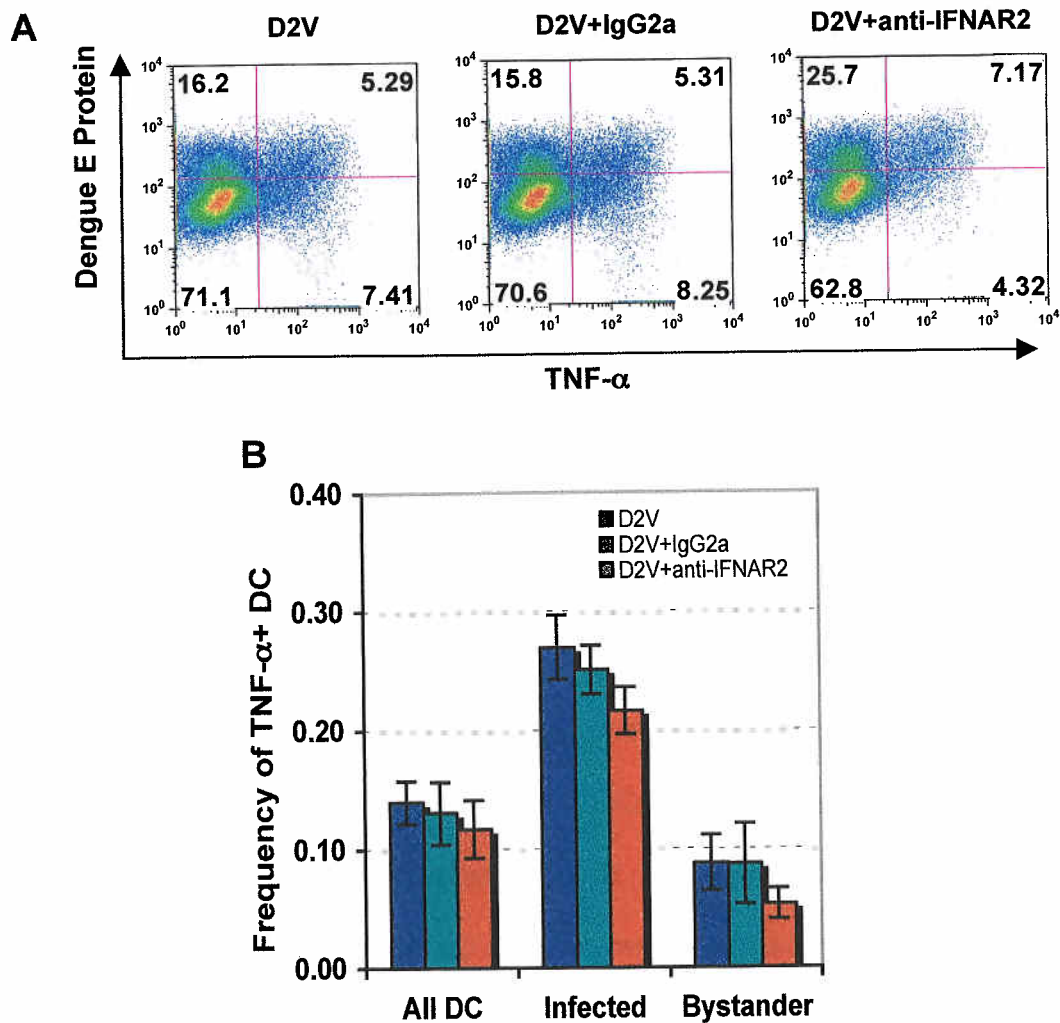


Figure 15 Blocking endogenous type I interferon does not inhibit TNF- α production by DCs.

DCs were left untreated or were treated with IgG2a or anti-IFNAR2 antibody as previously described, followed by addition of D2V NGC at an MOI=2.

A) Representative flow cytometry plots from the same donor as in Figure 14 (IP10), showing intracellular dengue E protein and TNF staining. One of five similar experiments. **B)** Mean frequencies \pm SD of TNF-positive cells in the specified populations from five DC cultures.

compared infection rates, in addition to assessing the respective abilities of the two viruses to induce IP10 in both infected and bystander DCs.

We noted a clear distinction between IP10 responses to NGC and those to the low passage clinical isolate. Representative flow cytometry shown in *Figure 16A* demonstrates that NGC induced IP10 potently in bystander DCs, and weakly in infected cells. By way of comparison, the Thai C0166/96 isolate largely failed to induce IP10 in either infected or bystander cell populations (*Figure 16B*). The percentage of DCs infected varied among donors for both viruses (*Figure 16C*), however overall infection rates were similar across the four experiments between NGC (mean±SD, 29.2±10.0%) and C0166/96 (26.5±8.6%) strains.

To determine if the relative lack of IP10 with low-passage strain infection at the 24-hour time point was explained by altered kinetics of IP10 production, we performed a 24-hour time course experiment. We treated DCs with brefeldin A for three different 8-hour intervals over the 24-hour infection period. DCs from each 8-hour timeframe (0-8 hours, 8-16 hours, and 16-24 hours) were harvested and fixed immediately following their respective incubation period (8, 16, or 24 hours). DCs from all samples were simultaneously stained following completion of the experiment. Although dengue E protein was detected earlier with C0166/96 than with NGC infection, IP10 was not (*Figure 16D*).

Together, these data indicate that our low-passage D2V strain was deficient in IP10 induction when compared with the laboratory-adapted strain NGC. This effect was neither related to differences in susceptibility to infection, nor was it due to kinetic differences in antigen or IP10 expression between viruses for this time period.

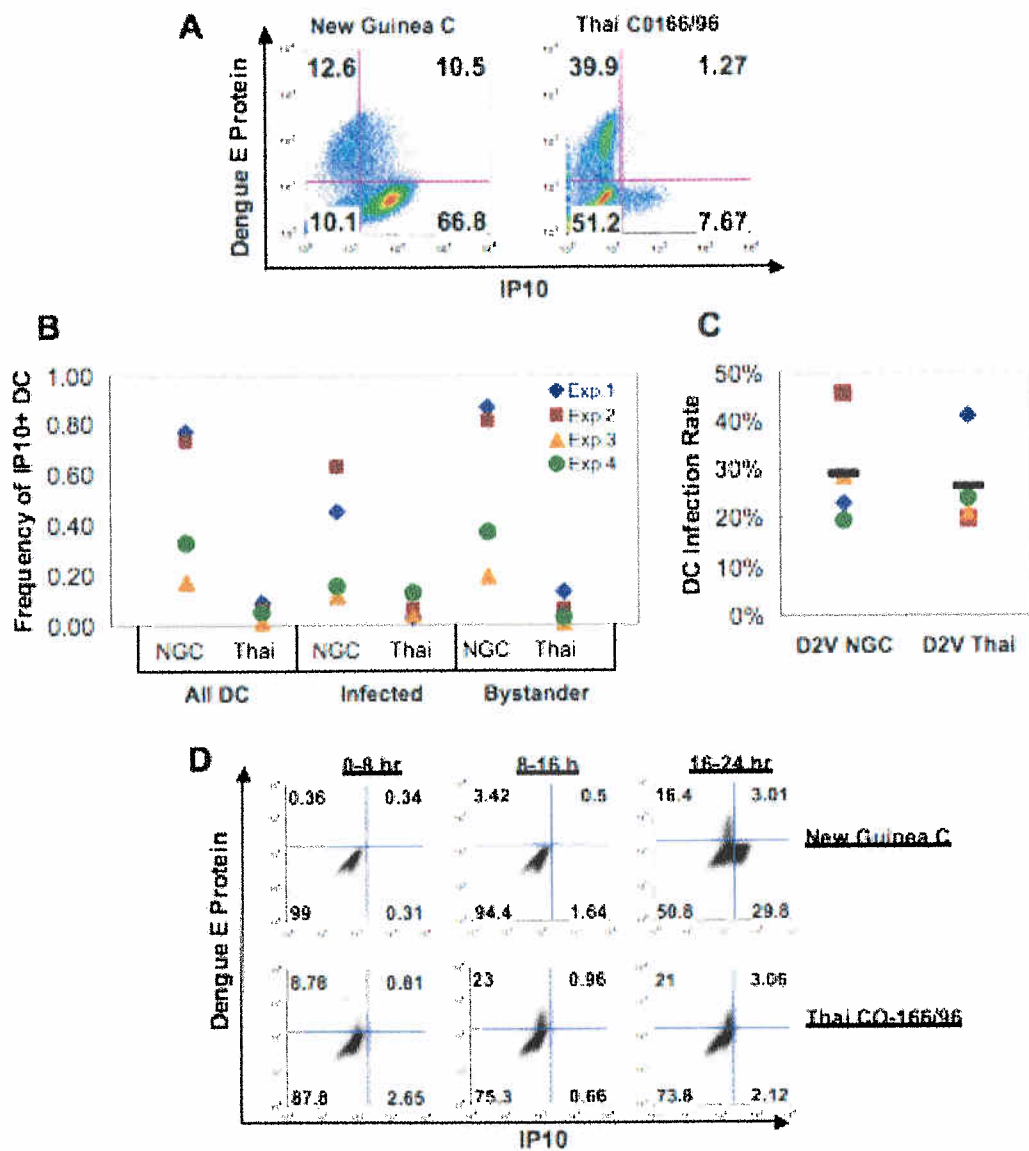


Figure 16: D2V strains differ in their ability to induce IP10 production in DC: DC were infected as previously described with dengue 2 strains New Guinea C or the low-passage Thai DF patient isolate, C0166/96, both at an MOI of 2. **A)** Representative flow cytometry plots. Brefeldin A was added at 18 hours, and the cells harvested and intracellularly stained for IP10 and dengue E protein at 24 hours. **B)** Frequencies of IP10-expressing cells in the specified populations in four experiments as described in (A). **C)** Infection rates as calculated by frequencies of DV E Protein+ DC in the four experiments using NGC and low-passage Thai strains shown in (B). **D)** In one experiment using two donors, cells were treated with brefeldin for 8-hour intervals, and harvested immediately at the end of the specified time period. Data shown are from one of two donors with similar results.

■ CHAPTER SUMMARY

In this series of experiments, we found that D2V stimulated DC secretion of a wide range of inflammatory cytokines and chemokines. Initial experiments comparing mock-infected DCs to D2V-infected DCs revealed that in terms of absolute amounts the chemokines IP10, MCP-1, MIP-1 α , and RANTES were the most potently induced molecules assayed. D2V infection of DCs also consistently induced the secretion of inflammatory cytokines such as TNF- α , IL-6, and IL-1; three of four donors also responded with IFN- α secretion. D2V was not a potent stimulus for the release of IL-12 p40, as this cytokine was only weakly induced in two independent series of experiments, and IL-12 p70 was not a factor in the DC response to D2V.

Results regarding IL-10 were inconsistent, as D2V did not induce this cytokine in two sets of analyses, while in virus dose-response experiments we detected IL-10 secretion, albeit low in most donors. A substantial IL-10 response was observed in only one of seven donors' DCs in the dose-response experiments. UV-inactivated D2V induced similar levels of MCP-1, IP10, as did live D2V, and IL-12 p40 and IL-6 levels were also similar with both treatments. DC secretion of the chemokines MIP-1 α and RANTES, as well as the cytokines TNF- α and IFN- α , was lower in UV-inactivated DV-infected DCs than DCs infected with live virus.

Using increasing amounts of input virus, we found that MIP-1 α , TNF- α , and RANTES secretion were dose-dependent, although one of seven donors' DCs responded most potently at low MOIs for all three molecules. In contrast, IFN- α secretion peaked before the highest MOI (of 5) in five of seven donors, with substantial variation in dose-

responsiveness and absolute amounts detected between donors. IP10 was the only analyte measured in this experiment that was consistently secreted at high levels following treatment with the lowest virus dose; in all donors, IP10 secretion reached a plateau early, and in some donors production decreased as MOI increased.

Intracellular cytokine staining from 16- or 18- to 24 hours demonstrated that TNF- α and IP10 production during this time period depended on replicating virus, and that the production profiles of these molecules in DCs were also replication-dependent. Specifically, D2V infection induced potent IP10 production in uninfected bystander cells, while infected DCs had low, and often absent, staining for IP10, suggesting that autocrine/paracrine activation through a soluble mediator was responsible for triggering IP10 and that signaling by this molecule was inhibited by active D2V infection. TNF- α production, on the other hand, was significantly more common in infected cells, implicating replication as the stimulus for expression. Blocking experiments demonstrated a role for endogenous IFN- α/β in both the inhibition of viral replication and in the stimulation of IP10 production, although not in the regulation of TNF- α production. In addition, experiments comparing NGC and a low-passage Thai patient isolate, C0166/96, demonstrated an absence of IP10 production with C0166/96 infection of DCs. This effect was not related to the efficiency of infection, or viral replication kinetics, and in light of the results demonstrating IFN- α/β dependence for IP10 secretion by NGC-infected DCs, suggests that C0166/96 does not induce IFN- α/β secretion following infection of DCs.

CHAPTER V

EFFECTS OF DENGUE INFECTION ON THE ABILITY OF DENDRITIC CELLS TO PRIME ALLOGENEIC RESTING CD4 T CELLS

■ OUTCOME OF DENGUE INFECTION ON DENDRITIC CELL PRIMING OF RESTING CD45RO⁻ CD4 T CELLS

Our studies on expression of cell surface molecules indicated that DV was an activating stimulus for DCs following infection, although infected and bystander cells had different levels of expression of a number of the surface molecules we tested. Similarly, we found that although DCs were induced to produce and secrete a number of cytokines and chemokines in response to infection, infected and bystander cells were again distinct in their activities. Therefore, we were interested in examining the effect of DV infection of DCs on the T cell priming capabilities of these cells. Priming of CD4 T cells is a major function of DCs in the stimulation of adaptive immune responses, through both cell-surface-bound and soluble mediators.

To investigate whether iDCs gain T cell priming function upon exposure to dengue virus, we compared DCs for their ability to stimulate proliferative responses following mock infection, D2V-infection or cytokine-mediated maturation, using co-cultures with allogeneic, negatively selected, resting CD45RA⁺ CD45RO⁻ CD4 T cells from CD14⁻ PBMC. Isolated cells were typically >90-99% CD3⁺, of which 95% were CD4. Of the CD3⁺CD4 cells, 98% or greater were CD45RA⁺ CD45RO⁻. We cultured these T cells with allogeneic DCs 24 hours after mock infection, treatment with TNF- α

plus IFN- α , or infection with D2V strain NGC. We measured T cell proliferation on day 4 or 5 by tritiated thymidine incorporation.

As expected, we noted a dose-dependent increase in CD4 T cell proliferation with increasing numbers of DCs; regardless of DC treatment (*Figure 17A*). In comparison with mock infection, D2V increased the T cell priming capacity of DC cultures. This effect was statistically significant ($P \leq 0.03$) at T: DC ratios of 20: 1 and 40: 1, and was associated with the highest values for stimulation index (SI, calculated as [cpm DV-infected]/[cpm mock-infected]); (*Figure 17B*) between 2500 and 313 DC/well, corresponding to T: DC ratios between 20: 1 and 160: 1.

DCs matured with the addition of exogenous TNF- α /IFN- α were likewise superior to mock-infected DCs as stimulator cells, at all T: DC ratios ($P \leq 0.03$), with SI values highest when the number of added DCs was lowest (T: DC ratio, 80:1; mean=34.0, range, 4.6-58.6 and T: DC ratio, 160:1; mean=36.4, range, 5.7-144.4). T cell proliferation induced by cytokine-matured DCs was also statistically significantly higher than that observed with D2V-infected DC with addition of 2500 or fewer DC (corresponding to 20: 1 and higher T: DC ratios; $p \leq 0.03$). These results demonstrate that dengue infection of DC cultures enhances their ability to stimulate CD4 T cell alloresponses, in concert with the observed changes in cell phenotype. Again reflecting previously observed differences in cell surface phenotype, DCs infected with D2V were somewhat less effective than DCs treated with exogenous cytokines at inducing CD4 T cell proliferation.

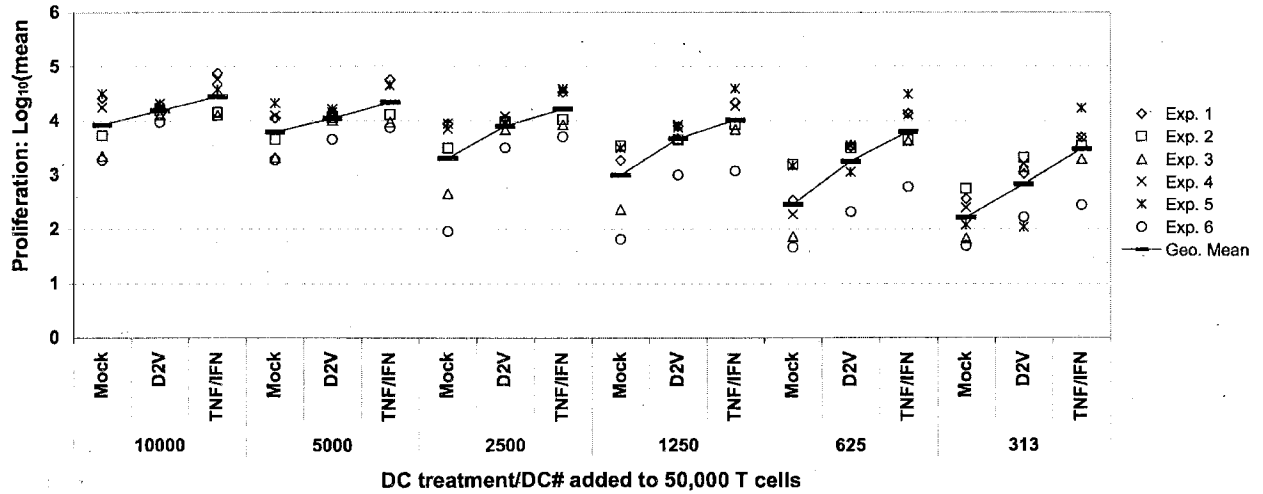
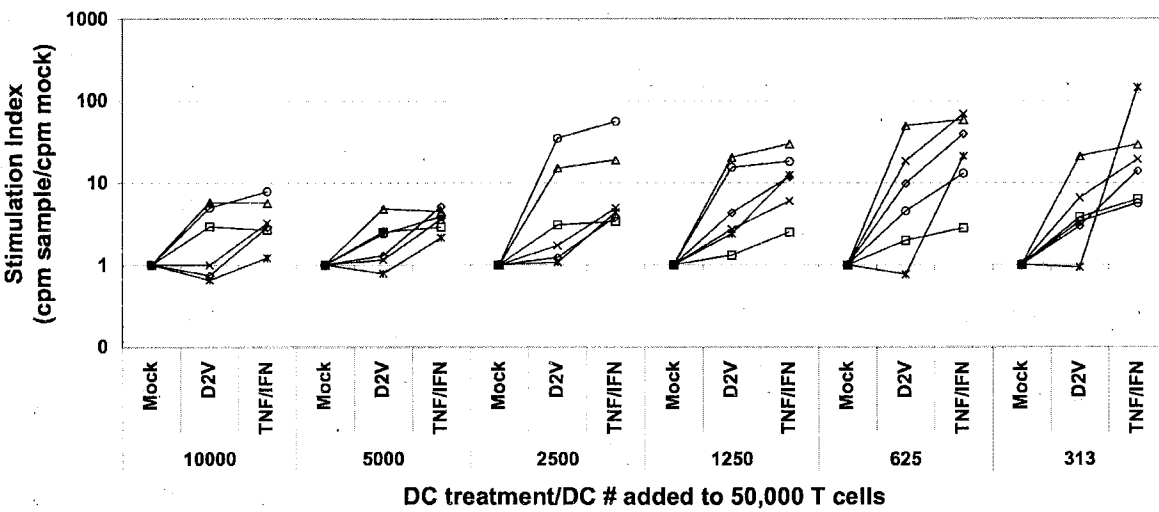
A**B**

Figure 17: Dengue infection of DC cultures facilitates priming of allogeneic resting CD4^T cells: DCs were mock infected (Mock) or infected with D2V NGC strain at an MOI=2 (D2V) as previously described. Cells were washed and returned to culture for 24 hours. Some mock infected cells were stimulated by the addition of exogenous recombinant human (rh)TNF- α (50 ng/mL) plus rhIFN- α (1000 U/mL=5 ng/mL) (TNF/IFN). After 24 hours in culture, DCs were harvested, washed, counted, and two-fold serial dilutions performed. DCs were added to 50,000 allogeneic CD45RA⁺CD45RO⁻ CD4 T cells and proliferation quantitated by tritiated thymidine incorporation on day 4 of co-culture using a β -counter. Data points represent means from quintuplicate wells. **A)** Proliferation responses from six independent experiments at a range of T: DC ratios from 5:1 to 160:1. Data are presented as log₁₀ [mean cpm (counts per minute) from quintuplicate wells] for clarity. Black lines represent the geometric mean values for all six experiments. **B)** Stimulation indices (SI values) were calculated from the data in (A) by normalizing to mock treatment for all experiments. The calculation is as follows: SI= cpm (treatment)/cpm (Mock).

■ EFFECTS OF INCREASING MULTIPLICITY OF INFECTION ON DC CAPACITY FOR INDUCING T CELL PROLIFERATION AND T_H1 OR T_H2 DEVELOPMENT

In previous experiments, we noted differences between infected and bystander DCs in cytokine/chemokine production profiles and surface molecule expression. Therefore, we postulated that the efficiency of T cell priming would also be related to the level of infection within DC cultures exposed to D2V. To test this hypothesis, we infected DCs with D2V using a range of MOIs from 0.04 to 5 and co-cultured them with T cells using a T: DC ratio of 20:1. In seven experiments, DC infection was dose-dependent, albeit with considerable variability between DC donors at any given MOI (*Figure 18A*; data also shown in *Figures 3 and 10*).

In this series of experiments, we found the lowest proliferative responses to DCs infected at an MOI=5, whereas DCs infected at an MOI=0.04 induced the greatest proliferative responses (*Figure 18B*). CD4 T cell proliferation responses to DCs infected at MOI=0.04 or 0.2 (mean±SD cpm 20261±4207 and 17515±2895, respectively) were higher than that to mock-infected DCs (MOI=0; cpm 10919±6739), although these differences were not statistically significant. However, DCs infected at an MOI=1 (11545±5073) induced lower T cell proliferation than did DCs infected at an MOI=0.04 or MOI=0.2, a statistically significant effect ($p<0.02$). Proliferative responses to DCs infected at an MOI=5 were lowest of all conditions tested (7982±4815), and was statistically significantly lower when compared to virus infection at lower MOIs ($p<0.008$). These results indicate that actively infected DCs are poor stimulators of T cell proliferation when compared to bystander DCs.

Two previous reports from the same group suggested that IL-10 production was responsible for reduced T cell proliferative responses to DV-infected DCs, but that CD40-CD40L interactions enhanced IFN- γ secretion in co-cultures of resting CD4 T cells with DV-infected DCs (Palmer et al., 2005; Sun et al., 2006). Therefore, we assayed supernatants from this series of experiments in order to determine if increasing DC infection with D2V resulted in a characteristic T_h response, by measuring IL-10, IL-13, and IFN- γ (*Figure 18, panels C-E*) using multiplex cytokine analysis. We found minimal IL-10 in the majority of co-culture supernatants, regardless of the MOI used to infect DCs. Both IL-13 and IFN- γ were produced at similar levels, and neither cytokine showed a consistent response to increasing the MOI. These results indicate that co-culture of resting CD4 T cells with D2V-infected DCs is not associated with substantial IL-10 production, and suggests that increasing numbers of infected DCs do not skew alloresponsive CD4 T cells towards a T_h1 or T_h2 phenotype during priming.

In order to determine if viral replication was responsible for the reduced proliferation noted at high MOIs, we compared T cell proliferation in co-cultures with DCs infected using either live dengue virus at an MOI=5, or the same virus inactivated with UV-irradiation. In four of five co-culture experiments where T cell proliferative responses to live virus infected DCs were reduced relative to mock infection, we did not note a similar effect for DCs infected with UV-inactivated virus; in one co-culture, the results were similar for both treatments (*Figure 18F*). These data demonstrate that viral replication is the determining factor in reduced allostimulatory functions of directly DV-infected DCs.

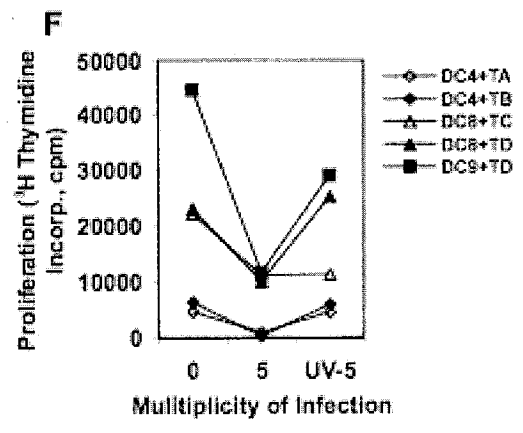
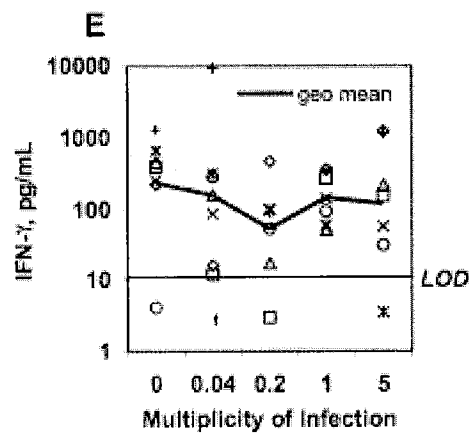
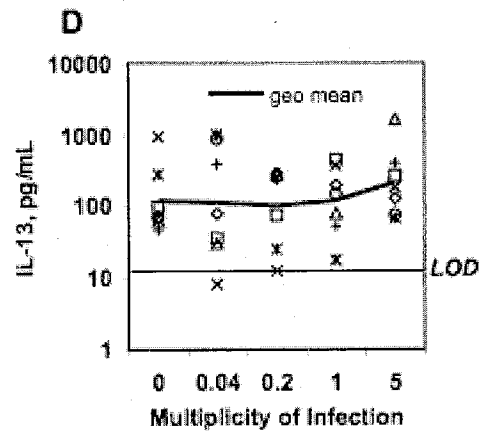
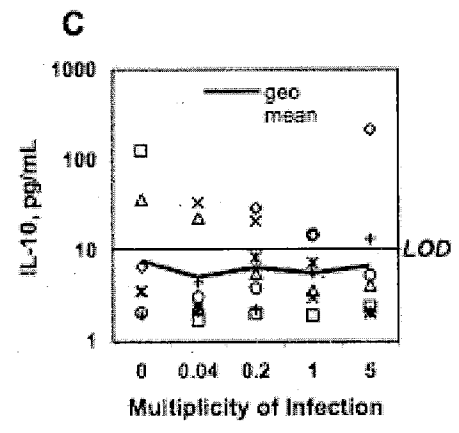
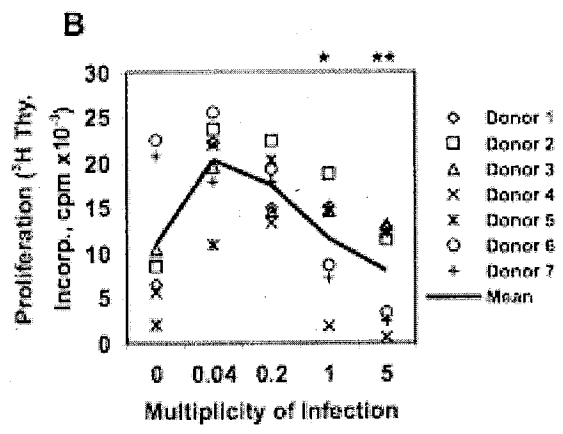
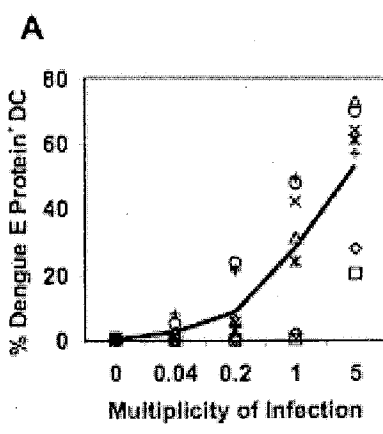


Figure 18: Higher rates of DV infection in DC are associated with decreased resting allogeneic CD4T cell proliferation without T_h skewing. DCs were mock-infected or infected at varying MOIs by using 5-fold serial dilutions. Following 24 hours of culture, DC were harvested, washed, counted, and added to 50,000 negatively-selected allogeneic CD4 T cells at a T:DC ratio of 20:1. **A)** DC from each experiment were intracellularly stained to determine infection rates (results previously shown in figure 3). **B-E)** After 4 days of co-culture, supernatants were collected and frozen at -70°C, 1μCi of tritiated thymidine was added per well, and the cells incubated for a further 18-20 hours. Co-cultures were harvested and quantified using a β-counter. **B)** Proliferative responses in seven T-DC co-cultures. Data points represent the mean cpm from quintuplicate wells. DC from donors 4 and 5 were each tested against T cells from two donors. For these experiments, mean values were calculated as described above for each T cell donor, and the mean of the values obtained from both T cell preparations plotted. Symbols in the legend to the right in (B) represent the same donors' DCs in each panel A-E. *p<0.05 vs. MOI=0.04 and 0.2. **p<0.05 vs. MOI=0.04, 0.2, and 1. Co-culture supernatants were analyzed using a multiplex cytokine analysis for production of **C)** IL-10 **D)** IL-13 and **E)** IFN-γ to determine T_h skew. Solid lines represent geometric mean concentrations. LOD=limit of detection. **F)** Comparison of proliferative responses in T-DC co-cultures where DCs were mock infected (0), infected with UV-inactivated D2V at an MOI of 5 (UV-5), or infected with live D2V at an MOI of 5 (5). Shown are five T:DC combinations where live virus treatment induced lower proliferation than mock treatment alone. DC4 represents donor 4 from panels A-E; DC8 and DC9 are additional donors.

Because response patterns were variable, particularly with regards to baseline proliferation induced by mock-infected DCs, we sought to determine the relative contributions of DCs and T cells to inter-experimental variability. When DCs from two donors were each tested against two different allogeneic T cell preparations, both T cell response curves to a given DC donor were highly concordant (*Figure 19*). These results demonstrate that the proliferative responses observed in this series of assays are largely a product of the qualities of the DCs utilized in the assay, and less so the characteristics of the T cells.

■ FAILURE OF HETEROSPECIFIC DENGUE IMMUNE PLASMA TO ALTER DV EFFECTS ON DC PRIMING FUNCTION

The presence of antibodies from a prior dengue infection has been proposed to contribute to severe disease in secondary dengue infections by increasing infection of FcγR-positive cells, particularly monocytes and monocyte-derived cells (Morens and Halstead, 1990). In addition, antibody-complexed Ross River virus (an alphavirus) has been demonstrated to inhibit antiviral responses and induce IL-10 production in the mouse macrophage line RAW 264.7 (Mahalingam and Lidbury, 2002). Although previous studies reported that heterologous dengue antibody failed to enhance dengue infection of DC (Wu et al., 2000), we hypothesized that DC functions might be modified by infection with DV-antibody complexes.

We infected DC with D2V (MOI=1) alone, D2V combined with control (dengue-naïve donor) plasma, or D2V combined with dengue 3 immune plasma. Final plasma dilutions were 1:20, a concentration previously shown to enhance D2V infection of K562

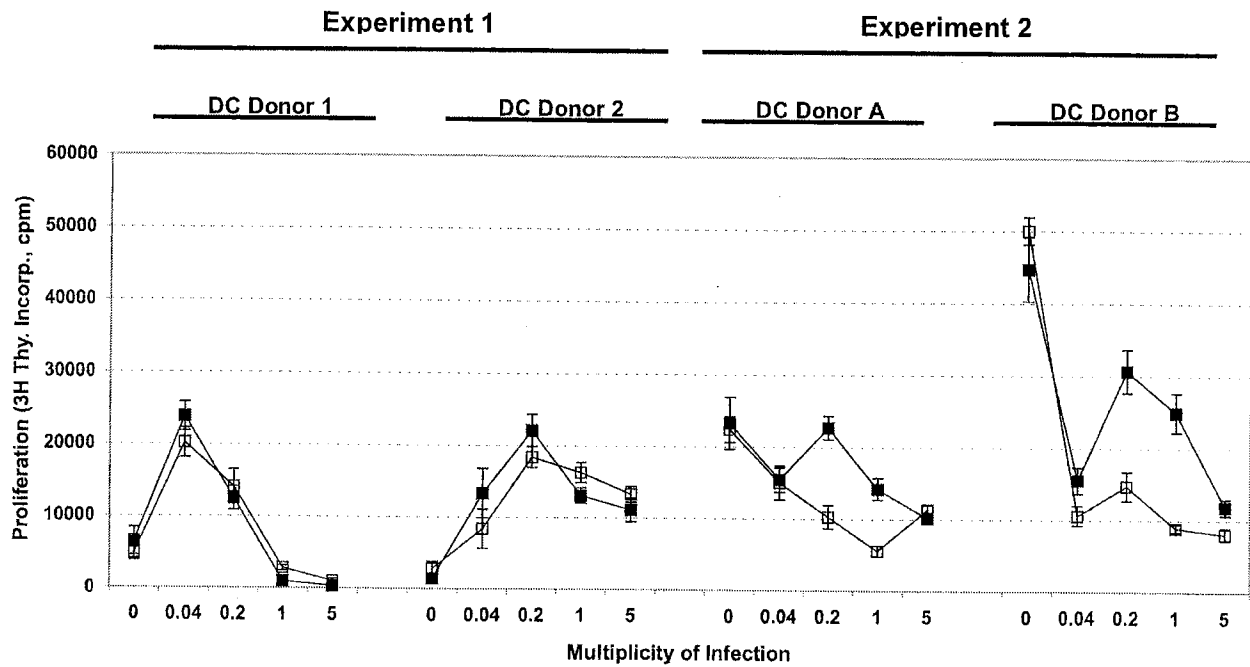


Figure 19: DCs largely determine the qualitative aspects of alloresponsive resting CD4 T cell proliferation. In each experiment, DCs from two separate donors were simultaneously generated, infected, and co-cultured with CD45RO⁻ CD4T cells freshly prepared from two allogeneic donors in a 2x2 matrix. Shown are the results from two independent experiments. Data are presented as proliferation response curves to both T cell preparations (open or closed squares) in a given experiment with each donor DC. Values are mean±SEM cpm of quintuplicate wells.

cells (Laoprasopwattana et al., 2005) and monocytes (unpublished data). Plasma and virus were pre-incubated for 30 minutes prior to addition to DC.

In experiments using four separate donors' DCs, we noted minimal differences in infection rates between DCs infected with virus alone or virus pre-incubated with either control plasma or D3V-immune plasma (*Figure 20A*). When these DCs were co-cultured with CD45RO⁺ CD4 T cells and assayed for proliferation, we again noted no differences. DCs infected with D2V alone, D2V plus control plasma, and D2V plus D3V-immune plasma all demonstrated enhanced allostimulatory function compared to mock infection in the presence or absence of control plasma or immune plasma (*Figure 20B*). These results indicate that this dengue heterospecific immune plasma neither enhanced infection of DCs nor altered DC priming functions following infection with D2V.

■ CHAPTER SUMMARY

In this series of experiments, we demonstrated that infection of DC cultures enhanced the ability of these cells to stimulate alloproliferation in resting CD4 T cells over mock infection alone. This effect became increasingly pronounced as the levels of alloantigen (i.e., the number of DCs) became limiting, and was similar to the effects seen when DCs were pretreated with a combination of TNF- α and IFN- α prior to co-culture, although the proliferation induced by infected DC cultures was clearly less pronounced than with cytokine treatment. Despite the increase in proliferation of allogeneic CD4 T cells noted in co-cultures with DCs that had been exposed to live virus, further experiments revealed that increasing the MOI of D2V used to infect DCs resulted in a reduction in the ability of the DCs to stimulate T cells at a T: DC ratio (20:1) that demonstrated a significant

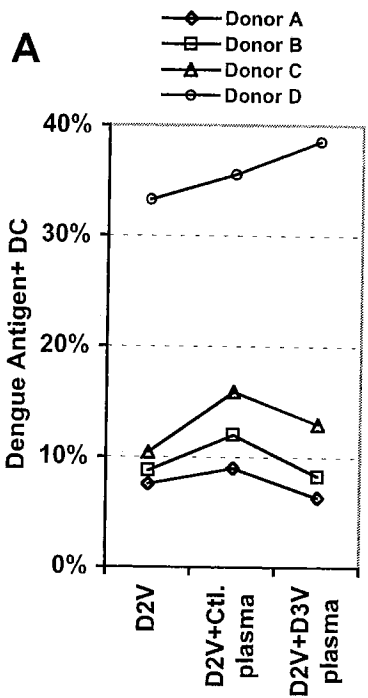
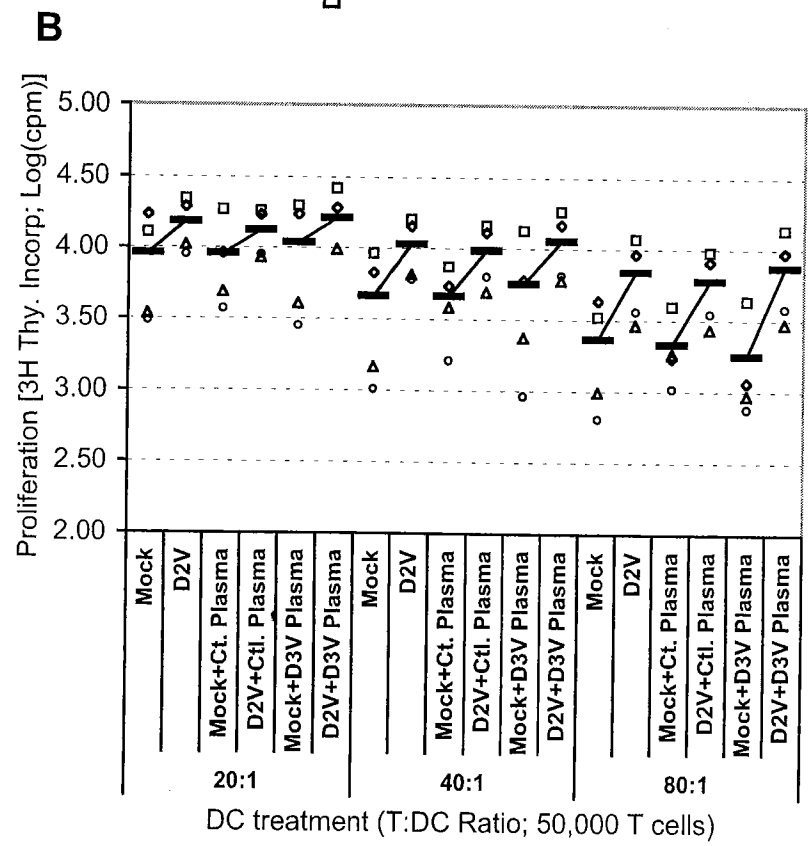


Figure 20 DC exposure to D2V in the presence of heterotype-immune plasma does not alter DC infection or T cell priming.

DC were mock infected or infected with live D2V NGC strain at an MOI=1 as previously described. For some samples, virus or mock treatment were combined with either control (Ctl.) plasma or plasma from a D3V vaccine recipient (D3V) at a final dilution of 20:1, and incubated on ice for 30 min, prior to adsorption. **A)** Percentages of dengue E protein-expressing DCs as determined by flow cytometry in experiments with four DC donors (A-D), following infection under the specified conditions for 24 hr. **B)** DCs from (A) were co-cultured with CD45RO⁺ CD4 T cells at T:DC ratios of 20:1, 40:1, and 80:1 and proliferation measured by tritiated thymidine incorporation as previously described. Symbols correspond to the respective DC donors shown in (A). Lines and bars indicate mean values from the four experiments.



increase in stimulatory functions of infected DCs in our first series of experiments. Thus, increasing MOI (and increasing infection rate) was associated with a reduction in the T cell allostimulatory activity of DV-infected DCs. This effect depended on replication-competent virus, since UV-inactivated virus at an MOI of 5 did not suppress DC stimulatory functions in comparisons to live virus. Experiments in which two T cell donors were tested against two DC donors revealed that DCs were largely responsible for determining the qualitative aspects of the responses we observed (i.e., virus-dose dependent stimulation or suppression). In addition, cases of reduced proliferation in T-DC co-cultures could not be explained by IL-10 production, since we detected only low levels of this cytokine in nearly all T-DC co-cultures. Finally, increasing MOI (increasing infection rate) did not have an effect on the T_h-type skewing of responding T cells, since the levels of secreted IFN- γ and IL-13 measured in co-culture supernatants were stable across the range of MOIs used to infect DCs.

CHAPTER VI

DISCUSSION

■ In this thesis work, I sought to identify the properties that DCs adopt following exposure to D2V and to elucidate the direct and indirect roles that D2V plays in determining the responses of D2V-exposed DCs. Exposure of whole DC cultures to D2V infection resulted in cellular activation, as measured by enhanced expression of cell surface molecules, secretion of inflammatory mediators, and increased capacity for eliciting allospecific CD4 T cell proliferation. However, measuring these effects was complicated by the fact that direct cellular infection has its own unique consequences for the functional properties of targeted DCs. The challenge, therefore, was to discriminate between multiple responses: 1) the response of all DCs to the virus particle, 2) the response of infected cells to internal viral replication, 3) the response of all DCs in culture to products secreted following infection, and 4) the virally-modified secondary responses of infected cells. The experimental plan was to observe whole culture responses to infection and then to determine which responses required viral replication and, where possible, to discriminate between the functions of infected and bystander cells. I applied this methodology to three levels of DC function:

- phenotypic maturation
- cytokine and chemokine production and secretion
- CD4 T cell priming

DCs in DV-exposed cultures demonstrated phenotypic maturation by increasing the expression of immunoregulatory molecules in comparison to mock-infected cells. Since UV-inactivated virus increased expression of several phenotypic markers, part of that effect was attributed to binding and/or internalization of virus particles. Replication-competent DV was required for the full effect. In cultures exposed to live virus, both infected (dengue antigen positive) and uninfected (dengue antigen negative) DCs demonstrated an activated surface phenotype when compared to mock-treated cells. However, the densities of surface molecules induced on infected versus bystander cells in the same culture were different. Of eight molecules tested, DV-infected cells had significantly lower surface expression than bystander cells for CD80, CD86, PD-L1, and MHC I, and trended towards lower CD83 expression. On the other hand, infected DCs had greater expression of PD-L2 and MHC II, and in the case of CD40, expression levels were equivalent. Therefore, although DV activated surface molecule expression on all cells, replicating virus altered the phenotype of actively infected cells.

To determine if DV stimulated a characteristic DC cytokine and chemokine secretion pattern in response to infection, we measured a range of inflammatory mediators in culture supernatants following DV exposure. I observed that live DV was a potent stimulus for secretion of multiple cytokines and chemokines. By contrast, UV-inactivated virus induced only a partial secretory response. Furthermore, the secretory response that DV infection elicited differed both qualitatively and quantitatively from the defined TLR4-mediated signaling of LPS. Examination of the dose-responsiveness of DCs to DV infection revealed that TNF- α , RANTES, and MIP-1 α production increased with increasing virus dose. IFN- α and IL-10 responses were more variable, and IP10

secretion was high even at the lowest MOI. When responses were analyzed on the single-cell level, infected DCs had dramatically reduced intracellular staining for IP10 when compared with uninfected bystander cells; conversely, infected cells had enhanced TNF- α expression. DV infection resulted in DC release of biologically active IFN- α/β , with consequences for both infected and bystander DCs. Endogenous IFN- α/β produced during infection process inhibited further infection of DCs and was the major factor driving DC IP10 production. IP10 production differed between D2V strains, suggesting strain-dependent differences in IFN- α/β induction. This series of experiments indicated that DV causes DCs to secrete a broad range of inflammatory cytokines and chemokines, but the specific responses of infected and bystander DCs to DV were distinct. Both direct and indirect effects of virus mediated secretory responses, effects that were distinct in a comparison of culture-adapted and wild type dengue viruses.

One main function of DCs is to initiate and direct primary immune responses; a series of experiments was designed to quantify DC priming function using multiple resting CD4 T cell activities as the readouts. Introducing D2V into DC cultures generally increased the ability of DCs to stimulate T cells; however, increasing the virus dose weakened or even abrogated this effect. When compared to mock-infected DCs, in most cases DV-infected DC cultures induced greater proliferation of resting allogeneic CD4 T cells. These effects were observed consistently across a range of T:DC ratios and were most pronounced when DC numbers were limited. The effect was less pronounced, however, when compared with the differences between cytokine-matured DCs and mock-infected DCs. In another set of experiments, DCs infected at lower MOIs were consistently better stimulators than cells infected at higher MOIs. In cases where DCs

suppressed T cell proliferation at high MOI, replicating virus was required since UV-inactivated virus did not have an equivalent effect. IL-10 induction during co-culture did not explain the reduced alloproliferation observed at high MOI, since I detected minimal IL-10 secretion in T-DC co-cultures. Nor was increasing MOI associated with T_h-type skewing, since similar levels of IL-13 and IFN- γ were detected in co-culture supernatants and did not show MOI-dependent effects. Infection of DCs with DV in the presence of serotype-heterospecific antibodies neither enhanced infection of these cells, nor altered their ability to induce proliferation in co-culture with quiescent CD4 T cells. My results indicate that exposure to DV stimulates proliferative priming function in bystander cells, an effect that is not influenced by infection with immune-complexed DV. By comparison, DV-infected DCs appear to be relatively poor CD4 T cell stimulators and do not potentiate the development of a specific T_h1 or T_h2 response.

■ GENERATION OF DENDRITIC CELLS AND INFECTION WITH DENGUE VIRUS

Positive selection of CD14-expressing monocytes and subsequent culture with GM-CSF and IL-4 allowed us to generate DC cultures with high conversion rates to CD1a-expressing cells. This result was in accordance with previous literature reports, including those addressing dengue infection (Wu et al., 2000; Palmer et al., 2005). Monocyte-derived DCs have the advantages of being primary cells, are readily generated, use established laboratory culture methods, and result in generally high purity of the desired cell population. However, being primary cells they must be isolated from multiple donors, thus introducing a substantial element of experimental variability into the system.

Following exposure of DCs to dengue virus, we noted peak antigen staining prior to 48 hours, in agreement with previous work using D2V NGC (Wu et al., 2000). Therefore, we used a 24-hour time point post-infection for our subsequent analyses. In several other studies the investigators utilized a 48-hour time point post-infection (Libraty et al., 2001; Palmer et al., 2005; Sanchez et al., 2006). The 24-hour time point in our studies was also selected based on a report that suggested increasing apoptosis in mock-infected cells with prolonged culture (Ho et al., 2001). Since this could potentially artificially skew our observations in favor of greater differences between mock infection and DV infection at later time points, we chose an earlier time than in previous studies. Additional studies indicate that DV infection has either no effect on, or actually reduces, apoptosis in DC cultures infected at an MOI of 1 (Palmer et al., 2005; Sun et al., 2006). The finding that dengue antigen staining was greater at 24 hrs than at 48 hours post-infection suggests either selective attrition of infected cells or reduced virus production, for example, as a consequence of the paracrine effect of IFNs. We were interested in examining DCs during the period of peak viral replication, when these cells would be expected to be migrating *in vivo*, and arriving in secondary lymphoid organs. Temporal effects are reported to have significant influence on the functions of DCs following their stimulation (Langenkamp et al., 2000).

New Guinea C strain was selected based on original experiments with DV infection of DCs (Wu et al., 2000), and the ready availability of virus stocks. We found that, as expected, infection with NGC was dose-dependent, as infection rates increased with increasing MOI. For most experiments, I utilized an MOI of 2, similar to previous reports (Wu et al., 2000; Ho et al., 2001; Libraty et al., 2001; Palmer et al. 2005). In my

analyses, there was substantial variation in the susceptibility to infection of DC derived from different donors, which was evident even in cultures infected on the same day. High variability in infection rate between DC preparations was previously reported, suggesting that this is a common effect in the DV-DC system (Cologna and Rico-Hesse, 2003; Sanchez et al., 2006).

The question of donor-to-donor variability in susceptibility to infection is an interesting one. Regarding our findings, the variability we noted could not be explained solely by the virus stock used or the day of infection, since donors' DCs infected on the same day (see *Figure 3*, donors 1-3) or donors infected using a single virus stock (see *Figure 3*, donors 1-5) still demonstrated variability in infection. In contrast, infection rates were similar for separate cultures of DCs from the same donor infected on the same day with and without addition of control IgG_{2a} (refer to *Figure 13*), or control human plasma (refer to *Figure 20 A*). Therefore it is unlikely that the variation we observed in the infectivity of DCs derived from separate donors was due solely to intra-experimental effects inherent to the infection procedure itself. These results suggest that donor-specific traits of DCs have substantial influence on the variability in this experimental system. This conclusion is supported by a report that promoter activity for DC-SIGN appears to be protective against DF (Sakuntabhai et al., 2005). This observation suggests that relative DC-SIGN expression levels between individuals may determine the development of symptomatic disease through influence on DC infectivity. Additional downstream responses, such as donor-dependent efficiency of IFN- α/β production by DCs in response to DV infection, the activity of PDCs in a given individual, or the

sensitivity of an individual's conventional DCs to IFN- α/β signaling likely also play a significant role in determining DC susceptibility to infection, either *in vitro* or *in vivo*.

Donor-dependent variability in DC responses to infection (and in other responses, including cytokine production, which is addressed later in this discussion) is an important observation for future research in the role of DCs in dengue disease etiology. The occurrence of outliers among donors might highlight rare aspects of DC responses to DV that either enhance or reduce the risk for disease pathology. This may be argued to be critical in the study of dengue, since individuals developing the most severe disease represent only a small fraction of those who contract the virus. Thus, "common" responses observed in studies such as ours, and others using similar systems which identify trends consistent between donors, may be more likely to reveal global responses associated with protective, rather than pathological, immunological behavior.

Because NGC is a laboratory-adapted strain, we addressed the issue of infection with other dengue viruses. Similar to previous reports, we report the susceptibility of DCs to multiple serotypes of DV (Cologna and Rico-Hesse, 2003; Wu et al., 2000; Ho et al., 2001), which in our study included low-passage clinical isolates of American and Asian strains of D2V. Our results, combined with the observations of others, indicate that DC infection with NGC performed in this body of work can be readily reproduced with other dengue viruses. Work with multiple viruses is important, since differences in cellular responses (such as cytokine/chemokine induction, which we noted) may reflect mechanisms of viral adaptation that contribute to virulence, and pathology.

Although analyses of functional differences between viruses were limited in the scope of this thesis, one literature report began to address this question by comparing DC

infection with both parent and attenuated vaccine strains of D2V, reporting differences between the two strains in DC responses as determined by surface molecule expression and cytokine production (Sanchez et al., 2006). Another study examining the replicative capacity of different D2V genotypes in DCs found that genome structures of American D2V strains were associated with reduced infection and virus production, as compared with those of Asian strains (Cologna and Rico-Hesse, 2003). Future investigation into the role that virus strain plays in DC susceptibility to infection, and the comparison of responses that different viruses elicit from DCs will prove an exciting avenue to pursue. In particular, comparisons of DV strains from individuals with severe disease against strains which are known to be attenuated, or are isolated from individuals with very mild disease, will facilitate the understanding of the mechanisms by which DV infection of DCs contributes to disease pathogenesis. Important molecules to address are IFN- α and IL-10, among others. Differences between strains in their effects on DC functions in alloreactions may also reveal important strain-dependent differences in quantitative and/or qualitative priming of T cells.

■ EFFECTS OF DENGUE VIRUS EXPOSURE ON DENDRITIC CELL SURFACE PHENOTYPE

DV exposure enhanced the expression of a panel of immunoregulatory surface proteins in both infected and bystander DCs. In a comparison of D2V to known DC activators (TNF- α /IFN- α and LPS), although D2V was the weakest stimulus, surface molecule expression changes seen with live virus were generally similar to those seen with high-dose cytokine treatment. This was apparent despite the fact that the concentrations of exogenous cytokines added were more than 5-10 fold those detected in culture following

infection (specifically, TNF- α at 50 ng/mL and IFN- α at approximately 5 ng/mL). D2V infection was most consistent with TNF- α /IFN- α treatment in the induction of CD40, PD-L1, PD-L2, CD86, and HLA A, B, C, and relative changes in CD80 were also somewhat similar between D2V infection and TNF- α /IFN- α treatment. The comparison to defined activators such as TNF- α /IFN- α and LPS were not addressed in previous related studies (Ho et al., 2001; Libraty et al., 2001; Palmer et al., 2005; Sanchez et al., 2006). Thus, D2V infection of DCs was not only able to activate phenotypic maturation of DCs, but the magnitude of the effect observed was substantial when viewed in light of the degree of change inducible in this system.

In experiments comparing UV-inactivated D2V to live virus, replication was not required for the induction of surface molecule expression, since significant increases in CD80, CD86, PD-L1, and PD-L2 expression occurred following treatment with UV-inactivated D2V as compared with mock infection. However, we observed greater expression levels in DCs infected with live virus when compared to UV-inactivated virus, indicating a role for viral replication in enhancement of phenotypic maturation. Although most differences between live and inactivated virus were not statistically significant, increases in expression levels following live virus treatment were statistically significant in comparison to mock infection for all molecules assayed, while UV-inactivated virus induced statistically significant changes in B-7 family molecules only. Therefore, increased surface marker expression on infected DCs as compared with UV-inactivated virus infected DCs likely represent real differences related to activation occurring secondary to viral replication.

Since I confirmed that there was no detectable residual infectious virus in the UV-inactivated samples, these data suggest that the binding and/or uptake of virus particles alone, possibly via E protein recognition by DC-SIGN, causes signaling within the DC, resulting in the observed changes in DC phenotype. Two studies found that DC-SIGN ligation by DC-SIGN specific antibodies did not result in phenotypic changes in DCs (Geijtenbeek et al., 2000b; Caparros et al., 2006), although the second study found phosphorylation of the signaling kinases extracellular regulated kinase (ERK)1/2 and Akt, without p38 mitogen-activated protein kinase (MAPK) activation, and noted PLC- γ phosphorylation and transient calcium influx. However, the structural epitope recognized by DC-SIGN specific antibodies influences their effects on signaling, as antibodies with both activating and ligand-blocking functions have been identified (Hodges et al., 2007). Therefore, DV binding to DC-SIGN could be responsible for the changes in cell surface phenotype that we observed.

Alternatively, virion internalization may be necessary to elicit cellular responses. DC-SIGN has internalization motifs, which are responsible for the uptake of HIV (Kwon et al., 2002). Antibody blocking of DC-SIGN binding prevents infection-induced phenotypic changes to HIV, however, abrogation of DC-SIGN internalization motif function does not prevent DV uptake, suggesting a role for additional molecules in DV internalization (Lozach et al., 2005; Tassaneetrithep et al., 2003). Therefore, virion binding alone to DC-SIGN may fail to induce, or induce weak and/or transient, activation that could be amplified when the DV particle is internalized. In one report, DC-SIGN-mediated human papilloma virus (HPV) pseudovirion (virus-like particle, VLP) binding to DCs, and antibody blocking of DC-SIGN specifically inhibited HPV VLP-induced

MHC I induction (Garcia-Piñeres et al., 2006). The lack of nucleic acid in VLPs demonstrates that the effects seen were dependent on protein structural components alone, although these findings cannot rule out a role for additional signaling mechanisms secondary to VLP binding by DC-SIGN. DC-SIGN binding by multiple pathogens, including mycobacteria, measles, and HIV-1, has also been demonstrated to activate signal transduction through Raf-1 mediated acetylation of NF- κ B p65, although this required prior TLR-mediated NF κ B activation (Gringhuis et al., 2007). Therefore, additional receptor-ligand interactions (or activation of intracellular pathways) may be essential for signaling following DV binding as well. These findings also suggest that DC-SIGN binding by DV may regulate later activation and intracellular signaling events that are dependent on replication. It should be noted, as discussed in the introduction, that multiple cellular receptors for DV have been postulated; the studies discussed above highlight the importance of determining additional cell-surface targets for both DV internalization and signaling beyond DC-SIGN.

It is possible that C6/36 cellular contaminants in the virus inoculum, such as heat-shock proteins, are partially responsible for DC activation in these experiments. Nevertheless, the observed differences in responses of DC to live virus and UV-inactivated virus show that DCs respond specifically to dengue viral replication and additional intracellular signaling events. We observed viral protein production in response to NGC infection between 8 and 16 hours (refer to *Figure 16 D*), consistent with previous reports (Wu et al., 2000). Therefore, phenotypic responses to replicative virus and resulting autocrine/paracrine cytokine signaling would likely become evident at 24 hours post-infection and later time points.

One study utilizing UV-inactivated DV suggested that viral particles themselves did not induce phenotypic maturation of DC, although the data were not shown (Libraty et al., 2001). Other studies using formalin inactivation or heat inactivation reported similar results to those of Libraty et al. (2001), although again, the data were not shown (Palmer et al., 2005; Sanchez et al., 2006). Our data are in contrast to these findings, but demonstrated a reproducible and statistically significant effect. One possible explanation for the disparity between our studies and those described above is the magnitude of the response to UV-inactivated virus observed in our system, which was low for PD-L2, CD40, and MHC molecules (although statistically significant in the case of PD-L2). We observed larger expression level changes in CD80, CD86, PD-L1, and CD83 (all of which were statistically significant), indicating that the choice of surface markers analyzed would affect conclusions regarding the effects of UV-inactivated virus. Specifically, none of the aforementioned studies addressed PD-L1 or PD-L2 expression, both of which we found to be significantly enhanced following UV-inactivated virus treatment of DCs. Low numbers of replicates, differences in viral strains, and temporal effects (with our studies performed at 24 hours, and previous work performed at 48 hours (Libraty et al., 2001; Palmer et al., 2005; Sanchez et al., 2006) may all be contributing factors in the discordance of our data and the work of other groups, although we can only speculate on the former, as none of those studies presented data on the effects of inactivated virus on DC surface molecule regulation.

Ideally, to answer the question of dengue particle-mediated effects on DCs, a highly purified preparation of virus could be utilized for infection, allowing comparison in the absence of potential medium contaminants including cellular debris. Studies of

dengue VLPs in comparison to UV-inactivated whole virus would allow for differentiation between structural protein and nucleic acid-mediated effects. Heat inactivation could also be used to denature virion components (or cellular protein contaminants) that may be responsible for signaling. Our findings indicate that DCs are partially activated following exposure to non-replicative virus, and therefore warrant further investigation into the mechanisms by which the DV particle itself induces signaling. One group of candidate DC-expressed molecules, which are involved in pathogen recognition and may be involved in DV-induced signaling, are the Toll-like receptors (TLRs). TLR recognition has been shown to be involved in the responses of murine DCs to YFV, a close relative of dengue (Querec et al., 2006).

When we examined the phenotypes of infected and bystander DCs based on dengue E protein expression, our data included the novel finding that DV induces expression of both PD-L1 and PD-L2, the known ligands for programmed-death-1 (PD-1). PD-1 is expressed on activated T cells, and binding to either of its ligands results in co-suppressive signals which inhibit T cell proliferation and cytokine production *in vitro* using primary human cells (Brown et al., 2003; Cai et al., 2004). We also found enhanced expression of CD40, CD83, CD80, CD86, and MHC class II molecules on both infected and bystander cells, while only bystander cells up-regulated MHC class I. These data demonstrate that both actively infected cells and uninfected bystanders increase expression of surface molecules involved in immune regulation following live DV infection of DC cultures.

In a contrast of infected to bystander DCs following DV exposure, PD-L1, CD80, CD86, and MHC Class I expression were lower in infected DCs; conversely PD-L2 and

HLA DR surface levels were enhanced. Using murine cells, PD-L1 has been shown to effectively inhibit both T_H1 and T_H2 responses in antigen-specific T cells, while PD-L2 preferentially inhibited T_H1 responses alone (Latchman et al., 2001). In experiments with human DCs in co-culture with CD4 T cells, blocking PD-L1 and PD-L2 had additive effects, although in this case, PD-L2 was the more potent cytokine and proliferation suppressor (Brown et al., 2003). The fact that both of these molecules were differentially regulated on infected versus bystander DCs in our experiments suggested that priming functions between these two populations may differ, particularly with regard to T_H -skewing, although we did not confirm such an effect (refer to *Figure 18, panels C-E*). IFN- α induces PD-L1 in cultured DC and renal tubular cells (Schreiner et al., 2004; Waeckerle-Men et al., 2007), and we observed up-regulation of both PD-L1 and PD-L2 in response to IFN- α treatment of DC (data not shown). Type I interferons (IFN- α/β) have been implicated in the regulation of other B7-family molecules in DCs, and enhance the expression of MHC Class I (Luft et al., 2002; Mohty et al., 2003). We also found that MHC Class II expression on DC could be induced by IFN- α (not shown); although DV infection has been shown to inhibit IFN- α/β signaling within infected cells (Munoz-Jordan et al., 2003; Munoz-Jordan et al., 2005; Ho et al., 2005), HLA DR and PD-L2 expression were selectively enhanced on infected DCs when compared to bystander cells in our system. These findings indicate that the observed differences between infected and bystander DC surface molecule expression could not be explained on the basis of DV-mediated IFN- α/β inhibition alone. Therefore, additional regulatory pathways affecting surface molecule expression, attributable to replicating virus, are active in DCs following infection. This is not surprising in that both dengue NS1 and

NS5 are implicated as mediators of intracellular signaling (Chua et al., 2005; Medin et al., 2005).

There is general agreement in the literature that bystander DCs have enhanced expression of co-stimulatory and MHC molecules following dengue exposure (Libraty et al., 2001; Palmer et al., 2005; Sanchez et al., 2006). In one study, CD80 and CD83 expression appeared to be slightly enhanced in infected DCs when compared to bystander cells, although this effect was not statistically significant (Sanchez et al., 2006). In that study, infection and culture were carried out at 32°C and used both a D2V attenuated vaccine strain, PDK53, and the parent prototype strain 16681. In a second study using D2V strain 16681, the authors reported that CD83, CD40, CD80, CD86, and MHC I and II molecule expression were all enhanced versus mock infection in both infected and bystander DCs, although infected cells had lower expression of CD83, CD80, CD86, and MHC I relative to bystanders (Libraty et al., 2001). Another study reporting on the same panel of markers found that DCs infected with the D2V prototype strain 16803 showed no induction of surface molecule expression when compared to mock infection, despite increases in surface expression of all of the molecules assayed in bystander DCs, and concluded that there was potent virus-specific suppression of responses (Palmer et al., 2005). Our findings were in contrast to the report by Palmer et al. (2005), being most consistent with those of Libraty et al. (2001), although we found enhanced expression of MHC Class II on actively infected DCs. In summary, including our work, three of four studies indicate differences in phenotypic maturation between infected and bystander DCs, and three of four studies report statistically significant phenotypic responses specifically in infected cells compared with mock infection. The differences in findings

regarding surface molecule expression between studies likely reflect differences between virus strains, genetic polymorphisms between DC donors, and the kinetics of surface molecule expression.

■ **WHOLE CULTURE DENDRITIC CELL CYTOKINE AND CHEMOKINE RESPONSES TO DENGUE 2 VIRUS INFECTION**

When we infected DCs with dengue virus, we noted a broad inflammatory cytokine and chemokine response, with the most striking changes being in chemokine production. Previous work in our laboratory indicated that DV induced DC expression of the chemokines MIP-1 α , MIP-1 β , IL-8, RANTES, and MCP-1 (Medin et al., 2005). Here we report similar findings, along with the observation that dengue also induces high-level secretion of IP10 from monocyte-derived DCs. This additional finding is potentially significant, since IP10 and not the other CXCR3 ligands MIG (monokine induced by gamma interferon, CXCL9) and ITAC (interferon-inducible T cell alpha chemoattractant, CXCL11) was reported to be critical for survival in a murine model of primary dengue illness (Hsieh et al., 2006).

Similar to previous reports (Ho et al., 2001; Libraty et al., 2001; Palmer et al., 2005), we found that dengue was also a potent inducer of inflammatory cytokines IFN- α and TNF- α , and that IL-12 p70 was not induced in response to DV infection. We did find increased IL-12 p40 induction by some donors' DCs in response to dengue infection in several experiments (Figures 7, 8, and 9), suggesting that failure to produce IL-12 p70 is also related to a failure to produce the IL-12p35 subunit. One potential alternative pathway for IL-12 p40 is IL-23, which utilizes the p40 subunit along with IL-23-specific

p19 (Oppmann et al., 2000). While IL-23 is reported to induce IL-17 secretion in CD4 T cells (Aggarwal et al., 2003), in preliminary experiments, we did not note any differences in IL-17 levels in co-cultures of T cells combined with mock-infected versus dengue-infected DC, suggesting that dengue did not specifically enhance IL-23 secretion by DCs, however (data not shown). DV infection was also a stimulus for the release of several additional inflammatory mediators, including IL-6 and IL-1 α . Infection also stimulated the release of IL-3, which is a growth factor for plasmacytoid dendritic cells (PDCs) (Strobl et al., 1998). PDCs are potent IFN- α producing cells, which are activated through TLR7 following exposure to DV (Wang et al., 2006). In a study of hospitalized children with febrile illness, those who developed DHF did not demonstrate elevated PDC frequencies in their PBMC, while those with DF had increased PDC numbers early during illness (Pichyangkul et al., 2003b). That same study reported that total PBMC IFN- α secretion in response to PDC-specific stimulation is dramatically reduced during acute dengue illness as compared with healthy controls (Pichyangkul et al., 2003b). Thus, PDCs appear to play an important role in dengue illness, and IL-3 production might be involved in the early proliferation of these cells following infection.

Similar to our findings on surface molecule regulation, UV-inactivated virus was capable of inducing the secretion of several cytokines and chemokines, including IL-6, MCP-1, RANTES, and IP10. These results are not surprising, in that DC cytokine responses to non-replicative VLPs and inactivated virus have been reported. HPV VLP binding to DCs induced secretion of TNF- α , IL-6, and RANTES (Garcia-Pineros et al., 2006), while UV-inactivated YFV 17D was able to elicit TNF- α and MCP-1 secretion from DCs (Querec et al., 2006). We found that secretion of most molecules, notably

IFN- α and TNF- α , as well as RANTES, were greater following stimulation with live virus than with UV-inactivated virus, indicating (similarly to surface molecule regulation), that D2V exposure stimulates DC functional activity, while replication enhances this activation. The finding that IFN- α and TNF- α production were increased specifically by live virus treatment likely reflects dependence on viral protein production or intermediates such as dsRNA formed during viral replication. As previously mentioned, DCs produce TNF- α in response to NS1-mediated signals (Chua et al., 2005), and dsRNA activates IFN- α/β production through multiple pathways (Cella et al., 1999; Yoneyama et al., 2004). Autocrine/paracrine signaling by these molecules could then further influence the secretion of other products.

Our findings with New Guinea C strain reflect previous literature reports indicating that DV either does not induce, or induces low levels of, IL-10 production in DCs from most donors (Libraty et al., 2001; Palmer et al., 2005). Specifically, we found low or absent IL-10 production in response to dengue infection in independent experiments (refer to *Figures 7 and 8*), results that were consistent with those of Libraty et al (2001). DCs did consistently produce IL-10 in response to LPS stimulation (refer to *Figure 8*), demonstrating their functional capacity to produce this cytokine. In other experiments where we did detect IL-10 (refer to *Figure 10 D*), responses were low for all but one donor across a range of virus inocula tested, and reflected levels reported by Palmer et al. (2005) at 24 hours. This assay had the best low-end sensitivity of our three multiplex analyses, as samples were diluted 2-fold for dose-response experiments, as opposed to 7.5-fold in experiments comparing D2V to LPS (See Materials and Methods). Only one of seven donors' DCs (donor 4) showed potent IL-10 secretion, which was

dose-dependent. IL-10 induction has been reported to occur late following DC stimulation, when compared with other cytokines (Langenkamp et al., 2000), suggesting that any differences between our results and those of Palmer et al. (2005) may be temporally related. However, the kinetics of DV infection did not appear to be responsible, since in our original analysis (refer to *Figure 7*), we measured high levels of intracellular dengue E protein staining at 24 hours post-infection, ranging from 33-90% (not shown). Therefore, in our system, low or absent IL-10 induction was not simply due to poor viral replication. Sanchez et al. (2006) reported some secretion of IL-10 by DCs following DV infection, but were unable to detect IL-10 during intracellular cytokine staining assays, further demonstrating the inconsistency of IL-10 induction in the DV-DC system. Donor-dependent cytokine responses should be considered, and would represent an interesting area for further research.

Further studies regarding the role of DC in IL-10 secretory responses to dengue viruses are needed, particularly given our finding that potent IL-10 induction by DV infection of DCs is an exception, and not the rule. IL-10 levels in plasma from patients with acute dengue infection are elevated with more severe disease (Perez et al., 2004; Green et al., 1999b), similar to findings in fatal Ebola virus infection of humans (Baize et al., 2002). APCs derived from the PBMC of acute dengue patients are unable to stimulate T cell alloproliferation and recall responses and show poor IFN- α secretory function (Mathew et al., 1998; Pichyangkul et al., 2003a). Together, these findings suggest a role for IL-10 in acute APC dysfunction during dengue illness. Individuals whose DCs are predisposed to produce IL-10 in response to dengue infection might therefore be at high risk of developing severe disease. In particular, early IL-10

production might suppress antiviral IFN activity and co-stimulatory molecule expression in APCs (Ito et al., 1999; Ding et al., 1993), as well suppressing cytokine synthesis in T_H1 cells (Fiorentino et al., 1989). This would inhibit effective memory activation, and failure to eliminate infected DCs would allow for continued viral replication, triggering ADE and leading to even higher viremia. The resulting antigen load could cause a “rebound” over-stimulation of adaptive responses, activation of pathological T cell subsets, and T cell death. Such effects may be reflected by the increased levels of soluble T cell-associated receptors (CD8 and IL-2R) and CD8 T cell apoptosis seen during acute dengue infection (Green et al., 1999a; Mongkolsapaya et al., 2003).

In additional analyses examining the secretory responses of DCs to increasing input levels of D2V, we found that production of TNF- α , MIP-1 α , and RANTES showed similar patterns, essentially responding in a dose-dependent manner. Experiments utilizing UV-inactivated virus revealed that replication had a substantial enhancing effect on the secretion of each of these products. Also germane to the virus-dose responsive nature of the secreted products noted above was the observation that TNF- α production occurred far more frequently in infected cells than in bystanders during our ICS assays (see *Figures 11 & 12*). Together, these data support the conclusion that the dose-dependent increases in TNF- α , MIP-1 α , and RANTES secretion were driven by increasing DV infection of DCs, particularly in light of the fact that UV-inactivated virus only marginally induced these products when compared to mock infection. These data also suggest that MIP-1 α and RANTES may have similar profiles in intracellular staining assays as compared with TNF- α .

On the other hand, variability of IFN- α levels in relation to virus dose and the induction of IP10 at low concentrations of virus are very likely related. This is particularly evident when viewed in the context of our finding that IFN- α/β receptor blockade inhibited IP10 production in intracellular staining assays (refer to *Figure 14*). The fact that IFN- α/β receptor blockade mediated biological effects indicates that, during culture, DCs are binding IFN- α produced in an autocrine/paracrine fashion. Therefore, quantitative measurement of IFN- α in supernatants is confounded by constant uptake, in addition to the potential effects of IFN inhibition by DV (Munoz-Jordan et al., 2003; Munoz-Jordan et al., 2005; Ho et al., 2005). Viral inhibition of IFN signaling might reduce IFN- α secretion through blocking effects on IFN- β signaling, particularly as infection rates and viral protein production increase. IP10 secretion appears to suffer some degree of inhibition as well at the highest MOIs, when we noted the highest cellular infection rates. This is not surprising, again, when viewed in light of the intracellular staining data, which indicate that infected DCs are particularly poor producers of IP10 (refer to *Figure 12*).

■ DIFFERENTIAL INDUCTION OF IP10 AND TNF-ALPHA FOLLOWING DENGUE

2 VIRUS INFECTION AND THE ROLE OF IFN-ALPHA

While we found a wide range of secreted products in response to dengue infection, intracellular staining revealed distinct cytokine production phenotypes for infected and bystander DCs. IP10 production was potently induced in bystander cells, whereas DCs with detectable dengue E protein levels demonstrated uniformly low levels of staining for IP10 (see *Figure 12 A*). In multiple experiments, the frequencies of IP10⁺ cells in the

infected cell population were significantly lower than those in bystander cells, an effect that was statistically significant (*Figure 12 B*). On the other hand, TNF- α production was more prominently induced in infected DCs (see *Figure 12 C*), and frequencies of TNF- α ⁺ cells were higher in the infected cell population than in the uninfected bystander cell population, also a statistically significant effect (see *Figure 12 D*). Our findings with IP10 are novel, while Sanchez et al. (2006) reported similar results with TNF- α using an attenuated dengue 2 strain. There are multiple potential effects for these observations, based on the biological activity of these two molecules.

The receptor for IP10 is CXCR3 (Loetscher et al., 1996). Interestingly, it was reported that CXCR3 is expressed on “central memory” helper T cells that selectively become T_h1-type cells producing IFN- γ on activation (Rivino et al., 2004). IP10 production by DCs has been shown to be important for clustering and retention of responding T_h1 cells in the hepatic lymph nodes in a murine model of granulomatous liver disease. In that study, blockade of IP10 resulted in hepatic infiltration of T_h1 cells and liver injury (Yoneyama et al., 2002). Failure of infected DC to produce substantial IP10 suggests that *in vivo* these cells are relatively weak recruiters for T cells expressing CXCR3. Therefore, it is possible that DV-infected DCs fail to recruit and to retain IFN- γ producing T_h cells from the memory compartment. Such an effect has clear implications for DC function, since IFN- γ -mediated signaling appears to be intact in DV-infected DCs (Ho et al., 2005; Libraty et al., 2001). Failure to recruit and activate T_h1 cells would prevent further DC activation and allow for unchecked viral replication in DCs. Secondly, a lack of IFN- γ in the local environment would potentially interfere with the “instructive” IFN- γ stimulated IFN- γ production in responding T cells, negatively

affecting downstream antiviral mechanisms, including the “conditioning” of DCs to induce cytotoxic CD8 T cell activity in the absence of immediate (i.e., in contact with the DC) T cell help (Ridge et al., 1998). Pathology might be further enhanced by ineffective retention of dengue-specific T_H cells in the draining lymph nodes of affected tissues such as liver, skin, or bone marrow, resulting in organ infiltration and immune-mediated damage.

The selective induction of TNF- α by infected DCs, on the other hand, may be directly involved in apoptotic signaling in responding T cells. In particular, CD8 T cells have been shown to be sensitive to TNF-mediated killing, while CD4 T cells are susceptible to Fas ligand (FasL) (Zheng et al., 1995). TNF-mediated cell death would reduce the number of DV-specific T cells generated during primary infection, and cause attrition of responding memory cells during secondary infection. In fact, CD8 T cell apoptosis has been demonstrated for DV epitope-specific MHC-tetramer⁺ cells during secondary infection, indicating that these cells are receiving death signals (Mongkolsapaya et al., 2003). During primary infection, CD8 T cell death may be of relatively little consequence, since cross-priming and presentation of apoptotic cells could eventually provide a sufficient response to clear the virus. The absence of ADE and generation of a serotype-specific antibody response would effectively neutralize circulating virus. However, during secondary infection, killing of dengue-specific T cells, particularly CD8 T cells, would have adverse consequences, since early effective responses may be crucial to control dissemination of the virus by infected DCs. Failure to eliminate these cells (as we also noted a lack of MHC Class I upregulation in infected DCs) would allow for viral replication and dissemination. At that point, contributing

factors to the development of severe disease such as ADE effects and pathological T cell expansion would be facilitated. It would therefore be of interest to examine the responses of dengue-specific CD8 T cell clones to autologous DV-infected DCs in the absence or presence of antibodies directed against TNF- α , to determine if antigen-specific activation followed by TNF-mediated apoptosis occurs in these cells.

IFN- α/β was reported to inhibit DV infection of human cells *in vitro*, but required pre-treatment to be effective (Diamond et al., 2000). Monocyte-derived DCs have been described as weak IFN- α producers when compared with plasmacytoid DCs (Izaguirre et al., 2003; Colonna et al., 2002), and IFN- α levels detected in supernatants from our experiments were low compared with a number of other DV-induced cytokines and chemokines. Nevertheless, autocrine and/or paracrine signaling by endogenous IFN- α/β was active in controlling ongoing DV infection in DC cultures. Blocking experiments revealed that IP10 production in response to virus was largely IFN- α/β -dependent. DV has been shown to suppress IFN actions in multiple *in vitro* systems (Munoz-Jordan et al., 2003; Munoz-Jordan et al., 2005; Jones et al., 2005; Ho et al., 2005). In light of those findings, viral inhibition of IFN- α/β signaling is likely the cause of weak IP10 induction in infected DCs.

In control experiments, anti-IFNAR2 antibody completely blocked IP10 induction in response to approximately 10-fold higher levels of IFN- α than we measured in culture supernatants from DV-infected DCs (refer to *Figure 14 C*), suggesting the production of additional regulators of IP10 in this system. These regulators may include IFN- γ , which we detected at very low levels in DC supernatants following infection, or the recently described IFN- λ s (Pekarek et al., 2007).

Unlike IP10, TNF- α production was not affected by IFN- α/β blockade, and appeared to represent a direct response to ongoing viral replication in infected DC. Results with Sendai virus infection of murine DCs were similar to ours, in that IFN- α was not involved in TNF- α production (Lopez et al., 2003). In addition to the report by Chua et al. (2005) implicating dengue NS1 in TNF- α production, intracellular signaling mechanisms responding to viral products are likely also active in infected DCs, including the RIG-I-MAVS pathway (reviewed by Hiscott et al., 2006). Alternatively, TNF- α may be regulated similarly to IL-8 induction, through the dengue NS5 protein (Medin et al., 2005).

We also found that DCs infected with the low-passage Thai DF patient isolate C0166/96 essentially failed to induce IP10 production at all following infection (*Figure 16*) when compared with NGC, despite the fact that these two viruses resulted in similar infection levels. That the C0166/96 strain and not NGC was associated with early dengue antigen expression indicates that this observation was not due to a consequence of delayed viral replication and therefore delayed secondary activation of IFN- α/β production in DCs infected with the C0166/96 strain. These findings suggest that the C0166/96 strain does not induce IFN- α production upon infection, or may act to selectively ablate IFN- α production. Interestingly, both RSV and measles strains which are able to prevent IFN- α production from plasmacytoid DCs in response to agonistic stimuli have been identified (Schlender et al., 2005). Variant strains of Sendai virus 52 (low production) and Cantell (high production) have also been shown to have vastly different capacities to induce IFN- α/β in murine DCs (Lopez et al., 2003). Given the important role of IFN- α/β in control of viral infection in general, and in dengue infection

specifically, investigation of the cause of this phenomenon is an extremely important avenue of future research.

■ STIMULATION OF T CELLS IN CO-CULTURE WITH DENGUE 2 VIRUS-INFECTED DENDRITIC CELLS

The experiments we performed on DCs in the absence of secondary stimulation indicated that D2V activates whole-culture responses. Therefore, we sought to determine if the activation of phenotypic and secretory function in these cells was reflective of enhanced function during interaction with CD4 T cells, as a measure of adaptive immune stimulatory capacity. Specifically, I was interested in determining if DV infection of DCs facilitated their T cell priming function. In my initial experiments, in accordance with the activation of DC surface molecule expression, most D2V-infected DC cultures stimulated allogeneic resting CD4 T cell proliferation. This effect was particularly striking as DC numbers, and background proliferation stimulated by mock-infected DCs, decreased (see *Figures 17 A and B*). At lower T: DC ratios, e.g. 5:1 and 10:1, some DV-infected DC cultures (but not cytokine-matured DC cultures) showed slightly reduced allostimulatory function. Specifically, although at both aforementioned ratios, three of six experiments showed enhanced T cell proliferative responses to DV-infected DC cultures when compared with mock-infected cultures, two cultures at 5:1 had reduced responses and one showed essentially no effect; one co-culture at 10:1 had a reduced response, and two more essentially no change. Even at 20:1, responses in three of six cultures were unchanged or only marginally enhanced (see *Figure 17*, experiments 1, 4, and 5). Therefore, under some conditions, particularly with high antigen load (high DC numbers), DV infection of DC cultures has minimal, even negative, effects on T cell

allostimulation. Interestingly, the variability in SI values (*Figure 17B*) for DV-infected DCs co-cultured with T cells appears to be a function of the substantial variability in absolute responses (i.e., cpm values) to mock-infected DCs between experiments (see *Figure 17A*, Mock), as absolute responses to DV-DCs tended to be consistent between experiments (*Figure 17A*, D2V). Therefore, the intrinsic capacity of mock-infected DCs to stimulate the T cell preparation used in a specific experiment has clear weight in interpreting the results following alternative treatments.

In consistent agreement with our surface phenotyping results, DCs that were matured with the addition of exogenous cytokines (TNF- α /IFN- α) gained in their ability to stimulate CD4 T cell proliferation. We saw moderately greater levels of surface markers on cytokine-matured DCs as compared to DCs infected with D2V, most clearly for CD83, but also for HLA DR, CD40, and CD80 (refer to *Figure 4*). Although absolute differences between mean expression levels of HLA DR and CD40 in this comparison were small, in both cases, they represented a substantial fraction of the “inducibility” within the system, as determined by the maximal stimulus for all molecules tested, LPS. In addition, PD-L1 and PD-L2 expression levels between DCs from D2V-infected cultures and cytokine-matured cultures were similar. Together, this indicates that the balance of co-stimulation versus co-suppression was more favorable in cytokine-matured DCs as compared to D2V-infected DCs. Not surprisingly, TNF- α /IFN- α treatment was a more effective and consistent stimulus for enhancing the ability of DCs to induce resting CD4 T cell proliferation than was infection with D2V (see *Figures 17A and B*). A comparison of SI values shows that in essentially all cases, cytokine maturation resulted in enhanced allostimulatory function, while in some instances, particularly at lower T:

DC ratios (greater alloantigen levels), D2V did not. However, in this experiment, failure of DCs to increase their allostimulatory activity following DV exposure was an infrequent finding when considering the sum of responses across the range of T: DC ratios. This argues against the conclusion that DV infection of DC cultures suppresses DC allostimulatory activity as reported by Palmer et al. (2005) and Sun et al. (2006). Although not as potent a stimulus as cytokine maturation, D2V infection generally resulted in improved allostimulatory function in DC cultures.

These results indicate that D2V infection of DC cultures does not induce an inherently allosuppressive culture-wide effect, as has been reported for MV (Fugier-Vivier et al., 1997; Grosjean et al., 1997; Schnorr et al., 1997; Dubois et al., 2001) and RSV (de Graaff et al., 2005; Bartz et al., 2003). Rather, results from *Figure 18B* suggest that direct infection of a given DC in culture appears to inhibit the allostimulatory capability of that specific cell, relative to bystander DCs within the same culture. This conclusion is drawn from the observation that higher multiplicities of infection, reflected in increasing infection rates (see *Figure 18A*), were associated with reduced proliferation (see *Figure 18B*) when compared to lower MOIs. Surprisingly, the results in this series of experiments comparing DV-DCs to mock-infected DCs appeared to be discordant on some levels with the data presented in *Figure 17*. Infection at lower MOIs in this series of experiments tended to enhance allostimulatory responses, similar to the results seen in the experiments comprising *Figure 17*. However, at higher MOIs that were more in keeping with the *Figure 17* data, mean allostimulation (i.e., the values for all seven experiments combined) tended to be similar to, or lower than, that seen with mock infection. This effect was not statistically significant, however, and the range of values

obtained in response to mock infection was highly variable, as previously described for *Figure 17*.

In the series of experiments presented in *Figure 18*, proliferative responses to DV-infected DCs in four of seven co-cultures were greater than responses to mock-infected DCs, while in three co-cultures responses were suppressed. Notably, in two of the co-cultures with reduced responses (donors 6 and 7), proliferative responses to mock-infected DCs were unusually high. DCs from these donors, and from donor 4 (which also demonstrated reduced allostimulatory function when compared with mock infection) had the greatest infection rates at an MOI=1, and all demonstrated infection of greater than 50% at an MOI=5. Also, in six of seven co-cultures, proliferation induced by DV-DCs at the lowest MOI (=0.04) was greater than that induced by mock-infected DCs, suggesting that activation of DCs in the absence of high percentages of infected cells (i.e., activation in the presence of a vast majority of bystander cells) is a consistent stimulus for enhanced alloproliferative activity.

As alluded to above, the failure of DCs infected at high MOIs to induce alloproliferation in some of the experiments presented in *Figure 18* could also be reflective of the high infection rates noted for this series of DC infections; the experiments in *Figure 17* could not be correlated to DC infection rates. In fact, four further experiments performed comparing mock-infected DCs to DV-infected DCs (MOI=1; shown in *Figure 20*) found enhanced allostimulatory activity in DV-infected DC cultures as compared with mock infected DCs, reminiscent of the results in *Figure 17*, albeit with generally lower infection rates than in *Figure 18*. When taken as a whole, reduced T cell proliferation to DV-infected DC cultures was rare (for example, only 3 of

17 co-cultures at a T:DC ratio of 20:1 and MOI=1 or 2; see *Figures 17, 18, and 20*).

Again, I interpret these data to mean that bystander DCs in D2V-infected cultures gain in allostimulatory activity, while the data in *Figure 18* argue that directly infected DCs have generally poor relative allostimulatory function.

Although the T: DC ratio in the *Figure 18* experiments was 20:1, in most cases the cpm values were more reflective of the cpm values obtained for T:DC ratios of 5:1 or 10:1 than those at 20:1 shown in *Figure 17* (compare responses to mock-infected DCs and DCs infected at an MOI=1 or 5 in *Figure 18B* to mock-infection or D2V infection in *Figure 17A* with addition of 10000, 5000, and 2500 DC). Furthermore, these data argue that increased baseline proliferation (e.g., with high DC numbers or in donors whose mock-infected DCs have unusually high allostimulatory function) reduces the ability of the system to detect increases in proliferation, while likely increasing the system's ability to detect suppression. While most pronounced at low T: DC ratios, such an effect appears to extend to the 20:1 T:DC ratio chosen for the experiments in *Figure 18*, indicating that further similar experiments at a higher T:DC ratio may have substantial value in clarifying the results in this experimental series in light of the additional experiments I have presented here. Thus, a general increase in the proliferative responses to mock-infected DCs and the presence of outliers with aberrantly high responses to mock-infected DCs appears to be contributing to the discordant nature of the results comprising *Figure 18*, as viewed in light of all co-culture data. However, a global assessment of all T:DC co-cultures performed in the experiments comprising *Figures 17, 18, and 20* indicates that D2V-infection of DC cultures enhances the culture allostimulatory potential, while *Figure 18* specifically suggests that increasing infection

rates, and thus a higher infected: bystander DC ratio independently reduces allostimulation. Variability in the susceptibility of donors' DCs to direct suppression by internally replicating virus likely contributes to the range of effects (e.g., stimulation in the face of infection versus frankly reduced proliferation) seen in these experiments.

The system I utilized for my co-cultures was unable to directly compare T cell proliferative responses to infected versus bystander DCs, since surface expression of dengue proteins was inadequate to effectively sort viable cells. However, in the series of experiments shown in *Figure 18*, in a minority of donors (4, 6, and 7), I did observe decreased T cell proliferation to DV-infected DCs in co-cultures as compared to mock-infected DC at the highest MOIs (*Figure 17 B*), and even at low MOIs (donors 6 and 7), similar to previous reports (Palmer et al., 2005; Sun et al., 2006). Reduced allostimulation in these experiments was dependent on replicating virus, since equivalent reductions did not occur with UV-inactivated D2V infection of DCs (*Figure 18 E*).

In donor 4, I found high-level IL-10 production during the initial 24-hour infection period (*Figure 10 D*), reflecting the mechanism proposed by Palmer et al. (2005). However, in T-DC co-culture, DCs from this donor had enhanced allostimulatory function at MOIs of 0.04 and 0.2 (*Figure 17 B*). This was despite the greatest IL-10 production of seven donors' DCs at all MOIs, as measured in supernatants from the 24-hour period post-infection, prior to use in T cell stimulation assays (*Figure 10 D*), indicating that IL-10 induction alone in this donors' DCs was not sufficient to prevent them from developing enhanced allostimulatory capability. Both donors 6 and 7 also showed increases in IL-10 production during the infection period, prior to co-culture, particularly at an MOI=0.2. Although IL-10 levels detected in supernatants from these

donors' DCs decreased at higher MOIs, the DCs became progressively less functional as infection rates increased (*Figures 17 A and B*), with reduced proliferation at an MOI=0.2 and higher. Also, donor 1 showed a dose-dependent increase in secreted IL-10 levels to levels similar to those of donors 6 and 7, yet DCs from donor 1 remained stimulatory, relative to mock infection, even at an MOI=5 (although T cell stimulation peaked at MOI=0.04). Together, these results suggest that, in some cases, D2V infection results in IL-10 production and associated reduction in DC allostimulatory function, although the magnitude of the effect appears to be highly variable, possibly reflecting the variability in IL-10 induction. Notably, this did not appear to be a dominant effect, since in four of seven co-cultures performed across the range of MOIs, we found greater proliferation at each MOI as compared with mock infection.

Both previous studies reporting reduced alloproliferative responses to dengue-exposed DCs showed data in which proliferation decreased as MOI increased (Sun et al., 2006; Palmer et al., 2005). This contrasts with the data we present here, which shows that at lower MOIs, DC stimulatory function is enhanced, while as MOI increases, DC stimulatory function tends to decrease, a statistically significant effect at an MOI=1 or 5, as compared with all other conditions using infected DC. The authors of the aforementioned previous studies interpreted their findings as indicative of D2V-mediated suppression of DC function, although even at the highest MOIs, proliferative responses were between 50% and 75% of the responses seen with mock infection (Palmer et al., 2005; Sun et al., 2006). Both reports by this group included only representative data, failing to address the consistency of their results (Palmer et al., 2005; Sun et al., 2006). Clearly, the effect noted in those studies, and in similar observations in our studies, is

mild when compared to results reported for either MV or RSV (Grosjean et al., 1997; Fugier-Vivier et al., 1997; Dubois et al., 2001; de Graaff et al., 2005). In addition, Palmer et al. (2005) reported enhanced apoptosis of DCs infected with high MOIs of D2V. Therefore, in that study, reduced T cell proliferation may have been secondary to increased attrition of infected DCs in culture.

Also in contrast to findings by Palmer et al. (2005), we noted little or no detectable IL-10 in supernatants from T-DC co-cultures, suggesting that this cytokine was not produced in response to the alloreaction, and thus does not play a role in suppressing T cell responses during the course of T-DC co-culture. Sun et al. (2006) reported IFN- γ production during T-DC co-culture, an observation I also made. Here, I report that T-DC co-culture results in the secretion of IL-13 as well. Mean IFN- γ and IL-13 levels were equivalent across the range of DC MOIs (*see Figures 18D and E*). In addition, neither IFN- γ nor IL-13 showed a dose-responsiveness to DC MOI, indicating that activation of resting CD4 T cells in co-culture with DV-infected DCs primes a mixed T_h1/T_h2 response, similar to mock infection. Increasing MOI was associated with increasing infection rates, and in most co-cultures, DV-infected DCs represented the major fraction of DCs added.

These results indicate that co-culture of DV-infected DCs with CD4 T cells does not skew responses towards either a T_h1 or T_h2 phenotype. This, in turn, suggests that following DV exposure, infected DCs do not develop intrinsic properties for skewing CD4 T cell responses. These findings contrast with those using RSV, in which case CD4 T cells co-cultured with infected DCs exhibited minimal production of IFN- γ or IL-13, although both of these cytokines were produced at substantial levels in T-DC co-cultures

where DCs were matured using exogenous cytokines, poly I:C, or InfV (de Graaff et al., 2005). Notably, in that study, InfV was a particularly potent stimulator of T_h1 -skewing, as IFN- γ levels were substantially higher than those of IL-13. On the other hand, results in our experiments were reminiscent of that noted for YFV 17D infection in mice, which facilitated the induction of both T_h1 and T_h2 -type responses (Querec et al., 2006).

Although these experiments did not allow me to discriminate the source of IL-13 and IFN- γ , additional experiments are possible to determine this. Restimulation of T cells with a non-specific stimulus such as phorbol esters (PMA) and ionomycin after 1 week in culture coupled with intracellular staining would reveal specific functional activities in CD4 T cell subsets. Analysis of CD4 T cell surface expression of molecules such as the IL-7 receptor would provide information regarding the ability of cells developed during co-culture with infected DCs to respond to homeostatic cytokine signaling. Also, functional studies using blocking antibodies to Fas or TNF- α would provide information as to whether or not these molecules were inducing death signals during co-culture of CD4 T cells with infected DCs. Performing a similar series of experiments with CD8 T cells as those presented in this thesis using CD4 T cells would yield information regarding the stimulatory activity of infected DCs for cytotoxic T lymphocyte function. Finally, testing autologous PBMC and T cell clones specific for DV against DV-infected DC targets will yield specific information regarding how DV infection affects T cell memory responses to infected DCs. Such activities might include DC lysis, T cell cytokine production, and DC-mediated T cell apoptosis (potentially through Fas or TNF- α as previously mentioned).

Our findings regarding T cell responses in co-culture with DCs infected with a range of MOIs clearly demonstrated variable results, despite the consistent trend for decreased proliferative responses at the highest MOIs. This suggests that donor-dependent effects may have a strong influence on DC allostimulatory activity, in keeping with the variability we noted in infection rates and the secretion of soluble factors. Testing of DCs and T cells in a 2x2 factorial design suggested that the DC source had the greater influence on the pattern of proliferation responses (refer to *Figure 19*). However, the characteristics of the T cells are likely also important. We used negatively selected CD45RO⁻ “resting” CD4 T cells. In contrast, Palmer et al. performed proliferation assays using positively selected CD3⁺ T cells, which included both naïve and memory CD4 and CD8⁺ T cells. These cells showed higher proliferation responses to mock-infected DC and may be more readily activated to express PD-1, thus becoming susceptible to inhibition by PD-L1/PD-L2 expression on the DC (Vibhakar et al., 1997; Latchman et al., 2001). In the report by Sun et al. (2006), which used naïve CD4 T cells selected in a similar manner as in our studies, the effect of DV infection on DC stimulation was more modest than that reported by Palmer et al (2005).

The presence of antibody during primary infection is thought to influence disease severity in infants, putatively through ADE of monocytes or macrophages. We tested the effects of dengue-immune plasma on the infection of DCs and their allostimulatory function. This experiment was in response to the question of whether immune-complexed DV demonstrated distinct effects on DC priming capacity as compared to DV in the absence of cross-reactive antibodies. In this experiment, we found no effect of D3V-immune plasma on either DC infection rates, or on the ability of these cells to

stimulate allogeneic CD4 T cell proliferation. This result indicates that DV in the context of immune complexes does not have intrinsic properties that differentiate it from naked virus as related to the induction of DC allostimulatory function. This finding argues against a role for immune-complex-mediated effects on DC function in disease pathology during primary infection of infants, or secondary infection in older children and adults.

■ **FINAL WORDS ON DENDRITIC CELL INFECTION AND TRANSLATING IN VITRO RESPONSES TO IN VIVO/CLINICAL FINDINGS**

An important point to recall when examining the effects of both measles and RSV infections *in vivo* versus *in vitro* in DCs is that the consequences *in vivo* are rarely as profound as might be predicted by those *in vitro*. This might be appreciated most simply by recalling that living organisms employ myriad semi- (and fully-) redundant mechanisms to cope with invading pathogens. Rather, the *in vitro* immunosuppression noted in T cell-DC co-cultures with these viruses may be reflective of a common survival and dissemination mechanism of acute cytopathic viruses, which replicate rapidly to high titers, induce potent immune responses, and thus must transmit themselves in as expeditious a manner as possible (reviewed by Hangartner et al., 2006). In such cases, immunosuppression may actually help to keep the host alive long enough to permit the virus access to a new host, rather than rapidly killing host (and thus virus). Examples of poorly human-adapted, emergent viruses such as Sin Nombre and Ebola, which “burn” fiercely within a host or community thus have either failed to adapt yet to facilitate transmission via the new host (humans), or induce such profound immunosuppression that the host is incapable of recovery (as appears to be the case with Ebola).

■ A PATHOGENESIS DISCUSSION: FUTURE WORK IN THE DV-DC (AND DV-APC) SYSTEM

In this thesis work, I found that DV infection of DC cultures activated a range of functions in these cells. Virion binding alone resulted in phenotypic and secretory functional responses, but these were quantitatively and qualitatively different in the presence of replicating virus. Viral replication stimulated IFN- α/β production, which drove bystander IP10 production, while production of this chemokine was inhibited in infected cells. On the other hand, infected DCs potently produced TNF- α . These data, combined with dose-response data, suggest that a number of cytokines and chemokines have a similar dependence on active replication. Experiments using DV-infected DCs to stimulate T cell responses demonstrated stimulation by infected cultures, although substantial evidence indicated that bystander DCs, and not infected DCs, were the more potent stimulators. I did not note IL-10 production in T-DC co-cultures, nor did I note T_h-skewing effects of infected DCs.

Since DCs have been demonstrated to be targets of DV infection *in vivo*, these data are extremely relevant to understanding dengue disease pathogenesis. A model proposed by Palmer et al. (2005) argued for a major role of IL-10 in DV infection of DCs, suggesting that DV-induced immunosuppressive effects facilitate the development of severe disease. However, any model that includes DCs must account for the fact that there is no difference in DC populations present between disease states (DF vs. DHF) or exposure histories (primary vs. secondary), suggesting that any potential pathogenesis arising from DC infection is related to downstream responses. Therefore, I argue that the Palmer model is inherently flawed, since, if all DCs produced IL-10 and caused

immunosuppression leading to severe dengue illness, DHF would be equally common in both primary and secondary infections. Instead, I propose that DCs play a role in the etiology of severe dengue disease through both typical, and atypical, responses to DV infection. A discussion of the infection process in light of the data presented in this thesis follows, with particular attention to the events following inoculation, and the roles that DCs may play in primary versus secondary infection.

Despite purported susceptibility of a number of cell types to dengue infection, including those previously mentioned *in vivo*, dendritic cells have substantially greater susceptibility to infection when compared to other cells. However, DCs might be expected to be relatively rare cells in the tissue microenvironment immediately following a mosquito bite and subsequent inoculation. Furthermore, the tissue architecture of the skin likely determines the local susceptibility to infection for resident cell types. Specifically, the superficial keratinizing stratified squamous epithelium of the skin is separated from both vascularized papillary dermis and deeper reticular dermis/cutaneous vascular plexi by a thick basement membrane. Insertion of the mosquito feeding apparatus transects the avascular epithelial layers, which contain Langerhans cells basally, and the physical barrier of the basement membrane. Because LCs are physically separated from the site of virus inoculation (which likely occurs most substantially at the deeper vascular layers) by the basement membrane, these cells might be expected to be much less common targets for natural infection than cells of the dermis, including conventional DCs (such as interstitial DCs or monocyte-derived DCs).

Following virus exposure, multiple cell types would be infected at various (but generally low) rates, based on previously discussed *in vivo* and *in vitro* studies. One

target that is of potential importance early after viral entry is the dermal fibroblast. Human foreskin fibroblasts are infected by dengue virus, and produce cytokines such as GM-CSF (Kurane et al., 1992). While the infection rate in these cells may be relatively low, their abundance in the dermis suggests that fibroblast infection may contribute substantially to downstream events early following exposure. Dermal fibroblasts may represent the earliest infected cell type, along with interstitial DCs, thus serving as an important source of virion production if, as might be expected, infected DCs migrated out of the local microenvironment following maturation. In addition, dermal fibroblasts would also serve in the recruitment of immune effectors in response to chemotactic factors and cytokines generated from cellular exposure to and/or infection by DV particles. In particular, recruitment of monocytes into the inflammatory microenvironment in the presence of factors such as GM-CSF may provide large numbers of highly susceptible target cells as viral replication begins to accelerate. Infection of these cells would become more efficient as they differentiated into a DC morphology.

Work in the murine system suggests that two major subsets of blood monocytes, distinct based on expression levels of the chemokine receptor CX₃CR1, represent precursor cells with divergent functions. Furthermore, analogous subsets are also present in humans, in which phenotypic distinction can be made based on the human homologue, also CX₃CR1 (Geissmann et al., 2003). The CX₃CR1^{lo} population serves as a short-lived “acute response” to local inflammation, while the CX₃CR1^{hi} population is longer lived, and migrates to tissue in the absence of inflammation. Both subsets are capable of differentiation into DCs, *in vitro* and *in vivo*, and have T cell priming capability, with the

former ostensibly recruited to local sites of infection in order to augment immunity in the face of an invading pathogen, and the latter likely serving as tissue resident interstitial DCs (Geissmann et al., 2003).

DCs are considered to be critical during viral infections because of their abilities to cross-prime, or present exogenous antigens in the context of MHC I molecules (reviewed by Bevan, 2006). Although there is considerable disagreement in the literature regarding the role that monocyte precursors from blood have in this process, it is possible that during viral infection, monocyte-derived DCs may serve as antigen transporting cells, carrying endogenously expressed viral epitopes in the context of MHC I following peripheral exposure to secondary lymphoid tissues, where antigen transfer may occur either through plasma membrane transfer to T-cell priming DCs, or through uptake of apoptotic monocyte-derived DCs (Randolph et al., 2007). An additional mechanism may be that viral transport to secondary lymphoid tissues results in local infection, with locally resident DCs either infected by the virus, or presenting exogenously acquired viral particles through a cross-presentation pathway. CD4 T cell stimulation would likewise be stimulated following exogenous particle uptake, particularly in the presence of specific antibodies. CD4 T cell-mediated local IL-2 induction and would potentiate outgrowth of the antiviral CD8 T cell population, an effect that would be enhanced by an environment favoring IFN- γ secretion and further stimulation of DC priming function.

In the case of DV infection, monocyte-derived DCs are likely to play a critical role at multiple stages during infection, since this cell population represents a mobile, susceptible reservoir of target cells that would facilitate viral dissemination beyond the

site of inoculation, and may also be infected secondarily after viremia occurs and target organ systems become sites for viral synthesis. The high infection rates associated with monocyte-derived DCs suggests that a substantial portion of emigrating cells from the initial nidus of infection would carry replicating virus, effectively translocating infection from the inoculation site to secondary lymphoid tissue as the DCs received maturation signals from virus-triggered inflammatory mediators, or from the direct effects of viral replication within a given cell. Once present in secondary lymphoid tissue, the virus would have access to a highly cellular environment, with a relatively dense target cell population, and potential for egress into the bloodstream, facilitating generalized viremia.

A modifying factor that likely plays a role early during viral replication and dissemination is the presence of potent IFN- α/β producing cells. In this thesis work, I found that monocyte-derived DCs were able to produce functional type I IFNs that were then able to inhibit ongoing dengue virion production in infected cultures. However, as previously mentioned, the role of monocyte-derived DCs in production of type I IFNs is generally considered to be marginal, depending on the system used, when compared with PDCs (Izaguirre et al., 2003; Colonna et al., 2002). An important adjunct cell type for IFN- α production in response to DV infection is likely the PDC; activity of these cells in producing IFN- α would spare monocyte-derived DCs (and interstitial DCs) by inducing maturation and inhibiting viral replication. Interestingly, although PDCs express the IP10 receptor CXCR3, recruitment appears to depend not on the concentration gradient of IP10 (or the additional CXCR3 ligands ITAC and Mig), but rather constitutively expressed chemokines that signal at much greater effectiveness following CXCR3 binding on the PDC (Vanbervliet et al., 2003). Therefore, in a pathological cycle, weak

IP10 induction by infected MDDCs would hamper PDC migration into the inflamed stroma of DV-infected skin, resulting in greater susceptibility of conventional DCs to DV infection. Indeed, the clinical use of PDC stimulators such as the TLR7 agonist imiquimod likely facilitate clearance of viral infections of the skin, such as HPV, through PDC activation and IFN production.

PDCs would appear to have additional roles in the secondary lymphoid tissue during DV infection as well. Such activities may include lymph node homing, with IFN- α dependent protection of cellular constituents following the arrival of peripherally infected conventional DCs, and IFN- α mediated T_h1 skewing. In fact, in humans PDCs are recruited to lymph nodes following natural infection with influenza virus (Cella et al., 1999), severe dengue disease is associated with reduced PDC numbers in blood early during disease (Pichyangkul et al., 2003), and *in vitro*, PDCs support T_h1 priming (Cella et al., 2000). Reduced PDC numbers in the blood of severely ill patients, as opposed to the initial spike seen in patients with mild disease, may represent either ineffective stimulation of PDC growth and emigration from bone marrow, or possibly enhanced extravasation to sites of infection (skin, lymphoid tissue, liver, etc.). PDCs might also be infected directly with DV, although this has not been demonstrated, and seems unlikely given the potency with which DV stimulates IFN- α production in these cells (Wang et al., 2006), and the fact that IFN- α production appears to protect PDCs from virally-induced cell death (Cella et al., 2000).

The absence of an appropriate PDC response in individuals suffering from more severe infections suggests a functional defect in type I IFN-mediated immunity secondary to weak PDC activity. Failed type I IFN activity would predispose these individuals not

only to increased viral load, but would shift the monocyte-derived DC infected: bystander ratio towards higher proportions of infected DCs. In some cases, monocyte-derived DCs may produce IL-10 in response to DV infection, which would functionally blunt both PDC and conventional DC innate immune activity by reducing the effectiveness of secreted type I IFNs, as well as suppressing antigen-presenting activity. The consequences of either case would be weaker adaptive immune stimulation and delayed DV-specific responses. That PDCs can stimulate T_H1 priming (Cella et al., 2000) also suggests that PDC recruitment to secondary lymphoid tissue, in concert with monocyte-derived DCs, may be important in providing help to skew T_H responsiveness towards an IFN- γ producing paradigm, a capacity that was conspicuously absent in our experiments when MDDCs alone were co-cultured with CD4 T cells.

Following local infection, monocyte-derived DCs would likely represent the earliest and most substantial population of cells migrating to secondary lymphoid organs to stimulate adaptive responses. The previously mentioned role of monocyte-derived DCs during acute inflammation suggests that there is an increasing influx of monocyte-derived and interstitial DCs to immune tissues during the course of infection until the point where viral replication is controlled through adaptive immune mechanisms, including the generation of neutralizing levels of DV-specific antibodies. During primary infection, the lack of dengue-specific antibodies early during viral replication would allow for infection of a broader range of cell types than might be expected during secondary infection. In the case of secondary infection, Fc γ R-expressing cells, including macrophages and blood monocytes, would be infected at enhanced rates, while non-Fc γ R-bearing cells might demonstrate reduced susceptibility with partial viral

neutralization. During the phase of generalized viremia, either in primary or secondary infection, the importance of DCs in determining the outcome of infection would likely be reduced, since in the case of primary infection, adaptive immune responses would be expanding and combating infection of target organs, while in secondary infection, ADE would likely shift the APC target population to macrophages and undifferentiated blood monocytes.

Early, rather than late, events surrounding DC infection are likely the critical factor (with regards to DCs) in determining the course of ongoing dengue infection. Since initiation of adaptive immunity is presumably necessary for viral clearance, the ratio of infected to bystander cells providing virus-specific stimulation is potentially important in determining the magnitude, if not the quality, of the T cell response. Host and/or viral factors contributing to a state that predisposes the host to a high ratio of infected to bystander DCs in secondary lymphoid tissues would therefore reduce the effectiveness of T cell priming. Some examples include defects in PDC numbers, recruitment, or IFN- α production in response to DV. High DC-SIGN expression on conventional (particularly monocyte-derived) DCs, high-level IL-10 induction by monocyte-derived DCs resulting in suppression of IFN- α signaling, and high-affinity binding, low-IFN- α inducing, or particularly aggressively replicating strains of DV are additional examples. The delay in T cell expansion might be further exacerbated by reduced recruitment of cell types responsive to IP10 (memory T cells or PDCs) and capable of potentiating IFN- γ production. Lastly, TNF- α production by infected DCs could result in T cell killing, and the infected DC surface phenotype may favor T cell inhibition or, in the case of secondary infection, expansion of pathologic memory T cell

subsets. Pathological responses, however, are likely multifactorial, as in most cases dengue illness is effectively cleared. This suggests that in the vast majority of cases, DC infection (in opposition to the model proposed by Palmer et al.) appropriately directs downstream immunity.

Based on the findings in this thesis, in the case of common DC responses to DV infection, I propose that during primary exposure, DV infection of DCs in most individuals results in immunostimulation. Stimulatory DC function depends on a relative abundance of bystander DCs, low infection rates, and low DC numbers. Such a situation would facilitate mixed T_h priming, and the generation of appropriate mixed immune responses that include dengue-specific cellular and humoral immunity, which effectively clear the infection. The minority of individuals prone to high infection rates, IL-10 production, or suppressed T cell responsiveness to infected DCs would exhibit mildly enhanced pathology.

During secondary infection, suppressed IP10 production and MHC I expression combined with TNF- α production in infected DCs would result in poor DC recruitment of T_h1 -type memory cells (thus affecting IFN- γ secretion and further DC activation) and reduced CD8 T cell-mediated lysis (through inhibition of MHC I induction and TNF- α mediated killing of activated high-avidity dengue-specific CD8 T cells). The infected DCs are thus inefficiently eliminated from circulation, and migrate to secondary lymphoid tissues, where they serve as reservoirs for DV dissemination. At that point, virus produced by DCs would more efficiently infect monocytes and macrophages through ADE, targeting organs such as the liver (Kupffer cells), skin macrophages, and possibly peritoneal/serosal macrophages. ADE-mediated infection of this normally

infection-resistant group of cells would induce IL-10 secretion and inhibit the antiviral functions of IFNs, and shift the major epitope-presenting population away from DCs. This in turn would cause activation of non-protective or pathological T cell subsets (which are not present during primary infection) that are inefficient in their clearance of infected APCs, including DCs. This effect would be further exacerbated through the inhibition of APC function by circulating IL-10. Factors such as early DC IFN- α production, low DC susceptibility to infection, and resistance to infection-dependent blunting of DC function would protect against this pathological cascade. DC IL-10 production, higher susceptibility of DCs to infection, and high levels of TNF- α secretion by infected DCs would exacerbate early immune suppression, and predispose the individual to development of DHF.

CHAPTER VII

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