INTERFERING WITH INFLAMMATORY CYTOKINE PRODUCTION DURING ANTIBODY-ENHANCED DENGUE VIRUS INFECTION

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ABSTRACT

Justin Bryce Callaway: Interfering With Inflammatory Cytokine Production During Antibody-Enhanced Dengue Virus Infection (Under the direction of Jenny P.-Y. Ting)

Dengue virus (DENV) is the most burdensome arbovirus in the world, infecting nearly 400 million people annually. Patients develop lifelong immunity to the original infecting serotype but only transient immunity to the remaining serotypes. Over time, the cross-reactive immune response weakens and can cause severe dengue disease upon infection with a heterologous serotype of DENV. This creates high serum viremia and aberrant production of cytokines, potentially progressing to life-threatening hypovolemic shock due to vascular leakage. One contributing mechanism is antibody-dependent enhancement (ADE) increasing entry of DENV into Fc-receptor-bearing cells, increasing production of cytokines and virus.

One cytokine elevated in the profiles of many severe dengue patients is interleukin-1beta (IL-1beta). IL-1beta is uniquely regulated, requiring one stimulus to induce synthesis of precursor pro-IL-1beta and a second stimulus to activate assembly of the inflammasome. The inflammasome activates caspase-1, which cleaves pro-IL-1beta into its biologically-active form. We sought to identify the steps modulated by ADE to increase IL-1beta secretion. We found that ADE induces IL1B and pro-IL-1beta expression rapidly after inoculation, inducing secretion of mature IL-1beta by 4 hours post-inoculation (hpi). This required activation of a signaling axis consisting of spleen tyrosine kinase (Syk) and extracellular signal-regulated kinase 1/2 (ERK1/2). Interestingly, ADE did not detectably increase caspase-1 activation, but IL-1beta processing required caspase-1 activity. Importantly, activation of Syk by ADE also induced
expression of TNF and IL6, suggesting that interfering with Syk may reduce expression of many ADE-induced cytokines.

Additionally, we found that the cell-type used to propagate DENV in vitro impacted the induction of IL-1beta secretion by primary monocytes. Inoculation of monocytes with supernatants from DENV-infected Vero cells induced IL-1beta secretion independent of ADE. This was due to an inflammatory moiety produced by DENV-infected Vero cells. Purification of Vero-derived DENV eliminated this factor and made ADE required for IL-1beta secretion. Interestingly, DENV-infected mosquito cells did not produce a similar inflammatory component. ADE increased IL-1beta secretion when monocytes were inoculated with supernatant from DENV-infected mosquito cells without need to purify DENV. This study indicates that the source and purity of DENV preparations can significantly impact studies of the innate immune response to DENV.
To my family, whose love, support, and joy they brought to my life kept me going throughout this demanding journey
ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Jenny P.-Y. Ting, for her support and guidance throughout my years in her lab. The early years of these studies were fraught with hurdles that I would not have been able to overcome without her guidance. Additionally, she taught me that not every idea can be pursued, which kept me on track to investigating the most important questions. As I grew as a researcher, I was fortunate to have the resources and flexibility under Dr. Ting’s guidance that afforded me the opportunity to pursue unique ideas with the potential to lead to ground-breaking discoveries.

I would also like to the members of my committee, Drs. Aravinda de Silva, Mark Heise, Glenn Matsushima, and Lishan Su. They always had great insight and helpful suggestions whenever I had questions. Had I been wiser, I would have asked them so much more.

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<th>Description</th>
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<tbody>
<tr>
<td>ADE</td>
<td>Antibody-dependent enhancement</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase activation and recruitment domain</td>
</tr>
<tr>
<td>CLEC</td>
<td>C-type lectin</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger-associated molecular pattern</td>
</tr>
<tr>
<td>DAP12</td>
<td>DNAX-activation protein 12</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic-cell specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>DENV</td>
<td>Dengue virus</td>
</tr>
<tr>
<td>DF</td>
<td>Dengue fever</td>
</tr>
<tr>
<td>DIV crude supernatant</td>
<td>DENV-infectious Vero-cell crude supernatant</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>E</td>
<td>Envelope protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FcR γ chain</td>
<td>Fc receptor γ chain</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fcγ receptor</td>
</tr>
<tr>
<td>ffu</td>
<td>Focus-forming units</td>
</tr>
<tr>
<td>hpi</td>
<td>Hours post-inoculation</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFNAR</td>
<td>Type I interferon receptor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon-stimulated gene</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITAMi</td>
<td>Inhibitory ITAM</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibition motif</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial antiviral signaling</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma differentiation-associated protein 5</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular-weight cutoff</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding domain, leucine-rich repeat containing protein</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural protein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associate molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>prM</td>
<td>Pre-membrane protein</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptor</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene 1</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SHP-1</td>
<td>Src homology 2 domain-containing phosphatase-1</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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CHAPTER 1: INTRODUCTION

1.1 Dengue virus overview

Dengue virus (DENV), consisting of four genetically distinct but related serotypes (DENV-1–DENV-4), causes nearly 400 million infections per year globally (1,2). A first exposure to any DENV serotype may cause an asymptomatic infection or present with a self-limiting illness referred to as dengue fever (DF). The recovered patient develops long-term immunity to the infecting serotype. Additionally, the human immune response to DENV is highly cross-reactive between serotypes, and patients develop a transient immunity to heterologous serotypes in the immediate months following primary infection (3,4).

Paradoxically, weakening cross-reactive immunity can cause more severe disease upon exposure to a heterologous DENV serotype (1,5,6). Though components of cellular immunity contribute to severe pathology (7), cross-reactive antibodies to the primary DENV serotype can enhance entry of heterologous serotypes into Fc-receptor-bearing cells (1). This increased entry, known as antibody-dependent enhancement (ADE), is directly responsible for enhanced virus production during secondary infections and can generate pathologic cytokine production (1,8,9).

Much research focuses on delineating the driving forces behind ADE, which has further been categorized as extrinsic or intrinsic (10). Extrinsic ADE is the enhanced cellular entry by endocytosis of infectious immune complexes into Fc-receptor-bearing cells. Intrinsic ADE, still controversial to this point, describes a suppression of the innate immune response by DENV immune complexes that increases DENV replication. Important questions remain regarding how ADE may allow DENV to bypass detection by innate immune receptors or interfere with the
antiviral response. My work will focus on mechanisms of extrinsic ADE.

1.2 DENV structure and lifecycle

An understanding of the innate immune defense against DENV, and how ADE may alter these events, first requires an understanding of the structure and lifecycle of DENV. DENV is a member of the Flavivirus genus in the Flaviviridae family (1). It is a positive-sense, single-stranded RNA (ssRNA) virus with a genome of approximately 10.7 kb (11,12). Translation yields a single polyprotein that is post-translationally cleaved into 3 structural proteins, capsid (C), pre-membrane (prM), and envelope (E) proteins, and 7 non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Only structural proteins are packaged into the newly-formed virion, while the non-structural proteins serve crucial roles in viral replication and assembly and immune modulation (1,13).

DENV enters a target cell through clathrin-dependent, receptor-mediated endocytosis (14). Once internalized in an endosome, DENV exploits the maturation process of an endosome to initiate replication. Endosomes undergo a series of maturation and acidification steps prior to fusion with the harsh lysosome contents. Mild acidity in the endosome triggers conformational rearrangements of DENV E protein that allow the virion to fuse with the endosomal membrane and deposit its ssRNA genome directly into the cytosol (15-17).

After translation and processing of the polyprotein, replication occurs at the surface of the endoplasmic reticulum (ER), with the newly replicated capsid and genome budding into the ER, acquiring a host-derived membrane containing careful arrangements of prM and E proteins (18,19). The immature virions progress from the ER to the Golgi apparatus until being secreted from the cell as mature, infectious virions. Importantly, maturation, which involves cleavage of
prM from the virion surface by the host protease furin, is an inefficient process (20,21). Secreted DENV virions are thus a heterogeneous population of mature, partially-immature, and fully-immature virions based upon the extent of prM cleavage. This impacts receptor binding, antibody-mediated neutralization, and ADE, as remaining prM remains dimerized with E protein (17,22). This prevents formation of the receptor-binding conformation, concealing some antibody epitopes while exposing others.

1.3 The innate immune response to DENV infection

DENV is believed to primarily target cells of the myeloid lineage, namely dendritic cells, monocytes, and macrophages (13,23). Other cells reported to become infected by DENV in humans include hepatocytes, endothelial cells, muscle cells, and lymphocyte subsets (24-28). However, the involvement of these cells in promoting severe disease is less clear. For the purposes of innate immune defense against DENV replication, focus will be given to the myeloid cells widely accepted to support DENV replication and disease progression *in vivo*.

Innate immune cells have evolved many pattern recognition receptors (PRRs) designed to sense and respond to danger- or pathogen-associated molecular patterns (DAMPs or PAMPs, respectively) (29,30). PRRs trigger innate immune responses within the cell designed to suppress viral infection and block replication (31). Transcription factors activated downstream of PRRs induce the expression and secretion of inflammatory or regulatory cytokines. PRRs can be expressed at the cell surface, on the endosomal membrane or in other cellular organelles such as the mitochondria and peroxisomes, or in the cytosol (30). Importantly, PRRs vary in DAMP or PAMP recognition based on which stimuli are likely to be encountered in each location.

PRR recognition of DENV at the cell surface of myeloid cells is less understood than
other subcellular locations. DENV has been shown to bind membrane-bound C-type lectin (CLEC) receptors (CLR) on the surface of myeloid cells (32-35). A large family, CLR vary widely in their functions but share the characteristic of binding to carbohydrates, which are often found on microbial surfaces (36). Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) has long been recognized as an important attachment factor for DENV infection (32,37). DENV, which has carbohydrate moieties on its surface, can bind DC-SIGN and gain entry to cells that express it. Additionally, inducing expression of DC-SIGN into DENV-resistant cells will render those cells susceptible to infection (32). However, deletion of the intracellular signaling tail of DC-SIGN, which is required for DC-SIGN endocytosis, does not abrogate infection, suggesting that signaling activity of this CLR is dispensable for endocytosis of DENV (38). Less understood is whether DENV binding to DC-SIGN induces important signaling changes that activate the innate immune response against the virus. Importantly, though, DC-SIGN expression is restricted to certain subsets of myeloid cells. Its expression is high in immature dendritic cells but decreases with maturation (39). It can be found on certain macrophage subsets but is nearly absent on monocytes without induction (40).

Mannose receptor (MR), another CLR, has similarly been reported as an attachment factor for DENV, but whether it contributes to signaling important for innate immunity against DENV is not known (33).

One CLR shown to bind DENV and induce a consequential immune response is CLEC5A. DENV binds CLEC5A on human macrophages, inducing DNAX-activation protein 12 (DAP12) phosphorylation and increased secretion of TNF, IL-6, IL-8, IP-10, and MIP-1α (34). DENV-induced CLEC5A signaling was subsequently identified to activate the inflammasome and mediate IL-1β secretion in inflammatory human macrophages (35). However, CLEC5A
binding was dispensable for viral entry and replication, and CLEC5A signaling was not required for interferon (IFN) secretion. Notably, type I IFNs, IFNα and IFNβ, are beneficial for suppressing DENV replication (41). Thus, the role of CLEC5A in DENV infection could be considered pathogenic, as it elevates pro-inflammatory cytokine production without inducing beneficial type I IFN. This is reflected by CLEC5A blockade protecting mice from lethal DENV infection (34). Interestingly, the inability of CLEC5A to promote DENV internalization may be explained by the much weaker binding of DENV with CLEC5A than DENV with DC-SIGN (42). Regardless, CLR s have not been found to induce a protective innate immune response in DENV-infected myeloid cells.

Toll-like receptors (TLRs) are another set of membrane-bound PRRs and may be found at the cell surface or in the endosome (43,44). Unlike CLRs, protective roles against DENV infection have been ascribed to multiple TLRs in human innate immune cells, though the exact mechanisms are unclear. To this point, no definitive function has been ascribed to cell-surface TLRs during DENV infection.

TLR3 specifically localizes to endosomal compartments in most cells and recognizes double-stranded RNA (dsRNA) (44,45). The presence of dsRNA in the endosome can indicate viral infection, as many viruses contain either a dsRNA genome or dsRNA intermediates during the replication process. Thus, TLR3 activation triggers an antiviral response. Unlike other TLRs, which signal through myeloid differentiation primary response 88 (MyD88), TLR3 solely signals through TIR-domain-containing adapter-inducing interferon-β (TRIF) (46). Upon binding dsRNA, TLR3 dimerizes and is phosphorylated, promoting the recruitment of TRIF (47-49). TRIF activation activates a number of downstream signaling pathways, culminating in the activation of transcription factors IRF-3, AP-1, and NF-κB (45,50-52). This induces the
production of a number of cytokines, but most notably induces robust type I IFN production. Both types I and II IFN protect against DENV infection by preventing translation of DENV RNA (41).

A number of studies have indicated that TLR3 signaling provides protection from DENV. Overexpression of TLR3 promotes clearance of DENV in many cells while TLR3 deficiency impairs clearance and reduces cytokine production (53-55). However, the exact mechanism providing TLR3-mediated protection is unknown. As described earlier, the DENV genome is ssRNA, and dsRNA intermediates are only present during replication in the cytosol, a step occurring after viral fusion with the endosome and deposition of the genome into the cytosol. Importantly, studies have indicated that dsRNA, released by infected dying cells, can be endocytosed by neighboring cells and activate TLR3 in the endosome (56-58). Compared to ssRNA, dsRNA is very stable and can resist degradation extracellularly (59). Thus, TLR3 may provide bystander protection during DENV infection, with dsRNA released by infected dying cells sounding the alarm that danger is nearby. Importantly, TLR3 expression is high in DCs but virtually absent in undifferentiated human monocytes (60), suggesting its protective role in DENV infection is highly cell-specific.

In addition to TLR3, TLR7 is a second endosomal PRR (44) reported to suppress DENV infection. However, in contrast to TLR3, TLR7 recognizes ssRNA (61). As ssRNA is abundant in the cytosol during active gene expression by the host cell, the sub-cellular localization of TLR7 to the endosome is critical to preventing autoimmune activation against self ssRNA. Detection of ssRNA in the endosome activates TLR7, leading to the recruitment and activation of MyD88 (62). TLR7-mediated MyD88 activation leads to the downstream activation of transcription factors IRF-7 and NF-κB, again inducing the expression of both type I IFN and pro-
inflammatory cytokines (50,63).

DENV activates TLR7 in plasmacytoid dendritic cells (pDCs), a circulating dendritic cell that is functionally and morphologically distinct from conventional dendritic cells found in the tissue (64). Notably, pDCs are extremely potent producers of type I IFN and are thus considered to promote antiviral responses. Correspondingly, DENV-induced TLR7 activation in pDCs causes production of type I IFN (64). This activation required that tertiary structures of DENV RNA remain intact, as ultraviolet (UV) cross-linking of DENV RNA severely impaired type I IFN production. As pDCs are not the main cells propagating DENV replication, it is unclear how this robust type I IFN production impacts early defenses against DENV replication. However, defects in the pDC response in vivo have been correlated with severe disease in DENV patients (65). This suggests an important role for TLR7 signaling in pDCs in suppressing DENV replication, as serum viremia directly correlates with disease severity (1).

The detection of DENV ssRNA in the endosome by TLR7 faces similar questions as TLR3 detection of DENV dsRNA in the endosome. During the DENV lifecycle, its ssRNA genome is not exposed in the endosome but is deposited into the cytosol for replication. It has been proposed that degradation of some virions upon exposure to the acidic and proteolytic environment of the endosome could cause ssRNA to leak into the endosomal compartment (62). Interestingly, TLR7 recognition of ssRNA in pDCs has been reported to require active replication of viruses so that replication intermediates can be trafficked to lysosomes by autophagic trafficking (66). Recognition of DENV by TLR7, most highly expressed in pDCs, has not been described in other cell types. As with TLR3, this suggests that TLR7-mediated protection against DENV would be highly cell-type specific, apart from bystander effects induced by type I IFN.
Aside from recognition of DENV by TLR3 and TLR7 promoting antiviral effects, further support exists for a protective role by providing TLR agonists in conjunction with DENV infection. Activating TLR3 with poly(I:C) prior to or concurrently with DENV infection significantly reduced replication in HepG2 cells, a hepatoma cell line (67). Additionally, a combined therapy of TLR3 and TLR7/8 agonists after infection with DENV reduced viral replication and improved the humoral immune response in rhesus macaques (68).

Though definitive protective roles during initial infection cannot be ascribed to any membrane-bound PRR, multiple cytosolic PRRs induce antiviral responses during the DENV lifecycle. Further indicating its important role in signaling potential infections, dsRNA is detected by two different retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) in the cytosol (69,70). RIG-I and melanoma differentiation-associated protein 5 (MDA5) are both phosphorylated in uninfected cells, maintaining the receptors in an inactive state (71). Though both are activated by dsRNA, RIG-I and MDA5 bind distinct and unique structures and forms (72,73). After binding their cognate ligands, both receptors undergo dephosphorylation of their caspase activation and recruitment domains (CARDs) and receptor oligomerization (71). This allows recruitment and activation of the signaling adapter mitochondrial antiviral signaling (MAVS) (73-75). MAVS initiates signaling cascades through TNF receptor-associated factor 2 (TRAF2), TRAF5 and TRAF6 to activate IRF-3 and NF-κB to induce robust type I IFN and inflammatory cytokine production (76). RIG-I signaling additionally requires recruitment of the adapter protein stimulator of interferon genes (STING) into the complex with MAVS (77-79). STING, a direct sensor of cyclic dinucleotides, serves as a critical adapter downstream of many DNA and RNA sensors, but it is not needed for MDA5 signaling (80).

Most viruses with dsRNA stages are detected by either RIG-I or MDA5 (81).
Interestingly, both RIG-I and MDA5 detect DENV dsRNA during replication, and either receptor is dispensable individually (72). Simultaneous deficiency in both receptors severely impairs DENV clearance and antiviral response (54,71). Given the questions surrounding how DENV ssRNA and dsRNA reach the endosome, RIG-I and MDA5 likely represent the first PRRs encountered during the DENV lifecycle that definitively sense initial infection and mount a protective antiviral response. Correspondingly, this is reflected by evolution of evasive and inhibitory mechanisms by DENV to interfere with IFN production. The DENV NS2B/3 protease cleaves the adapter protein STING to interfere with RIG-I-mediated signaling, suppressing type I IFN production (82-84). Further, DENV has evolved several mechanisms to interfere with signaling cascades downstream of the type I IFN receptor (IFNAR) (85-87). Thus, DENV antagonizes both IFN production and response to evade the protective antiviral response initiated by RIG-I and MDA5.

A second set of cytosolic PRRs contributes to the innate immune sensation of DENV. The nucleotide-binding domain, leucine-rich repeat containing (NLR) proteins are a large family of cytosolic proteins with a diverse set of functions (88,89). A subset of NLRs oligomerize upon sensation of their stimuli and form the backbone of a multi-protein complex termed the inflammasome. Though the stimuli and requirement for adaptor molecules vary by NLR backbone, the assembled inflammasome recruits in multiple copies of the inactive zymogen pro-caspase-1 (90,91). Pro-caspase-1 proteins autocatalytically cleave one another into active caspase-1, which cleaves pro-IL-1β and pro-IL-18 at aspartic acid residues into their active forms (92). Caspase-1 is the predominant mechanism for pro-IL-1β processing, so the activation state of caspase-1 is of interest in any disease showing elevated levels of IL-1β. Studies of immune profiles from DENV patients have found elevated IL-1β levels in severe patients (93,94). These
studies have been further corroborated in vitro, with DENV inducing IL-1β secretion by primary myeloid cells (35,95,96).

Correspondingly, DENV was found to activate the NLRP3 inflammasome in primary human inflammatory macrophages (35). However, this activation of NLRP3 by DENV appears to be indirect, as DENV must bind and signal through CLEC5A for inflammasome activation. Blockade of CLEC5A reduced caspase-1 activation and secretion of IL-1β, and Stat1−/− mice that were also deficient for Clec5a no longer had detectable levels of IL-1β in their sera after DENV infection (34,35). Additionally, blocking caspase-1 activity in inflammatory macrophages reduced pyroptosis, an inflammatory mechanism of cell death mediated by caspase-1. DENV infection also activates the NLRP3 inflammasome in platelets, leading to caspase-1-dependent release of IL-1β-containing microparticles that correlate with vascular permeability (97).

How these studies may apply to monocytes is less clear. Though DENV induces IL-1β secretion by primary monocytes within 4 hours of inoculation, DENV does not reportedly activate caspase-1 in primary monocytes until 4 days post-inoculation (95,98). Further studies are needed to understand the cell-specific differences in caspase-1 activation induced by DENV.

1.4 When neutralization fails: ADE of DENV infection

A consideration of how ADE may manipulate the innate immune response first requires an understanding behind the mechanisms of antibody-mediated entry of DENV. ADE can simplistically be considered in the context of failing to achieve neutralization. Every antibody that can neutralize DENV has the potential to enhance infection at low concentrations (99-101). Additionally, antibodies without neutralization capacity can bind DENV and still enhance infection. In vitro analyses of the structural basis of neutralization have indicated that the
induction of ADE depends on factors such as antibody concentration, antibody affinity for an epitope, the number of epitopes available on the virion, and how accessible the epitopes are (1,99).

Though many variables can prevent the achievement of neutralization, the consequences of such failures are similar. A DENV virion complexed with multiple immunoglobulin G (IgG) antibodies forms an immune complex (1). Fc gamma receptors (FcγRs), most abundantly expressed by innate immune cells, bind the Fc region of IgG, and cross-linking of multiple FcγRs by immune complexes triggers receptor-mediated endocytosis (102,103). DENV immune complexes are thus delivered to an endosome, the cellular compartment required for the DENV lifecycle. If the antibodies facilitating entry do not interfere with virion fusion, DENV will fuse and deposit its genome into the cytosol before neutralization can occur (99).

The susceptibility of any cell to ADE is dependent upon not only the expression of FcγRs, but also the susceptibility to infection in the absence of antibody. Immature dendritic cells, which express FcγRs, are highly susceptible to DENV infection in the absence of antibody due to the expression of DC-SIGN (39). In this study, increasing DC-SIGN expression obscured ADE, indicating that antibody-mediated entry of DENV competes with entry that occurs after DENV binds with DC-SIGN. However, antibody-mediated entry becomes a major factor in other FcγR-bearing cells of the myeloid lineage, namely mature dendritic cells, monocytes, and macrophages (1,5,39,104). These cells are not as susceptible to DENV infection as immature dendritic cells. Most notably, many studies show monocytes to be extremely resistant to DENV infection (104-106), presumably due to lack of suitable attachment factors, such as DC-SIGN, or putative surface receptors. Thus, antibody-mediated entry is the most efficient entry mechanism in monocytes, not a competitive entry mechanism. The establishment of infection in monocytes
by DENV immune complexes has long been considered a driving force behind severe dengue 
(5,107). As a result, further information presented here will be considered in the context of 
circulating blood monocytes.

Many questions linger regarding ADE research. ADE was initially considered to simply 
increase the pool of infected cells but is now reported to increase DENV replication compared to 
entry through other receptors (10). The proposed intrinsic ADE phenomenon is more 
controversial than the better understood mechanisms of extrinsic ADE. The key to further our 
understanding of intrinsic ADE may well lie in the signaling pathways induced by FcγRs.

1.5 Signaling properties of FcγRs relevant to DENV

Binding of DENV immune complexes to FcγRs is a pivotal point in ADE, hence this 
section will provide a review of this family of receptors. The human FcγR family is divided into 
3 classes: FcγRI (highest affinity for IgG), FcγRII, and FcγRIII (lowest affinity) (103). Though 
most members are activating, FcγRIIb provides inhibitory signals and blocks ADE (108). All 
FcγRs must be cross-linked by an immune complex to activate and endocytose or phagocytose 
immune complexes (102,109). However, this is not an immunologically inert process. In 
contrast, FcγR cross-linking induces receptor clustering and protein phosphorylation, activating 
inflammatory processes such as antibody-dependent cellular cytotoxicity, reactive oxygen 
species (ROS) production, and chemokine/cytokine production (110-112).

Both FcγRI and FcγRIIa have been implicated in mediating ADE during DENV infection 
(113-115). Interestingly, FcγRI is reported to be more efficient at mediating DENV 
neutralization while FcγRIIa is more efficient at promoting its enhancement (113,116). However, 
most of our knowledge of FcγR use during ADE comes from in vitro studies that transfect each
FcγR individually into non-FcγR-bearing cells. Myeloid cells constitutively express both receptors, thus complicating studies. Activating signals for both FcγRs are transmitted by immunoreceptor tyrosine-based activation motifs (ITAMs) that contain two tyrosine residues for phosphorylation (112). FcγRIIa has an ITAM in its cytoplasmic tail, while FcγRI associates with the FcR γ chain, which also contains an ITAM. Cross-linking of FcγRs induces rapid phosphorylation of the cytoplasmic ITAMs by Src family kinases (117). Interestingly, this phosphorylation is indispensable for FcγR-mediated phagocytosis, but FcγR-mediated endocytosis occurs in the absence of ITAM phosphorylation (102). However, ITAM phosphorylation is critical for the activation of signaling cascades that mediate the inflammatory responses of FcγR cross-linking. The exact kinases and isoforms activated quickly diverge based upon which FcγR is cross-linked (118). However, one common kinase is important for both FcγRI and FcγRIIa.

Spleen tyrosine kinase (Syk) is an important kinase that is activated by FcγR engagement (110,119). Syk exists in an auto-inhibitory state in the absence of FcγR cross-linking (120,121). Upon cross-linking of either FcγRI or FcγRIIa, Src family kinases phosphorylate the ITAM residues of the FcR γ chain or FcγRIIa, respectively (110,122). Syk has two Src homology 2 (SH2) domains that bind the two phosphotyrosine residues of the ITAM (121). This changes the conformation of Syk and releases it from its inhibited state. Syk is then fully activated by phosphorylation of its tyrosine residues, either by Src family kinases or through autophosphorylation. Additionally, Syk can phosphorylate the ITAM tyrosine residues of FcγRIIa or the FcR γ chain, inducing a positive feedback loop to amplify its activation.

Syk serves as a central mediator of signaling by classical immunoreceptors bearing ITAMs (121,123). However, the precise downstream signaling cascades vary by
immunoreceptor. Upon activation, Syk can directly bind PI3K (p85α subunit), SLP65, SLP76, phospholipase isoforms, and VAV family members (121). A variety of important signaling intermediates may then be activated, including MAPKs, NF-κB, PKC, DAG, and TEC family kinases, among others (121,124). This induces activities as varied as actin rearrangements, ROS generation, cytokine synthesis, cell survival, and inflammasome activation (121,125,126).

Cross-linking of both FcγRI and FcγRIIa induce phospholipase activation and elevations in cytoplasmic calcium levels through Syk activity (127,128). Interestingly, the pathways diverge immediately. FcγRI activation requires phospholipase D1 to activate protein kinase C and calcium release, while FcγRIIa requires phospholipase Cγ1 (124). Though similar biological effects are induced, the exact pathways utilized differ.

As ADE is associated with increased cytokine production, signaling downstream of FcγRs likely induces inflammatory cytokines. As both FcγRI and FcγRIIa signal through Syk, the contribution of Syk to elevated cytokine production during ADE needs to be established. However, recent studies suggest that the cross-linking of FcγRs during ADE does more than allow cell entry and induce cytokine production (129,130). Could it be that DENV immune complexes suppress the innate antiviral response through FcγR signaling?

1.6 Suppression of the innate antiviral response by ADE

Though ADE has been described for decades, in recent years a secondary role for this entry pathway has been described. Intrinsic ADE is a term used to describe alterations in the cellular environment induced by ADE that cause infected cells to produce more virus (10). The first support for this in DENV infection came when ADE was reported to suppress the production of IFNγ and IL-12, block signal transducer and activator of transcription 1 (STAT1)
activation, and promote the production of IL-10 in THP-1 cells (129). The authors concluded this led to a suppression of the intracellular antiviral response and enhanced DENV replication. This was followed by a report that ADE promotes the downregulation of the antiviral RLRs RIG-I and MDA5 in THP-1 cells, and this was associated with elevated expression of ATG5-ATG12 (130). The ATG5-ATG12 complex was previously shown to inhibit RLR activation (131). Additionally, ADE was found to suppress TLR signaling by downregulating expression of TLRs and adapters and promoting negative regulators of NF-κB (132). For both studies, DENV replication was promoted by a downregulation of IFNβ production. The authors verified many of these phenotypes by screening PBMCs from severe dengue patients.

These findings are additionally supported by clinical data. IL-10 production is elevated in severe dengue disease, while IL-12 is often decreased (133-135). Additionally, a shift away from a protective Th1 immune response (promoted by IL-12) toward a Th2 response (promoted by IL-10 in conjunction with other cytokines) is considered to contribute to severe dengue disease (10,136). Though many cytokines, including inflammatory cytokines, are still induced by ADE, the differential regulation of several key cytokines appears to impair viral clearance.

However, the role of intrinsic ADE is not totally understood. In one study, ADE increased DENV production without enhancing IL-10 secretion or inhibiting type I IFN production (106). ADE has also modulated cytokine secretion in another study without affecting IL-10 production (39). These discrepancies may be explained by another study, in which it was found that modulation of type I IFN and IL-10 by ADE varies by cell type and donor (137).

As mentioned earlier, DENV non-structural proteins suppress the immune response by inhibiting type I IFN production and signaling (13,82,85). ADE has been suggested to increase uptake of both antibody-bound and free DENV (106). If true, this would lead to increased
translation of non-structural proteins, potentially amplifying the active suppression induced by DENV non-structural proteins. However, recent advances in the understanding of FcγR signaling have identified pathways that may contribute to intrinsic ADE.

1.7 How FcγR signaling may suppress antiviral immunity during ADE

Immunoreceptor signaling has traditionally been broken into ITAMs transmitting activating signals while immunoreceptor tyrosine-based inhibition motifs (ITIMs) transmit inhibitory signals (138). This suggests that ADE, which occurs primarily through ITAM-associated FcγRI and FcγRIIa, would be expected to only enhance immune responses. However, recent evidence clearly shows that ITAMs can modulate the immune response by providing inhibitory signals to heterologous pathways (138,139). These suppressive properties of ITAMs are now described as inhibitory ITAMs (ITAMi).

Binding of type I IFN to its receptor induces a protective antiviral response against DENV by signaling through the Janus kinase (JAK)/STAT pathway and inducing the expression of interferon stimulated genes (ISGs) (140,141). Thus, targeting STAT1 is one of the evasive mechanisms developed by DENV to suppress the antiviral response induced by type I IFN (13). Interestingly, cross-linking of FcγRI by immune complexes on primary human monocytes suppresses STAT1 phosphorylation, impairing the response to interferons (142). This inhibition of IFN signaling was due to activation of Src homology 2 domain-containing phosphatase-1 (SHP-1), an important protein that dephosphorylates proteins and negatively regulates immune responses (143).

The activation of SHP-1 after FcγR-cross-linking has long been recognized (143). However, this has typically been viewed as a means of negative feedback on FcγR activation
Extensive cross-linking of FcγRIIa leads to aggregation and clustering of the receptors, followed by phosphorylation of the two tyrosine residues in the ITAM of FcγRIIa (145). Syk is recruited to the ITAM and forms a stable interaction, subsequently mediating proinflammatory signals (144). In this same study, the authors found that SHP-1 was also recruited and activated, suggesting that strong aggregation of FcγRIIa induces inhibitory signals to downregulate its own activity.

However, a very recent report elegantly showed that weaker stimulation of FcγRIIa causes it to transmit ITAMi signals (146). In this study, cross-linking FcγRIIa into dimers (by using F(ab’)2 fragments targeting FcγRIIa specifically) induced only transient recruitment of Syk. This short interaction prevented Syk from achieving full activation but did allow Syk-mediated recruitment and activation of SHP-1. SHP-1 activated through this pathway was able to suppress lipopolysaccharide (LPS)-induced NF-κB activation and cytokine production and could also inhibit reactive oxygen species production. Importantly, in the same study, forming extensive FcγRIIa aggregates induced stable interactions with Syk. This ablated the inhibitory phenotype and recruitment of SHP-1, resulting in a proinflammatory phenotype. Even FcγRIII is now recognized to have ITAMi signaling capabilities (147).

As ITAMi is a developing topic within immunoreceptor research, how this applies to intrinsic ADE is unclear. However, SHP-1 activation can clearly interfere with interferon signaling by dephosphorylating STAT1 (142,148-150). Interfering with SHP-1 activity in models of intrinsic ADE could provide crucial insight into this phenomenon.

The published support for intrinsic ADE extends beyond dephosphorylation mediated by SHP-1. General FcγR cross-linking induced by incubating primary human monocytes with plate-bound IgG increased SOCS3 and IL10 expression, while also interfering with TLR signaling
These are all phenotypes suggested to contribute to intrinsic ADE (129,130,132). Further, dendritic cells from mice lacking the FcR γ chain, utilized by FcγRI and other immunoreceptors, have enhanced cytokine production in response to TLR activation (152). The developing field of ITAMi research continues to provide evidence that cross-linking of FcγRs during ADE may induce pathways that suppress heterologous receptors.

1.8 Concluding remarks

Innate immune recognition of DENV is a field with many lingering questions. PRRs at the cellular surface appear to be detrimental to the host by either serving as attachment factors (e.g. DC-SIGN and MR) or by transmitting inflammatory signals that induce pathologic inflammation (CLEC5A) (32-35). Protective roles have been attributed to the endosomal TLRs 3 and 7 (13). However, the exact step in the lifecycle at which they provide protection is unclear. The absence of DENV ssRNA, and especially dsRNA intermediates, in the endosome during the normal lifecycle suggest that these TLRs may provide protection to bystander cells that endocytose RNA from dying cells. This would reduce virus production by reducing the pool of susceptible cells.

In contrast, a more accepted protective role has been established for the widely-expressed cytosolic RLRs. Both RIG-I and MDA5 bind DENV dsRNA and provide protection against DENV replication by inducing robust type I IFN production (54,71,72). Secreted type I IFN can act back on cells in an autocrine matter or provide bystander protection to neighboring cells through IFNAR, inducing expression of protective ISGs through the JAK/STAT pathway (153).

The impact of ADE on IFN-mediated protection is unclear. Studies have reported that ADE suppresses antiviral responses, and enhances DENV replication, in both the presence and
the absence of reduced type I IFN production (106,130). This suggests that intrinsic ADE may mediate important effects independent of type I IFN production, which is consistent with STAT1 dephosphorylation. Indeed, many of the phenotypes described in intrinsic ADE are consistent with the seemingly paradoxical ITAMi signaling pathways. Dephosphorylation of STAT1, anti-inflammatory cytokine induction, and inhibition of TLR signaling pathways could collectively inhibit the protective functions of TLR3, TLR7, RIG-I, and MDA5 in DENV infection.

However, an important consideration is that experimental conditions may substantially impact any intrinsic ADE phenotype. Our understanding of FcγR activation is far from complete. The recent finding that the extent of FcγRIIa aggregation is a critical factor determining ITAM versus ITAMi signals may explain some discrepancies in the intrinsic ADE field (146). Conditions impacting the extent of receptor clustering may drastically impact whether ADE promotes inhibitory signals, with potential STAT1 interference and anti-inflammatory cytokine production, or induces proinflammatory activities without suppressing the antiviral response.

Interestingly, whether ADE is inducing proinflammatory responses or suppressing the antiviral state, Syk is likely to be a key mediator. Whether ITAM or ITAMi signals are induced by FcyRs, Syk is the key kinase recruited to the phosphorylated ITAMs (110). Thus, the role of Syk in both proinflammatory responses (“cytokine storm”) and immune suppression (intrinsic ADE) should be investigated in antibody-enhanced DENV infection.

The controversial phenomenon of intrinsic ADE likely stems from our incomplete understanding of FcγR cross-linking and activation under different conditions. However, evidence does exist that FcγRs can suppress antiviral activity in the right conditions. Attention should go to determining if ITAMi signals create the described intrinsic ADE phenotype and whether such a scenario is biologically plausible or promoted by laboratory conditions.
CHAPTER 2: SPLEEN TYROSINE KINASE MEDIATES IL-1β INDUCTION BY PRIMARY HUMAN MONOCYTES DURING ANTIBODY-ENHANCED DENGUE VIRUS INFECTION

2.1 INTRODUCTION

Dengue virus (DENV) is transmitted by the bite of infected *Aedes* spp. mosquitoes and is the most burdensome arthropod-borne virus in the world. The World Health Organization estimates that the 4 distinct serotypes of DENV (DENV-1–DENV-4) combine to cause 50–100 million infections per year worldwide (154). However, a recent cartographic-based analysis of DENV prevalence estimates that the true number of global infections is approximately 390 million per year (2). Approximately 96 million of these cases present with disease symptoms annually. Most symptomatic cases present with mild to severe flu-like symptoms, but a small percentage of patients progress to life-threatening severe dengue (154).

Severe dengue almost always occurs in conjunction with a secondary infection with a heterologous serotype, leading to the hospitalization of approximately 500,000 people yearly (3,154). The reason for this phenomenon is due to cross-reactive immunity from the original infection. Initially cross-protective against other serotypes in the months following a primary infection, the cross-reactive DENV immune response wanes in strength over time (1). Eventually, this cross-reactive response becomes too weak to neutralize heterologous serotypes and paradoxically can enhance both viral replication and the production of dangerous inflammatory mediators (3,155).

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It is believed that cross-reactive antibodies contribute to severe dengue during infection with a heterologous DENV serotype by a process termed antibody-dependent enhancement (ADE) of infection (1,107). The molecular mechanisms underlying ADE are incompletely understood. Cross-reactive antibodies may bind heterologous DENV serotypes, forming immune complexes when a DENV virion is bound by multiple antibodies (1). IgG immune complexes trigger endocytosis by cross-linking at least two activating Fc gamma receptors (FcγRs) on FcγR-bearing cells (117). Antibody-mediated neutralization of flaviviruses has strict antibody occupancy thresholds that must be reached to prevent endocytosed DENV from fusing with the endosome (99). When immune complexes are formed that do not meet the neutralization threshold, endocytosis of the complexes can promote increased cellular infection with DENV (4,5,115). Circulating blood monocytes are thought to be the primary target of ADE in vivo and are largely resistant to DENV infection in the absence of enhancing antibodies (104-106,156).

Immune dysfunction during severe dengue can progress to life-threatening hypovolemic shock due to hemorrhage and leakage of vascular fluid (157). It is believed that a “cytokine storm,” a massive and aberrant production of cytokines, contributes to this deadly pathology (9). Though much progress has been made in recent years elucidating the mechanisms of cytokine production during severe dengue, our understanding is incomplete, with many studies providing conflicting results (158).

One inflammatory cytokine seen elevated in the serum cytokine or gene expression profiles of many severe dengue patients is IL-1β (93,94,159). Notably, IL-1β increases vascular permeability, especially in conjunction with TNFα and IFNγ, two other cytokines elevated in many severe dengue profiles (93,160-162). Exaggerated vascular permeability, caused by biologic mediators and not structural damage, is a key feature of severe dengue (157).
Understanding the mechanism of IL-1β production during DENV infection in human monocytes is important for gaining insight into disease pathogenesis. IL-1β production by DENV-infected platelets was recently shown to correlate with vascular permeability in patients and promote permeability in endothelial cell layers in vitro (97). Thus, production of IL-1β by DENV-infected monocytes could be expected to enhance vascular leakage as well.

IL-1β is an extremely potent cytokine that is tightly regulated and can be induced by DENV in macrophages and monocytes (35,95,163). Induction of IL1B mRNA expression leads to the translation of inactive pro-IL-1β, which must undergo post-translational cleavage to mature IL-1β by activated caspase-1 (164,165). Caspase-1 activation is regulated by a second, independent stimulus, a multi-protein complex termed the inflammasome (90,166). Several nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs), as well as other innate immune receptors such as AIM2, can serve as basic building blocks of the inflammasome, and each shows differing specificity to pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) (91,167,168). With both transcriptional and post-translational regulatory mechanisms, IL-1β thus serves as an interesting cytokine to study differential cytokine modulation between unenhanced DENV infection and ADE.

Since monocytes circulate in the blood, they represent a first line of defense within the battleground of severe dengue. Human monocytes have long been known to secrete IL-1β in response to DENV inoculation, with elevated IL-1β secretion induced by ADE (95). No study has compared how ADE modulates the transcriptional versus post-translational regulation of IL-1β in primary monocytes, though. DENV inoculation in the absence of ADE has been studied and is found to activate caspase-1 in primary human monocytes 4 days after infection (98). However, monocytes secrete IL-1β within 4 hours of inoculation with DENV (95), suggesting
that DENV may induce IL-1β secretion before it causes this delayed caspase-1 activation. Consistent with this, monocytes are known to partially process synthesized pro-IL-1β in the absence of additional caspase-1 agonists (169-173), which may account for rapid IL-1β secretion after DENV inoculation. A study of the pathways activated by DENV immune complexes in human monocytes is needed to elucidate the regulation of IL-1β secretion during ADE.

This report studies IL-1β synthesis and caspase-1 activation during ADE by using clinically- and physiologically-relevant human anti-DENV mAbs isolated from previously infected patients. This is particularly informative because DENV does not cause relevant disease in immunocompetent mice unless extremely high inoculums are used (174-182). Using these valuable antibodies in ADE studies with primary human monocytes allows identification of important signaling pathways that represent potential therapeutic targets for the treatment of severe dengue disease. Here, we find that ADE rapidly enhances expression of IL1B and pro-IL-1β, leading to significant IL-1β secretion by 4 hours post-inoculation (hpi). Enhanced IL-1β secretion is independent of viral replication but instead requires activation of spleen tyrosine kinase (Syk) by DENV immune complexes. Elevated pro-IL-1β synthesis was not accompanied by a detectable increase in caspase-1 activation, suggesting that ADE enhances IL-1β secretion primarily by increasing IL1B transcription and pro-IL-1β synthesis. Importantly, ADE also induced Syk-dependent elevation of TNF and IL6 expression, suggesting that activation of Syk by DENV immune complexes may have broad implications for the cytokine storm. Finally, activation of extracellular signal-regulated kinase (ERK) 1/2 by Syk is required for an IL-1β response to DENV immune complexes, thus identifying two potential points of intervention for interfering with ADE-induced cytokine production.
2.2 RESULTS

**ADE of dengue virus infection increases viral replication in primary human monocytes**

Purified CD14+ monocytes were inoculated with mosquito-cell-derived supernatant containing a multiplicity of infection (MOI) of 50 DENV-2 16681 Vero-cell focus-forming units (ffu) per monocyte. One hour prior to inoculation, DENV was incubated with 2 μg/ml anti-DENV prM human mAb 5G22 to form DENV immune complexes or with control medium for non-ADE conditions. Mock-infected cells were inoculated with spent culture supernatant from uninfected mosquito-cell cultures. At 1 hour post-inoculation (hpi), cells were washed and resuspended in fresh medium until 24 hpi. Cells were stained with the anti-E-protein (pan-flavivirus) mouse mAb 4G2 conjugated to AlexaFluor-647 to measure intracellular expression of DENV E protein by flow cytometry. Detecting DENV antigen intracellularly by flow cytometry is a measure of viral replication (156). Inoculation of monocytes with DENV alone caused little shift in DENV E-protein expression compared to mock conditions (Fig. 2.1, A and B). However, inoculation with DENV that had been complexed with mAb 5G22 significantly increased the percentage of monocytes expressing DENV E protein. This agrees with previous literature that monocytes are largely resistant to DENV infection in the absence of anti-DENV enhancing antibodies (104,106,183).

**ADE increases secretion of IL-1β by primary human monocytes independent of viral replication**

Culture supernatants collected at 24 hpi from the above experiment were then assessed for the presence of human IL-1β by ELISA. Consistent with literature reports (95), inoculation of monocytes with DENV alone significantly enhanced secretion of IL-1β, and forming DENV immune complexes with mAb 5G22 further increased IL-1β secretion (Fig. 2.1C). This confirms
that DENV alone induces IL-1β secretion by primary human monocytes and that ADE further elevates IL-1β secretion. Interestingly, the degree of IL-1β enhancement by mAb 5G22 is not proportional to the enhancement of cells expressing DENV E protein. This suggests that DENV alone induces IL-1β production in primary monocytes by signaling through a pathway that does not promote increased cellular infection and DENV replication.

To determine the requirement for viral replication, we subjected DENV to shortwave UV exposure for 2 minutes, rendering the virus replication incompetent. UV inactivation decreased the infectious titer of DENV, as measured by immunoassay on Vero cells, by 10^5 fold (Fig. 2.1D). Consequently, UV inactivation prevented the expression of DENV E protein in primary monocytes at 24 hpi (Fig. 2.1E). However, UV inactivation only modestly reduced ADE-induced IL-1β secretion at 24 hpi (Fig. 2.1F). These data indicate that ADE-induced IL-1β secretion by primary monocytes can occur independently of viral replication.

ADE of DENV supernatant induces early elevated IL1B and pro-IL-1β expression

Detection of elevated IL-1β in culture supernatants could be due to elevated synthesis of pro-IL-1β, elevated caspase-1 activation, or both. To determine which steps were modulated by ADE, inoculated and control monocytes were assessed for IL1B gene expression differences at 2 hpi by real-time PCR. Inoculation of monocytes with DENV alone induced significant elevation of IL1B expression, while DENV complexed with mAb 5G22 further increased IL1B expression (Fig. 2.2A). These results were measured against the IL1B expression induced by a known agonist LPS. The IL1B expression induced by ADE at 2 hpi was even greater than the levels induced by stimulation with 100 ng/ml of LPS for 2.5 hours.

As elevated IL1B expression should increase pro-IL-1β synthesis, we assessed pro-IL-1β
expression by monocytes after DENV inoculation. Monocytes were lysed at 4 hpi and subjected to gel electrophoresis and immunoblot. As with IL1B expression, inoculation with DENV alone induced elevated pro-IL-1β expression, while DENV complexed with mAb 5G22 further increased this expression (Fig. 2.2B). Supernatants collected at 4 hpi for the corresponding experiment were assessed for IL-1β by ELISA (Fig. 2.2C). DENV complexed with mAb 5G22 induced significant elevation of IL-1β secretion by this early time point, while that induced by DENV alone fell just short of significance. As monocytes can secrete both pro-IL-1β and mature IL-1β (184), assaying supernatants by immunoblot is a way of specifically assessing the 17-kDa mature IL-1β protein. Thus, we again inoculated monocytes and collected supernatants at 4 hpi but assayed specifically for the presence of mature IL-1β by immunoblot (Fig. 2.2D). There was faint detection of mature IL-1β in the supernatant of cells inoculated with DENV alone. However, DENV complexed with mAb 5G22 induced robust mature IL-1β secretion. These data confirm that ADE-induced expression of IL1B and pro-IL-1β lead to elevated mature IL-1β secretion by 4 hpi.

**ADE of DENV does not cause elevated caspase-1 activation in primary human monocytes**

We next determined if ADE could enhance intracellular processing of pro-IL-1β to mature IL-1β by activating the inflammasome. The caspase-1 p10 subunit is an indicator of inflammasome activation and can be detected by immunoblot. However, we could not detect caspase-1 p10 by immunoblot under any mock or viral condition in 2 hpi lysates (Fig. 2.3A), despite the use of different sources of antibodies and protocols. Caspase-1 p10 could only be detected in the positive control, consisting of lysates of cells that had been primed with 200 ng/ml of LPS for 4 hours and then pulsed with 5 mM of the NLRP3 agonist ATP for 45 minutes.
Importantly, we could not detect caspase-1 p10 in response to viral inoculation even at very long exposures and in multiple experiments.

To measure caspase-1 activity in a different manner, we employed a caspase-1 activity assay in development by Promega (Fig. 2.3B). The assay uses a lytic reagent that contains a proteasome inhibitor and the caspase-1 substrate Z-WEHD conjugated to aminoluciferin. A luminescent signal develops when caspase-1 cleaves the substrate. At 2 hpi and 3 hpi, caspase-1 activity in mock- and ADE-inoculated cells was on par with cells treated with LPS only. In contrast, LPS and ATP induced a large increase in caspase-1 activity. These data confirm that ADE does not induce elevated caspase-1 activity in primary human monocytes in the early hours following inoculation. However this does not exclude the involvement of caspase-1.

**ADE-induced IL-1β secretion requires caspase-1**

Our results to this point are consistent with reports on IL-1β processing in human monocytes after induction of pro-IL-1β synthesis (169-173). This endogenous processing is inefficient, with less than 30% of synthesized pro-IL-1β cleaved to mature IL-1β (171), and processing is greatly enhanced by inflammasome agonists (171,173). More recent studies support these findings, indicating that primary monocytes and monocyte-like THP-1 cells have low levels of caspase-1 activity in the absence of inflammasome agonists (185,186).

This suggests the possibility that low levels of caspase-1 activity may mediate the processing of IL-1β in the current system. To test for this, we employed two irreversible caspase-1 inhibitors, Z-YVAD-FMK and Z-WEHD-FMK. Both caspase-1 inhibitors ablated ADE-induced elevation of IL-1β secretion (Fig. 2.4A). We also assessed caspase-1 dependency by immunoblot, measuring mature IL-1β present in supernatants collected at 5 hpi (Fig. 2.4B).
detected mature IL-1β in the supernatants collected from ADE-inoculated cells that were treated with DMSO vehicle. However, no mature IL-1β was detected in supernatants from ADE-inoculated cells treated with Z-YVAD-FMK. These data confirm that caspase-1 is required to process ADE-induced pro-IL-1β.

**Processing of DENV-induced pro-IL-1β can be enhanced with ATP**

To assess if processing of DENV-induced pro-IL-1β can be enhanced, we pulsed monocytes with 2 mM ATP to activate the NLRP3 inflammasome after DENV inoculation with and without mAb 5G22 (Fig. 2.4C). Exogenous ATP addition significantly increased the secretion of IL-1β induced by DENV and DENV immune complexes but had less impact on IL-1β secretion induced by mock conditions. These data confirm that caspase-1 activation is sub-optimal in monocytes inoculated with DENV alone or DENV immune complexes.

**ADE-induced Syk activation is dispensable for DENV replication**

To this point, our data indicate that ADE enhances transcription of *IL1B* mRNA and the synthesis of pro-IL-1β protein but does not significantly enhance caspase-1 activation. To determine the pathway utilized by ADE to elevate *IL1B* expression, we considered early signaling pathways likely to be activated by DENV immune complexes. Syk is a critical protein for initiating signaling cascades downstream of many classical immunoreceptors, including Fc receptors (121,122). Cross-linking of both FcγRI and FcγRIIa strongly activates Syk, which induces expression of numerous cytokines in monocytes (119,187). Thus, we first assessed if ADE of DENV infection induces elevated Syk activation in primary monocytes. At 4 hpi, inoculation with DENV complexed with mAb 5G22 induced strong Syk phosphorylation at
tyrosine 323 (Fig. 2.5A).

To assess the importance of Syk activation in ADE-induced infection and cytokine production, we interfered with Syk activation by employing the potent, cell-permeable Syk inhibitor BAY 61-3606. Importantly, BAY 61-3606 is very selective for Syk at the concentrations used (188). We first assessed the effects of Syk inhibition on DENV replication by measuring intracellular DENV E-protein expression in ADE-inoculated monocytes at 24 hpi (Fig. 2.5B). Syk inhibition with BAY 61-3606 did not reduce the detection of intracellular DENV E protein, indicating that Syk is dispensable for viral replication. This is in agreement with the report that Syk activation is not required for Fc-receptor-mediated endocytosis (102).

**Syk mediates IL-1β induction by DENV immune complexes**

We next sought to determine the impact of Syk inhibition on ADE-induced IL-1β synthesis and secretion. We first assessed the impact of Syk inhibition on IL1B expression. Monocytes were inoculated after pretreatment with DMSO vehicle or BAY 61-3606, and RNA was collected at 2 hpi for assessment by real-time PCR (Fig. 2.5C). Syk inhibition did not significantly alter IL1B expression induced under mock conditions. In contrast, inhibiting Syk prevented DENV immune complexes from inducing increased IL1B expression over DENV alone. These data suggest that Syk mediates ADE-induced IL1B expression and that impairment of Syk activation reduces IL1B expression to levels induced by DENV alone.

Interestingly, though we previously detected phosphorylated Syk only after ADE inoculation, Syk inhibition appeared to modestly suppress IL1B expression induced by DENV alone. To measure lower levels of Syk phosphorylation, we again pre-treated monocytes with BAY 61-3606 or DMSO vehicle and inoculated the cells. At 8 hpi, cells were lysed, and we
assessed Syk phosphorylation by immunoblot at both short and long exposures (Fig. 2.5D). A low level of Syk phosphorylation became evident in non-ADE samples at long exposures. This suggests that Syk has activity independent of FcγRs in the current study, which is not surprising due to the association of Syk with a multitude of signaling pathways (121). Importantly, BAY 61-3606 substantially reduced ADE-induced Syk phosphorylation.

We additionally assessed the impact of Syk inhibition on IL-1β maturation by assessing the supernatants from the above experiment by immunoblot (Fig. 2.5D). Interestingly, Syk inhibition almost completely ablated mature IL-1β secretion induced by both DENV and DENV complexed with mAb 5G22, despite only partial inhibition of IL1B expression by BAY 61-3606. The Syk pathway has been found to be important for DENV-induced inflammasome activation through C-type lectin receptors (CLRs) but has not been described for FcγRs (35,126,189,190). A recent study reported that Syk phosphorylates the inflammasome adapter ASC to promote caspase-1 activation (191). Thus, Syk activity may be critical for maintaining the sub-optimal caspase-1 activation in the current study. These data suggest that Syk exerts two levels of control on IL-1β during DENV infection in primary human monocytes, both at the level of IL1B transcription and at the level of IL-1β maturation.

As Syk is important for antibody-mediated signaling, we sought to verify that mAb 5G22 elevates DENV-induced IL-1β due to immune complex formation with the virus. This is especially important as FcγRI can bind monomeric IgG that is not bound to antigen (125). Thus, we employed an isotype-matched control antibody for side-by-side comparison with mAb 5G22. Serotype-specific for DENV-1, mAb 1F4 does not bind DENV-2 (192). Thus, as expected, mAb 1F4 did not enhance DENV-2 infection in monocytes (Fig. 2.6A). When assessing IL-1β secretion by ELISA, inoculating monocytes with DENV in the presence of mAb 1F4 only
modestly increased IL-1β secretion over the mock condition, just as was seen earlier with DENV alone (Fig. 2.6B). This strongly suggests that anti-DENV mAb only enhances IL-1β secretion when mAb and DENV form immune complexes.

**Syk inhibition ablates ADE-induced ERK1/2 activation**

We next sought to identify additional intracellular signaling events mediated by DENV immune complexes. FcγR cross-linking by immune complexes activates ERK1/2 through activated Syk, leading to induction of cytokine expression (193-196). Notably, cross-linking of FcγRs in THP-1 cells induces IL-1β secretion by activating NF-κB (197). In that study, FcγR-mediated ERK1/2 activation was necessary for activation of NF-κB by immune complexes.

Thus, we wanted to assess the relationship between Syk, ERK1/2, and NF-κB during a time course of DENV inoculation with and without immune complexes. Monocytes were inoculated after pretreatment with BAY 61-3606 or DMSO vehicle, and cells were collected and lysed for immunoblot analysis at 0.25, 1, or 2 hpi (Fig. 2.7A). As expected, DENV complexed with mAb 5G22 induced Syk phosphorylation, which was detected by 1 hpi. DENV inoculation in the presence of mAb 1F4 did not induce detectable Syk phosphorylation, confirming the need for immune complexes for strong Syk activation. BAY 61-3606 inhibited the Syk phosphorylation induced by DENV immune complexes. Additionally, only DENV immune complexes induced strong phosphorylation of ERK1/2, which was also ablated by Syk inhibition. This suggests that Syk mediates activation of ERK1/2 after inoculation with DENV immune complexes. Both DENV with mAb 1F4 and DENV complexed with mAb 5G22 enhanced activation of the NF-κB subunit p65 compared to mock conditions. Interestingly, in vehicle-treated cells, DENV complexed mAb 5G22 modestly increased p65 activation compared to
DENV with mAb 1F4. Syk inhibition diminished this difference but did not reduce p65 activation to mock levels. In total, these data suggest that DENV immune complexes signal through Syk and ERK1/2 as has been described for other immune complexes.

**Syk inhibition impairs ADE-induced elevation of other inflammatory cytokines**

As ADE is associated with increased cytokine production during severe dengue (9), it stood to reason that this Syk-dependent pathway could mediate elevated expression of other ADE-induced inflammatory cytokines. Thus, we assessed RNA isolated from monocytes at 2 hpi for TNF and IL6 expression by real-time PCR (Fig. 2.7, B and C). Inoculation with DENV complexed with mAb 5G22 significantly increased expression of both TNF and IL6 compared to DENV alone. However, Syk inhibition diminished the ADE-induced elevation of both inflammatory cytokines. These data suggest that Syk mediates ADE-induced expression of multiple inflammatory cytokines.

**Syk-mediated ERK1/2 activation is critical for ADE-induced IL-1β secretion**

As ERK has been shown to mediate IL-1β secretion in THP-1 cells after FcγR cross-linking (197), we sought to assess the involvement of ERK1/2 activation in ADE-induced IL-1β secretion. Thus, we employed the use of the potent ERK1/2 inhibitor, PD98059. PD98059 blocks MEK1 and MEK2, the upstream kinases that specifically activate ERK1/2, and is highly selective at the concentrations used here (198,199). Monocytes were inoculated with DENV after pretreatment with PD98059 or DMSO vehicle. At 4 hpi, cells were lysed and assayed by immunoblot (Fig. 2.8A). ADE-induced Syk activation remained intact in the presence of PD98059. However, PD98059 completely ablated ERK activation, confirming that ERK
activation is downstream of Syk. We next assayed supernatants collected at 4 hpi by immunoblot to determine the effects of ERK inhibition on mature IL-1β secretion (Fig. 2.8B). We could no longer detect mature IL-1β in the supernatant from ADE-inoculated cells that were pretreated with PD98059. These data confirm that Syk-mediated activation of ERK is critical for ADE-induced secretion of IL-1β by primary human monocytes.

2.3 DISCUSSION

In this study, we confirm that ADE of DENV infection elevates secretion of IL-1β by primary human monocytes and provide insight into the modulation of cytokine induction by ADE. DENV immune complexes activate a signaling axis containing Syk and ERK1/2 that is critical for cytokine induction but dispensable for DENV replication. ADE robustly induces pro-IL-1β synthesis but not caspase-1 activation, suggesting ADE primarily modulates IL-1β secretion by increasing IL1B expression and pro-IL-1β synthesis. Correspondingly, we find that ADE-induced expression of inflammatory cytokine genes TNF and IL6 is sensitive to Syk inhibition as well, suggesting that Syk activation may have broader implications for cytokine production during severe dengue.

Notably, the phenotype we describe here in primary monocytes differs from what is seen in a more-differentiated myeloid cell. In inflammatory human macrophages, DENV triggers release of mature IL-1β through induction of pro-IL-1β synthesis and activation of the NLRP3 inflammasome (35). NLRP3 activation is induced by binding of DENV with the CLR CLEC5A on the macrophage cell surface, requiring signaling through DAP12 and Syk. Lack of NLRP3 inflammasome activation in the current system suggests either divergent roles or differential expression of CLEC5A in monocytes versus inflammatory macrophages. Correspondingly,
macrophage differentiation is associated with increased expression of CLEC5A (200).

Similar to DENV binding CLEC5A in macrophages, Syk activation downstream of CLRs licenses inflammasome activation in response to fungal pathogens and helminthes (126,201,202). Additionally, malarial hemozoin is engulfed by phagocytes, inducing inflammasome activation through Syk (189). Thus, Syk-mediated inflammasome activation may be linked intimately to certain pathways, or even certain cell types, and may not be induced by FcγR cross-linking in primary human monocytes. Syk activation cannot be viewed as universally equivalent in its downstream effects. Even within members of the FcγR family, signaling pathways quickly diverge downstream of Syk (124).

Regardless, the absence of high levels of caspase-1 activation by ADE in the current study did not completely block mature IL-1β secretion. Instead, a low level of IL-1β processing after induction of pro-IL-1β synthesis did occur, and this process was caspase-1-dependent, as has been observed for monocytes (170-173,185,186,200,203). This inefficient processing is supported by the experiment showing that processing efficiency of DENV-induced IL-1β in primary monocytes was greatly enhanced by the addition of ATP, which provided the necessary second signal for inflammasome activation. We further showed that IL-1β maturation upon DENV inoculation and ADE is dependent upon Syk, as Syk inhibition ablated secretion of mature IL-1β. Thus, Syk can affect IL-1β at the level of mRNA as well as protein maturation.

Though the unique regulation of IL-1β makes it an interesting model to study, the implications of the current study may extend far beyond any individual cytokine. Our study provides the first evidence that Syk activation is critical for ADE-induced expression of multiple inflammatory cytokines in undifferentiated primary human monocytes. This identifies Syk as a potential therapeutic target to interfere with the pathogenic cytokine storm of severe dengue.
Interestingly, a recent report indicates that ADE itself inhibits early Syk activation in THP-1 cells and primary monocytes by co-ligating surface receptor LILRB1 (204). This suggests that exogenous addition of a Syk inhibitor would provide no additional effect. However, ADE-induced Syk inhibition in the Chan, et al., study was strongest at 10 and 30 minutes post-inoculation and appeared to decrease afterward. Consistent with this, we detected much higher levels of phosphorylated Syk between 2 and 8 hours after inoculation than at earlier time points. Additionally, the Chan, et al., study measured total tyrosine phosphorylation after immunoprecipitation of Syk, whereas the current study measured Syk phosphorylation specifically at Y323. As Syk contains many phosphorylation sites, this confounds direct comparisons (205).

Our findings indicate that ADE induces inflammation by signaling through a Syk–ERK pathway described in other studies investigating the cross-linking of Fc receptors (193-197). A DENV immune complex must have important viral binding epitopes exposed to be infectious, thus leading to ADE (17,99). This suggests that DENV complexed to enhancing antibodies may still ligate other signaling receptors. By activating a pathway common to immune complexes, ADE may provide an additional, as opposed to an alternate, pathway of inflammation that augments signaling induced by other receptors. This is reflected in the current study, as inhibiting ADE-induced Syk activation reduced inflammatory cytokine expression to levels induced by DENV alone. Interfering with this additional signaling axis activated by DENV immune complexes may be of therapeutic use in dengue patients. Since inhibitors of Syk and ERK have been considered as therapeutics for cancer and autoimmunity, these compounds could be repurposed in an attempt to interfere with the cytokine storm in severe dengue. An additional benefit is that such treatments may provide relatively rapid anti-inflammatory effects in a patient.
after illness has developed, unlike the pre-exposure immunity that must be induced by a vaccine.

2.4 MATERIALS AND METHODS

Ethics Statement

Mobilized peripheral blood mononuclear cells (PBMCs) were isolated from the blood of leukapheresed patients enrolled in a study approved by UNC IRB (Study #05-2860) after providing written informed consent. Samples were anonymized and provided as de-identified samples prior to use in the described studies. The UNC Office of Human Research Ethics determined that the use of the de-identified samples does not constitute human subjects research as defined under federal regulations [45 CFR 46.102 (d or f) and 21 CFR 56.102(c)(e)(I)] and does not require further IRB approval.

Cell isolation and cell culture

Mobilized PBMCs were isolated from leukapheresed blood using a 1.073 g/ml Ficoll-Hypaque Premium gradient (GE Healthcare) according to manufacturer’s instructions. After collection of PBMCs, CD14+ monocytes were purified via negative selection (as to prevent pre-activation by signaling through CD14) using the Dynabeads® Untouched™ Human Monocytes kit (Life Technologies). Monocytes were cryopreserved in 90% heat-inactivated FBS (FBS-HI) with 10% DMSO. Monocytes were thawed, washed twice, and placed at 37°C for 1-2 hours in RPMI with 10% FBS-HI, 1% L-glutamine, 1% NEAA, 1% penicillin/streptomycin, 20 mM HEPES Buffer, and 30 units/ml DNase. Cells were then counted and resuspended as needed in the same RPMI mixture (without DNase) for experiments.
**Virus stock growth**

All experiments used DENV-2 strain 16681, kindly provided by Dr. Robert Tesh of UTMB-Galveston. To generate large stocks for experiments, near-confluent T150 flasks of mosquito cell monolayers were inoculated with DENV supernatant at an MOI of 0.5 ffu per cell diluted into 2 total ml of MEM medium with 1% FBS-HI, 1% NEAA, 1% penicillin/streptomycin, and 20 mM HEPES Buffer. Flasks were placed in a 30°C incubator with 5% CO₂ and were rocked every 15 minutes. At 2 hpi, 15 ml of medium was added (MEM + 10% FBS-HI, 1% penicillin/streptomycin, and 20 mM HEPES buffer). At various days, typically days 3, 7, and 10, medium was collected from the flasks and spun down at 4,000 RPM for 10 minutes to clear the supernatant of cells, and the debris-cleared, infectious supernatant was aliquoted into tubes for storage. Fresh medium replaced the collected infectious supernatant. Except for figure 2.1, all virus was derived from C6/36 *Aedes albopictus* mosquito cells (ATCC). Experiments in figure 2.1 used virus derived from C7/10 cells, a line related to the C6/36 cell line. C7/10 cells were provided by Dr. Frank Scholle at North Carolina State University.

To enumerate the concentration of virus, we modified a previously described protocol (206). Briefly, Vero cells were inoculated with sequential 10-fold dilutions of infectious supernatants. 2 hpi, 150 µl of a 1.6% carboxymethylcellulose overlay (diluted 1:1 in Gibco 2X MEM and supplemented with 1% FBS-HI, 10 mM HEPES and 1X antibiotics) was added to each well to limit spread of virus. 72 hpi, supernatants were aspirated and cells were fixed with 200 µl of a 1:1 mixture of acetone and methanol. Viral foci positive for DENV E protein were visualized with VIP Peroxidase Substrate kit (Vector Laboratories) and expressed as ffu/ml.
**DENV-specific antibodies**

Crude mAb 4G2 supernatant for use in IHC viral titrations was provided by Dr. Frank Scholle. The generation and purification of human mAbs 5G22 (α-DENV-1–DENV-4 prM used for enhancement) and 1F4 (α-DENV-1 virion for isotype) were described in detail previously (192,207,208). Purified mouse mAb 4G2 for flow cytometry was produced by the Antibody Core Facility at UNC Chapel Hill.

**Reagents**

All IL-1β ELISAs were measured using the BD OptEIA™ Human IL-1β ELISA Set II (BD Biosciences #557953). LPS (#tlrl-ebplps), ATP, and the ERK1/2 inhibitor PD98059 were all purchased from Invivogen. The bioluminescent caspase-1 activity assay was kindly provided by Dr. Martha O’Brien of Promega to assist with product development. Z-YVAD-FMK and Z-WEHD-FMK were purchased from R&D Systems. Syk inhibitor BAY 61-3606 was purchased from Santa Cruz Biotechnology.

**Inoculation of PBMCs or monocytes**

One hour pre-inoculation, dilutions of enhancing antibodies, isotype-matched control antibodies, or control medium were plated into 96-well, round-bottom, non-tissue-culture treated plates. Crude supernatant from mosquito cells infected with DENV-2 strain 16681 was diluted to the appropriate MOI and mixed with antibodies or control medium. Plates were incubated for 1 to 1.5 hours at 37°C with 5% CO2 to allow immune complex formation. For mock infection, spent culture medium from uninfected mosquito-cell cultures was used instead of infectious supernatant. After immune complex formation, purified primary monocytes were added and
mixed by pipetting. For figure 2.1, at 1 hpi, cells were washed 2 times with PBS and resuspended in fresh culture medium until 24 hpi. Once IL-1β secretion was confirmed to be an early event, samples were collected at the earlier indicated time points without prior removal of inoculum and washing of cells. Supernatants were assayed immediately by ELISA, stored at 4°C short-term, or stored -20°C until ELISAs could be ran. For flow cytometry, cells were washed once with PBS and fixed with Cytofix/Cytoperm™ fixation/permeabilization solution (BD Biosciences) for 20 minutes at 4°C. For immunoblots, cells were washed once with PBS and lysed with RIPA buffer (Boston BioProducts) with 1X cOmplete Protease Inhibitor Cocktail (Roche) and 1X PhosSTOP (Roche) for at least 20 minutes at 4°C with rotation. For RNA isolation, unwashed cell samples were mixed at a ratio of 1 volume of culture supernatant to 5 volumes of RNAprotect Cell Reagent (QIAGEN) to immediately protect and stabilize mRNA. All inoculations were done with an MOI of 50 Vero-cell ffu of DENV per monocyte. Vero cell cultures are highly susceptible to DENV infection, while primary monocytes are not. Thus, this measurement is used to keep doses consistent between virus stocks and does not translate exactly to 50 times the inoculum required to infect 1 monocyte.

**Virus inactivation**

Crude infectious supernatant was inactivated using shortwave UV exposure. 100 μl/well of virus stock was added to each well of a 24-well plate, placed on ice, and exposed to shortwave UVB (254 nm) irradiation for 2 min at a distance of approximately 5 cm.

**Flow-cytometric analysis**

After fixation, cells were washed twice with Cytofix/Cytoperm Perm/Wash Buffer. Non-
specific binding was blocked by incubating cells for 15 minutes with Human Fc Receptor Binding Inhibitor (eBioscience) diluted 1:20 in 1X Perm/Wash Buffer. DENV E-protein was then stained with the pan-flavivirus E-protein mAb 4G2 conjugated to Alexa Fluor® 647 diluted into Perm/Wash buffer for 30 minutes at 4°C. Cells were analyzed on a Cyan ADP flow cytometer. For experiments utilizing inhibitors, cells were stained with LIVE/DEAD® Fixable Violet Dead Cell Stain (Life Technologies) per manufacturer’s instructions prior to fixation. Cells were initially gated on FSC-area vs. SSC-area, with single cells positively selected by gating cells on FSC-lin vs. Pulse Width followed by selecting cells on the diagonal of FSC-lin vs. FSC-area. When used, cells were then gated for low fluorescence of LIVE/DEAD dye on the Pacific Blue channel. Cells positive for DENV antigen were detected on the APC channel for intracellular E-protein expression, with positive gates set based on mock-infected cells.

**Immunoblot analysis**

Lysates (or supernatants to be assayed by immunoblot) were recollected into fresh tubes and appropriate volumes of 4X NuPAGE® LDS Sample Buffer (Life Technologies) and DTT reducing reagent were added to yield final concentrations of 1X sample buffer and 50 mM DTT. Samples were vortexed and heated at 97°C for 5 minutes. Protein samples were loaded into Novex NuPAGE® SDS-PAGE 4-12% Bis-Tris pre-cast gels (Life Technologies) using 1X NuPAGE® MES SDS Running Buffer (Life Technologies) and separated via SDS-PAGE. Gels were transferred onto Bio-Rad 0.2-μm nitrocellulose membranes under wet transfer conditions for 45–60 minutes at 100 volts using 1X transfer buffer (Boston BioProducts) containing 20% methanol. The following primary antibodies were used: 1:2,000 dilution α-IL-1β (Santa Cruz #sc-7884), 1:1,000 α-caspase-1 (Santa Cruz sc-56036) for pro-caspase-1, 1:500 α-p-Syk (Y323)
(Santa Cruz #sc-293118), 1:1,000 α-Syk (Cell Signaling Technology #13198), 1:1,000 α-p-ERK1/2 (Cell Signaling Technology #4370), 1:2,000 α-ERK1 (Santa Cruz #sc-93), 1:2,000 α-ERK2 (Santa Cruz #sc-154), 1:2,000 α-p-NF-κB p65 (S536) (Cell Signaling Technology #3033), 1:2,000 α-NF-κB p65 (Cell Signaling Technology #8242). Membranes were incubated for 1 hour at room temperature with HRP-conjugated goat-α-mouse (Jackson ImmunoResearch #115-035-062) or goat-α-rabbit (Jackson ImmunoResearch #111-035-144) secondary antibodies. Concentrations varied from 1:10,000 to 1:30,000 depending on primary antibody. Actin was measured with 1:15,000 α-actin conjugated to HRP (Santa Cruz #sc-1615 HRP). Caspase-1 p10 was measured using SuperSignal Western Blot Enhancer (Thermo Scientific #46640) according to manufacturer’s instructions, with α-caspase-1 (Santa Cruz sc-522) diluted 1:250 in the primary antibody diluent contained in the kit. Membranes were developed after 5-minute incubations with SuperSignal West Pico (Thermo Scientific #34080) or SuperSignal West Femto (Thermo Scientific #34096) Chemiluminescent Substrates.

**Real-time PCR**

RNA was purified using RNEasy Plus Mini Kit per manufacturer’s instructions (QIAGEN #74134). Cell samples preserved in RNAProtect were stored at 4°C until processing. RNA was eluted in a volume of 30 µl RNase/DNase free water. To generate cDNA, 16 µl of RNA eluate was mixed with 4 µl 5X iScript™ Reverse Transcription Supermix (Bio-Rad #170-8841) and incubated in a thermal cycler as detailed in manufacturer’s protocol.

For real-time PCR analysis, 4.5 µl cDNA was added to 0.5 µl 20X ABI TaqMan® Gene Expression Assay mix (Life Technologies #4331182) and 5 µl of ABI 2X TaqMan Universal PCR Master Mix (Life Technologies #4304437) in a 384-well plate. Each biological sample was
pipetted in at least duplicate. Plates were run on the ABI ViiA™ 7 Real-Time PCR machine using the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, and 40 repeats of 95°C for 15 seconds followed by 60°C for 1 minute. Fold change values were calculated using the ΔΔCt method, normalized to a control value set at 1. For all samples, 18s rRNA was used as the housekeeping-gene control.

The following ABI TaqMan® Gene Expression Assays were used for real-time PCR analysis of gene expression: assay Hs01555410_m1 for IL1B, Hs01113624_g1 for TNF, Hs00985639_m1 for IL6, and Hs03928985_g1 for RN18S1.

**Bioluminescent caspase-1 activity assay**

The assay reagent was mixed per manufacturer’s instructions. At indicated time points, unprocessed cell samples were mixed at a 1:1 ratio of culture supernatant to assay reagent in 96-well opaque plates. Each sample had a matching well in which 1 μM Ac-YVAD-CHO was added to the assay reagent, blocking caspase-1-mediated cleavage of the substrate. Cell-free controls were also run to control for background signal. After 1 hour of incubation at room temperature, luminescence was read on a standard plate reader. The appropriate cell-free background luminescence value was first subtracted from each sample. Then, YVAD control values (measuring caspase-1-independent peptide cleavage) were subtracted from the full assay values. The control-adjusted values represent relative caspase-1 activity.

**Data presentation and statistical analyses**

Unless otherwise noted, all graphs shown represent the mean ± SEM of 3 or more values. Statistical analyses were performed only when 3 or more biological replicates were present using
Prism 6.0 (GraphPad). Statistical significance was defined as p < 0.05. When unpaired, two-tailed t tests were used, asterisks represent actual p values. Ordinary one-way ANOVAs were used for comparisons within one independent variable. Ordinary two-way ANOVAs were used for comparisons containing two independent variables (e.g. presence or absence of virus and presence or absence of mAb 5G22). For figure panels using ANOVA comparisons, asterisks represent the multiplicity-adjusted p values within the multiple comparisons. Tukey’s test was used when all means were compared, and Bonferroni’s test was used when only means within one independent variable were compared (two-way ANOVAs only). For all cases, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. All data are representative of two or more independent experiments.
FIGURE 2.1 – ADE of DENV infection induces elevated IL-1β secretion independent of viral replication

A. Representative flow-cytometric histogram overlay showing DENV E-protein expression in primary monocytes. Cells were inoculated with mock supernatant (light gray region), DENV alone (black line), or DENV complexed with mAb 5G22 (dark gray line) for 1 hour at 37°C before being washed, resuspended in fresh medium, and incubated until 24 hpi, at which point cells were prepared for flow cytometry as described in Experimental Procedures. B. Cumulative percentages of all samples from experiment described in panel A. C. IL-1β ELISA of 24 hpi supernatants from experiment described in panels A and B. D. Measurement of infectious titer of DENV stock before and after exposure to shortwave UV irradiation for 2 minutes. Titer was determined based on immunoassay in Vero cells as described in Experimental Procedures. E. Flow-cytometric detection of DENV E-protein expression within monocytes 24 hpi after inoculation with live and UV-inactivated DENV. Cells again were exposed to inoculum for 1 hour prior to washing and resuspension in fresh medium. M = mock-treated, L = live virus, and
UV = UV-inactivated virus. F. IL-1β ELISA of 24 hpi supernatants from experiment described in panels D–E. “All n.s.” = all comparisons were not statistically significant. For all figures, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001, and “n.s.” = not significant. Statistical tests: Ordinary two-way ANOVA (Tukey’s) for all comparisons. See Materials and Methods for detailed information on statistical analyses.
FIGURE 2.2 – ADE rapidly induces IL1B and pro-IL-1β expression to elevate mature IL-1β secretion by 4 hpi
A. At 2 hpi, monocytes were lysed, and RNA was purified. No treatment (NT) and LPS (100 ng/ml) samples were collected at 2.5 hours post-stimulation (hps). Samples were prepared as described in Experimental Procedures. IL1B expression was measured using semi-quantitative real-time PCR. All data is normalized to mock inoculation with no mAb 5G22. Mock and DENV values are pooled from two independent experiments. NT and LPS conditions contain 2 values each and represent mean ± SD. B. At 4 hpi, monocytes were pelleted, washed, and lysed. Pro-IL-1β protein levels were then measured via immunoblot. C. 4 hpi supernatants corresponding to panel B were assessed by ELISA for IL-1β. D. In a separate experiment, supernatants from inoculated monocytes were collected at 4 hpi and assessed for mature IL-1β by immunoblot. Statistical tests: Ordinary two-way ANOVA (Tukey’s) (A and C).
FIGURE 2.3 – ADE does not induce detectable caspase-1 activation in primary monocytes

A. Monocytes were inoculated with indicated mock or viral conditions and allowed to incubate until 2 hpi when cells were lysed. No treatment (NT) and LPS (200 ng/ml priming for 4.25 hours) plus ATP (5 mM pulse for final 0.75 hours) control samples were collected and lysed at 5 hps in a separate experiment. Pro-caspase-1 and caspase-1 p10 were measured via immunoblot.

B. Monocytes were inoculated and mixed at a 1:1 ratio with bioluminescent caspase-1 activity assay reagent or reagent with YVAD (to block cleavage of substrate by caspase-1) at indicated time points. Graph represents relative caspase-1 activity after subtraction of cell-free background and YVAD controls. 100 ng/ml LPS was given to indicated cells. Cells receiving ATP received a 3 mM pulse 45 minutes prior to assay reagent addition.
FIGURE 2.4 – Inefficient processing of ADE-induced pro-IL-1β is caspase-1 dependent

A. Monocytes were treated with DMSO vehicle, 20 μM Z-YVAD-FMK, or 20 μM Z-WEHD-FMK at time of inoculation. Supernatants were collected at 5 hpi and assayed for IL-1β by ELISA. Vehicle-treated cells had 3 samples per condition, and inhibitor-treated cells had 4 samples per condition. Values not shown fell below the limit of detection. B. Monocytes were treated with DMSO vehicle or 10 μM Z-YVAD-FMK at time of inoculation. Supernatants were collected at 5 hpi and assayed for mature IL-1β by immunoblot. C. Monocyte supernatants were collected at 4 hpi after adding 2 mM ATP (final concentration) to the indicated wells 45 minutes before collection. Supernatants were then assayed for IL-1β by ELISA. Statistical tests used: Ordinary one-way ANOVA (Tukey’s) within DENV treatment was used for panel A since many mock values fell below the limit of detection; Ordinary two-way ANOVA (Bonferroni’s) (C).
FIGURE 2.5 – Activation of Syk mediates induction of IL-1β by ADE

A. At 4 hpi, monocytes were collected and lysed, with subsequent measurement of p-Syk and Syk via immunoblot. B. Monocytes were inoculated with DENV complexed with mAb 5G22 after pretreatment for 30 minutes with DMSO vehicle (solid line) or 0.5 μM BAY 61-3606 (dashed line). Cells were allowed to incubate without removal of inoculum until 24 hpi, at which point cells were prepared for DENV E-protein measurement by flow cytometry. C. Cells were pre-treated with DMSO vehicle or 0.5 μM BAY 61-3606 for 30 minutes before inoculation. At 2 hpi, cells were lysed and RNA was purified. *IL1B* expression was assessed by semi-quantitative real-time PCR, with normalization to mock and vehicle treatment. Vehicle-treated values are part of pooled values in figure 2.2A. Differences were analyzed with two separate ordinary two-way ANOVAs (Bonferroni’s), one for mock-inoculation and one for DENV-inoculation, with antibody and inhibitor conditions set as independent variables. D. Monocytes were pretreated with DMSO vehicle or 0.5 μM BAY 61-3606 for 30 minutes, inoculated, and allowed to incubate until 8 hpi without removal of inoculum. Supernatants were then collected, and cells were washed and lysed. Lysate p-Syk, lysate Syk, and supernatant mature IL-1β levels were then measured via immunoblot. Boxes indicate samples are non-adjacent lanes, but all samples are from same blot and exposure.
FIGURE 2.6 – Non-complexing antibody does not enhance DENV-induced IL-1β secretion

A. DENV was complexed with mAb 5G22 (dashed line) or incubated with an equal concentration of mAb 1F4 (solid line) prior to inoculation onto monocytes. At 4 hpi, cells were washed twice and resuspended in fresh medium until collection at 24 hpi and staining for flow-cytometric detection of intracellular DENV E protein. B. Cells were inoculated as indicated, and supernatants were collected at 4 hpi. IL-1β was then assessed by ELISA. Statistical test used: Ordinary two-way ANOVA (Tukey’s) (B).
FIGURE 2.7 – Syk activated by DENV immune complexes mediates ERK1/2 activation and inflammatory cytokine expression

A. Monocytes were inoculated after pretreatment with DMSO vehicle or 1 μM BAY 61-3606 for 30 minutes. At 0.25, 1, or 2 hpi, cells were lysed and assayed via immunoblot for indicated proteins. B and C. Cells were pre-treated with DMSO vehicle or 0.5 μM BAY 61-3606 for 30 minutes before inoculation. At 2 hpi, cells were lysed and RNA was purified. TNF (B) and IL6 (C) expression was assessed by semi-quantitative real-time PCR, with normalization to mock and vehicle treatment. Statistical tests used: Two separate ordinary two-way ANOVAs were used for each panel (Bonferroni’s) (B and C), one for mock-inoculation and one for DENV-inoculation, with antibody and inhibitor conditions set as independent variables.
FIGURE 2.8 – Syk activates ERK1/2 to mediate ADE-induced IL-1β secretion

A. Monocytes were inoculated after pretreatment for 1 hour with DMSO vehicle or 25 μM PD98059 ERK1/2 inhibitor. At 4 hpi, cells were lysed, and the indicated proteins were measured via immunoblot. B. Cells were inoculated after 1 hour pretreatment with DMSO vehicle or 25 μM PD98059. At 5 hpi, supernatants were collected and assayed by immunoblot for mature IL-1β. Boxes indicate non-adjacent lanes taken from the same blot and exposure.
CHAPTER 3: SOURCE AND PURITY OF DENGUE-VIRAL PREPARATIONS
IMPACT REQUIREMENT FOR ENHANCING ANTIBODY TO INDUCE ELEVATED
IL-1β SECRETION: A PRIMARY HUMAN MONOCYTE MODEL

3.1 INTRODUCTION

With an estimated 390 million global infections per year, dengue virus (DENV) is the most burdensome arbovirus in the world (2). The four distinct serotypes (DENV-1–DENV-4) are transmitted by the widespread, tropical Aedes aegypti and Aedes albopictus mosquitoes, and nearly half of the global population lives in DENV-endemic regions (209). A first infection with any serotype may cause an asymptomatic infection or a mild to severe flulike illness referred to as dengue fever (DF) (210). Patients typically recover without complication and develop long-term immunity to the same DENV serotype, but immunity to heterologous serotypes is transient (1,211). Upon later infection with a second serotype of DENV, a small percentage of patients progress to the life-threatening disease course of severe dengue (209,211). During severe dengue, a reversible permeability develops in the vasculature, causing hemorrhagic manifestations and potential hypovolemic shock (157,210). There is no specific cure or vaccine, but supportive therapy until the disease course passes can reduce mortality levels from greater than 20% to less than 1% (9,209).

It is now widely accepted that weakening immunity to a primary infection can increase disease severity during a heterologous DENV infection. For decades, cross-reactive antibodies

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2 This chapter is part of a manuscript submitted to PLOS ONE on 18 July 2014: Callaway JB, Widman DG, Smith SA, McKinnon KP, Scholle F, Dittmer DP, Crowe, JE Jr., de Silva AM, Ting JP. Source and purity of dengue-viral preparations impact requirement for enhancing antibody to induce elevated IL-1β: a primary human monocyte model.
have been identified as a risk factor for severe dengue (5). The human immune response during a primary DENV infection generates an antibody profile that is highly cross-reactive with other serotypes (4). As a patient becomes further removed from a primary infection, the concentration of cross-reactive antibodies drops below the level required to provide cross-protective neutralization. Instead, these antibodies may enhance infection of DENV into Fc-receptor-bearing cells by a mechanism known as antibody-dependent enhancement (ADE) of infection (4,210). Circulating CD14+ monocytes, which express high levels of Fc receptors, have been identified as the primary target of ADE among all peripheral blood mononuclear cells (PBMCs), and increased activation of monocytes is associated with more severe dengue disease (104,105).

The entire spectrum of pathologies contributing to severe dengue has not been completely defined. However, it is believed that a “cytokine storm,” a massive and aberrant upregulation of cytokine production, contributes to vascular permeability and hemorrhagic complications (9,210). A substantial number of patient studies have identified the upregulation of a wide array of cytokines during DENV infection (9,93,158,159,212-214). The lack of a consensus on the most damaging cytokines likely reflects the complicated nature of defining disease severity and achieving consistency between study parameters. As well, abundant evidence identifies the importance of the specific sequence of infecting serotypes, with a primary DENV-1 infection followed by a secondary DENV-2 infection carrying a much higher risk of severe disease development than other combinations (210). The sequence of infections varies both yearly and regionally.

One inflammatory cytokine that is elevated in many cytokine profiles of DENV patients is IL-1β. Higher levels of circulating IL-1β have been detected in the sera of severe dengue patients compared to DF patients (93,94). Also, 10-fold higher expression of the gene IL1B,
which encodes the pro-IL-1β zymogen, has been measured in PBMCs of severe dengue patients compared to DF patients (94,159). Further, monocytes infected in culture with DENV have been found to secrete IL-1β (95). As IL-1β is a potent inflammatory cytokine, its mechanism of production during ADE may provide insight into severe disease pathogenesis.

The unique regulation of IL-1β production and secretion is generally controlled by a two-step process. The first step is transcriptional and translational stimulation (such as via NF-κB activation downstream of TLR signaling) leading to increased expression of IL1B and translation of the pro-IL-1β zymogen (215). Alone, pro-IL-1β expression is not sufficient to induce secretion of active IL-1β. Instead, the second step of the control mechanism is the activation of the inflammasome (90). The inflammasome is a large, multi-protein complex typically containing one or more nucleotide-binding domain, leucine-rich repeat containing (NLR) proteins which, upon assembly, recruit in multiple copies of the pro-caspase-1 zymogen. Autocatalytic cleavage occurs when pro-caspase-1 proteins come into close association with one another, forming active caspase-1 (166-168). Active caspase-1 subsequently cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 (90,92). More recent studies have revealed that other, non-NLR proteins, such as AIM2 and RIG-I can also form an inflammasome (216,217). The stimuli that activate the inflammasome vary by specific NLR component. To date, the NLRP3 inflammasome is recognized to respond to the widest variety of pathogen- and danger-associated molecular patterns (91).

The purpose of this study is to assess the mechanisms of ADE-induced IL-1β production in primary human monocytes. That antibody enhances DENV-induced IL-1β secretion by primary monocytes has been known for years (95). However, no study has subsequently reassessed the mechanism of ADE-induced IL-1β production in primary monocytes since the
discovery of the inflammasome. We employed a highly-relevant physiological system utilizing not only primary human monocytes, but also monoclonal antibodies (mAbs) isolated from DENV-immune patients (207). Interestingly, we found that inoculation with crude supernatant harvested from Vero cells infected with DENV-2 strain 16681 induced equivalent IL-1β secretion by primary monocytes in the presence or absence of enhancing antibodies. Further studies indicated that the secretion of IL-1β was completely independent of antibody signaling and viral replication. Removal of DENV virions from crude supernatant preparations indicated that a soluble component present in the supernatant was responsible for the induction of IL-1β. In contrast to crude infectious supernatant from Vero cells, DENV purified from infectious Vero-cell supernatant induced elevated IL-1β secretion only in the presence of ADE. Similarly, ADE of crude supernatant harvested from DENV-infected C6/36 mosquito cells induced significant elevation of IL-1β secretion without the need to purify the viral preparation. Our data indicate that cell-line and purity selections during DENV preparation should be carefully considered before undertaking a study investigating DENV-induced cytokine production.

3.2 RESULTS

Primary human monocytes are the main blood cell type infected by DENV

We first sought to confirm that CD14+ monocytes were the target cell of dengue virus (DENV) in the current system. Mobilized PBMCs were isolated from leukapheresed blood and inoculated with a multiplicity of infection (MOI) of 50 focus-forming units (ffu) of DENV-2 strain 16681 that had been incubated with 1 μg/ml anti-DENV prM human mAb 5G22. Crude culture supernatant harvested from DENV-infected Vero cells was cleared of cellular debris and used as the inoculum, hereafter referred to as DENV-Infectious Vero-cell (DIV) crude supernatant. The human mAb 5G22, isolated from a DENV-immune patient, is known to
potently enhance DENV infection (207). At 24 hours post-inoculation (hpi), the inoculated PBMCs were analyzed by flow cytometry for both CD14 and DENV E-protein expression \textbf{(Fig. 3.1A)}. Intracellular DENV E protein detected by flow cytometry is a measure of viral replication (156). Cells negative for DENV E protein expressed varying levels of CD14, while all cells positive for intracellular DENV E protein also expressed high levels of CD14. These data confirm that CD14\(^+\) monocytes are the target of ADE in a mixed population of PBMCs. Thus, most of the subsequent experiments utilize cryopreserved CD14\(^+\) mobilized monocytes that were purified using negative isolation to prevent activation through CD14. The advantage of mobilized, leukapheresed blood is that it provided a large source of cells from a single blood draw (with two separate donors total), eliminating much of the variability associated with multiple blood draws from different volunteers over time. Key phenotypes were verified using CD14\(^+\) monocytes purified from fresh draws of non-mobilized blood.

**DIV crude supernatant induces IL-1\(\beta\) secretion by primary monocytes independent of ADE**

We next examined the induction of IL-1\(\beta\) in primary monocytes by DIV crude supernatant in the context of ADE. In the absence of mAb 5G22, no intracellular expression of DENV E protein was detected at 24 hpi in mobilized monocytes \textbf{(Fig. 3.1B)}. In contrast, pre-incubation of DIV crude supernatant with increasing levels of mAb 5G22 caused a dose-dependent increase in intracellular E-protein expression. Unexpectedly, DIV crude supernatant induced IL-1\(\beta\) secretion by mobilized monocytes independent of the presence of mAb 5G22, as measured by ELISA on monocyte supernatants collected at 24 hpi \textbf{(Fig. 3.1C)}. An identical pattern was observed in freshly-isolated monocytes from a non-mobilized donor, with mAb 5G22 required for intracellular E-protein expression but not for induction of IL-1\(\beta\) secretion.
We next inoculated mobilized monocytes with varying doses of DIV crude supernatant in the presence of a constant antibody dose or control condition. Increasing doses of DIV crude supernatant increased intracellular E-protein expression in the presence of 1 μg/ml mAb 5G22 (Fig. 3.1F). However, increasing doses of DIV crude supernatant induced elevated secretion of IL-1β regardless of the presence of mAb 5G22 (Fig. 3.1G). These data indicate a bifurcation in the dependence on anti-DENV antibody, with mAb 5G22 enhancing DENV replication but being dispensable for IL-1β secretion in primary monocytes inoculated with DIV crude supernatant.

To verify that the lack of ADE-induced IL-1β was not isolated to mAb 5G22, we tested additional antibody conditions with other human mAbs isolated from DENV-immune patients. Enhancement of DENV infection with 0.1 μg/ml mAb 2D22 and 2 μg/ml mAb 1C17 (indicated by peaks with higher DENV E-protein expression in the histograms) both failed to alter IL-1β secretion induced by DIV crude supernatant (Fig. 3.1H). In addition to enhancement, mAb 2D22 also strongly neutralizes DENV-2 infection at higher concentrations, as previously reported (207). Neutralization of infection with 2 μg/ml mAb 2D22 (indicated by the loss of elevated DENV E-protein expression compared to 0.1 μg/ml 2D22) also did not affect IL-1β secretion.

We next antagonized antibody binding by pre-incubating mobilized monocytes with an Fc-receptor binding inhibitor. Fc-receptor inhibition significantly reduced intracellular, ADE-induced E-protein expression (Fig. 3.1I) but did not affect the secretion of IL-1β (Fig. 3.1J).

To rule out the possibility that these outcomes are unique to the Vero cell line we used, crude supernatant was harvested from a second, independent line of Vero cells infected with DENV-2 strain 16681. IL-1β secretion induced by DIV crude supernatant harvested from this second Vero line also was independent of the presence of mAb 5G22 (Fig. 3.1K). In total, these
results indicate that anti-DENV antibodies significantly impact viral replication in primary monocytes but are completely dispensable for IL-1β secretion induced by DIV crude supernatant.

**IL-1β secretion induced by DIV crude supernatant precedes viral replication**

As enhancing viral replication had no effect on IL-1β secretion, we wanted to determine if the kinetics of IL-1β release differ from the kinetics of viral replication. All previous experiments had assessed IL-1β production at 24 hpi, so we initiated a time course with sample collections at 2, 8, 16, and 24 hpi. Mobilized monocytes inoculated with DIV crude supernatant secreted significantly more IL-1β as early as 8 hpi compared to mock conditions (Fig. 3.2A). However, viral replication only began to elevate significantly at 16 hpi. Intracellular DENV E-protein expression could not be detected until 16 hpi (Fig. 3.2B). To further assess replication of the virus, neither the amount of infectious virus present in the supernatant (Fig. 3.2C) nor intracellular presence of DENV genome copies (Fig. 3.2D) significantly increased until 24 hpi compared to the 2 hpi measurements. We next assessed IL-1β secretion at a time point between 2 hpi and 8 hpi. DIV crude supernatant induced ADE-independent IL-1β secretion by 4 hpi in both mobilized monocytes (Fig. 3.2E) and fresh, non-mobilized monocytes (Fig. 3.2F). These data indicate that IL-1β secretion occurs early after inoculation with DIV crude supernatant and precedes measurable viral replication.

**IL-1β secretion induced by DIV crude supernatant is independent of viral replication**

Since IL-1β secretion was elevated before replication could be detected, we sought an alternate way to determine whether infectious DENV was required to induce IL-1β secretion. To
do so, we inactivated DIV crude supernatant by incubation with formalin or exposure to shortwave UV irradiation prior to inoculation onto mobilized monocytes. Both formalin and UV inactivation ablated intracellular DENV E-protein expression in mobilized monocytes (Fig. 3.2G). However, each inactivated infectious supernatant induced significant elevation of IL-1β secretion compared to mock conditions (Fig. 3.2H). These data indicate that the infectivity of DENV is dispensable for IL-1β induction by DIV crude supernatant.

**DIV crude supernatant induces IL1B and pro-IL-1β expression in primary monocytes**

Elevated secretion of IL-1β can be caused by increased pro-IL-1β expression, increased inflammasome activation, or both. To assess the mechanism of IL-1β induction by DIV crude supernatant, we first considered known cellular expression of key genes reported in the online database BioGPS (Fig. 3.3A). CD14+ monocytes express high baseline levels of CASP1 and NLRP3, which encode the inflammasome components caspase-1 and NLRP3, respectively. However, basal expression of IL1B is low, indicating it likely needs induction. Thus, we measured IL1B expression after inoculation of mobilized monocytes with DIV crude supernatant (Fig. 3.3B). IL1B expression increased rapidly 2 hours after inoculation with DIV crude supernatant, compared to mock conditions, and gradually reduced over time. Correspondingly, we detected a strong induction of 31-kDa pro-IL-1β expression in the cell lysates of mobilized monocytes collected 3 hours after inoculation with DIV crude supernatant (Fig. 3.3C). Additionally we detected a strong elevation in the 17-kDa mature IL-1β, indicating successful intracellular processing of pro-IL-1β.
Caspase-1 and NLRP3 are required for IL-1β secretion induced by DIV crude supernatant

As mobilized monocytes processed IL-1β after inoculation with DIV crude supernatant, we next sought to assess inflammasome involvement. We were unable to reproducibly and reliably detect active caspase-1, a notoriously difficult protein to detect in human cells. However, pre-treatment of monocytes with the irreversible caspase-1 inhibitor Z-WEHD-FMK significantly reduced the IL-1β secretion induced by DIV crude supernatant (Fig. 3.3D). To test for NLRP3 dependency, we transfected cells for 24 hours with either a negative control siRNA or one targeting NLRP3 prior to inoculation with DIV crude supernatant (Fig. 3.3, E and F). At the time of viral inoculation, NLRP3 expression was reduced approximately 63% by the specific siRNA compared to the negative control (Fig. 3.3E). Expression of NLRC4, an alternate NLR family member, was not affected. Knockdown of NLRP3 expression significantly reduced the secretion of IL-1β by mobilized monocytes after inoculation with DIV crude supernatant (Fig. 3.3F). These data indicate that caspase-1 and NLRP3 are required for IL-1β secretion induced by DIV crude supernatant.

Depletion of antibody-bound virions does not reduce IL-1β secretion induced by DIV crude supernatant

We next sought to determine the reason that anti-DENV antibodies can enhance DENV replication without altering the secretion of IL-1β. We considered the possibility that a soluble factor not associated with the virion could induce signaling in the monocytes. To test this, we developed a method of depleting antibody-bound virions from DIV crude supernatant (Fig. 3.4A). Antibodies targeting several DENV epitopes were incubated with DIV crude supernatant individually or in combination. Un-depleted control tubes (fraction C in schematic) received an equal volume of PBS in lieu of beads. Magnetic protein G beads were incubated in depletion
tubes. Subsequently, depletion tubes were placed on a magnet, sequestering beads, and all bead-bound components, to the side of the tube. Residual supernatant (fraction R) could then be collected free of bead-bound components. Finally, the bead-bound fraction (fraction B) was resuspended to the original volume for analysis. Depleting monoclonal antibodies had specificity for prM, EDI/II, or EDIII, three main antibody targets found on the surface of the DENV virion, as described previously (218). Control, residual, and bead-bound fractions were then assessed by immunoblot for efficiency of DENV depletion (Fig. 3.4B). Under mock conditions with all three antibodies, DENV E and prM antigens were not detected, as expected. Human IgG was not detected in the residual fraction depleted of bead-bound components (lane R), while there was strong detection of human IgG Fc in the bead-bound fraction (lane B). This indicates that the protein G beads efficiently removed all human IgG. In a second control containing DIV crude supernatant in the absence of antibodies (DENV + PBS), DENV E protein and prM were only detected in the residual fractions (lane R). This indicates that beads did not non-specifically bind and deplete DENV virions. Next, each antibody was used individually at 3 μg/ml with DIV crude supernatant. This resulted in varying levels of DENV depletion, as assessed by the depletion of DENV E or prM proteins from the residual fractions (lane R). Importantly, the mAb targeting DENV EDI/II removed nearly all detectable virus from the residual supernatant. Similar results were obtained when all three antibodies were added at 1 μg/ml each with DIV crude supernatant. By contrast, anti-prM and anti-EDIII mAbs only partially removed DENV virions when compared to their corresponding control supernatants.

Subsequently, both the un-depleted control supernatants (lane C) and antigen-depleted supernatants (lane R) were inoculated onto mobilized monocytes at an approximate pre-depletion MOI of 50 (Fig. 3.4C). No depletion condition altered IL-1β secretion compared to its respective
un-depleted control supernatant. These data suggest that DENV virions are not responsible for the IL-1β secretion induced by DIV crude supernatant.

Several potential factors eliminated as IL-1β-inducing component of DIV crude supernatant

To identify the component in DIV crude supernatant that could induce IL-1β secretion, we investigated several possibilities. First, though most DENV nonstructural proteins are neither present in an infectious virion nor secreted from infected cells, nonstructural protein 1 (NS1) is secreted from infected cells as a soluble hexamer (219). To determine if soluble NS1 present in DENV supernatant is responsible for the induction of IL-1β secretion, we depleted NS1 from viral supernatant using antibody-mediated depletion with protein G beads. Using an anti-NS1 antibody, we obtained near complete removal of NS1 from DIV crude supernatant (Fig. 3.4D). However, inoculating mobilized monocytes with NS1-depleted DIV crude supernatant had no impact on the secretion of IL-1β.

We next verified that DIV crude supernatants were not contaminated with LPS, a potent IL-1β agonist. Two independent DIV crude supernatant samples were sent to the UNC Tissue Culture Facility for LPS screening by a limulus amebocyte lysate assay. Both samples were reported to contain less than or equal to 0.10 Endotoxin Units/ml, thus excluding LPS contamination as a factor.

To investigate the possibility that DENV-infected Vero cells release inflammatory RNA or DNA, we treated DIV crude supernatant with DNase or RNase prior to inoculation onto mobilized monocytes (Fig. 3.4E). Neither nuclease prevented IL-1β secretion induced by DIV crude supernatant. To assess if complement or another heat-sensitive component had a role, DIV crude supernatant was heated for 30 minutes at 56°C prior to inoculation onto mobilized
monocytes (Fig. 3.4F). Heat-inactivated DIV crude supernatant still induced elevated IL-1β secretion over mock conditions.

To test if cytokines secreted by infected Vero cells, which originated from African green monkey kidney, could induce IL-1β secretion by primary human monocytes, we filtrated the DIV crude supernatant. Ultrafiltration of human plasma using a pore with a 60-kDa molecular-weight cutoff (MWCO) has been shown to effectively remove IL-1β, IL-6, TNFα, IL-10, and IL-8, as these cytokines are smaller and able to pass through the pore (220). Thus, we chose to employ centrifugal filtration with a MWCO of 100 kDa to allow flow-through of cytokines but not DENV or other larger components. The flow-through fraction, containing everything less than 100 kDa (approximately), and the concentrated retentate (components above 100 kDa) were collected and brought to equal volumes. Subsequently, equal volumes of both fractions and a mock condition were inoculated onto mobilized monocytes (Fig. 3.4G). Only the fraction containing components larger than 100 kDa induced the secretion of IL-1β by primary monocytes. This excluded many key cytokines present in Vero-derived supernatant as a contributing factor to IL-1β induction.

**ADE is essential for DENV-induced IL-1β secretion by primary monocytes infected with purified virus**

Though the identity of the inflammatory component present in DIV crude supernatant remained unclear, we wanted to assess if purification of DENV virions away from other supernatant components would alter the secretion of IL-1β. To purify the virions, DIV crude supernatant was concentrated and subjected to ultracentrifugation through a 10% – 40% continuous sucrose gradient (221). Subsequently, mobilized monocytes were inoculated with either purified virus alone or purified virus pre-incubated with mAb 5G22. Similar to the finding
with DIV crude supernatant, intracellular DENV E-protein was only detected in monocytes at 24 hpi in the presence of mAb 5G22 (Fig. 3.5A). However, unlike that induced by DIV crude supernatant, purified DENV induced significant elevation of IL-1β secretion only in the presence of mAb 5G22 (Fig. 3.5B). Interestingly, the level of IL-1β induced by purified DENV is lower than that induced by DIV crude supernatant shown in earlier experiments, consistent with the presence of an additional inflammatory component in the crude supernatant.

We next cleared both viral preparations of DENV virions and assessed the IL-1β induction by residual filtrates. Both DIV crude supernatant and purified DENV preparations were passed through 0.2-μm and 0.02-μm syringe filters. DENV virions have an approximate diameter of 50 nm, suggesting that DENV should not be able to pass through a 0.02-μm filter (222). As expected, DENV passed through the 0.2-μm filter but not the 0.02-μm filter, as measured by immunoblot for DENV E protein present in the different fractions (Fig. 3.5C, top). Further, the 0.02-μm filter removed infectious DENV as measured by immunoassay in Vero cells (Fig. 3.5C, bottom). Consistent with this, after incubation of the filtrates with mAb 5G22 and inoculation onto mobilized monocytes, we could only detect DENV E-protein expression in monocytes inoculated with 0.2-μm filtrates (Fig. 3.5D). These fractions were then tested for their IL-1β-inducing activity compared to mock supernatant. Both 0.2-μm and 0.02-μm filtrates of DIV crude supernatant induced significantly elevated IL-1β secretion compared to the mock condition (Fig. 3.5E). The crude filtrate cleared of DENV virions maintained inflammatory activity compared to mock conditions, consistent with the presence of an additional inflammatory factor. In contrast, using purified DENV filtrates, only 0.2-μm filtrates induced elevated IL-1β secretion compared to the mock condition. Removal of DENV virions by filtration from the purified preparation prevented significant IL-1β secretion compared to the
mock condition. These data further support that an inflammatory factor is produced by Vero cells that is eliminated by purification of the DENV preparation.

As all previous experiments utilized DENV propagated in Vero cells, we next tested DENV propagated through mosquito cells, which represent the natural vector of DENV. Crude supernatant harvested from DENV-infected C6/36 mosquito cells was inoculated onto mobilized monocytes in the presence or absence of mAb 5G22. Similar to Vero-derived DENV, mAb 5G22 enhanced the intracellular expression of DENV E protein in mobilized monocytes 24 hours after inoculation with crude supernatant from DENV-infected C6/36 cells (Fig. 3.5F). Interestingly, in contrast to DIV crude supernatant, ADE with mAb 5G22 significantly enhanced DENV-induced IL-1β secretion when mobilized monocytes were inoculated with crude supernatant from DENV-infected C6/36 cells (Fig. 3.5G). Importantly, the presence of mAb 5G22 did not impact IL-1β secretion induced by crude supernatant from uninfected C6/36 cells. These data suggest that, unlike Vero cells, C6/36 mosquito cells do not produce a potent inflammatory component during the propagation of DENV in culture.

### 3.3 DISCUSSION

This work shows that while all DENV preparations display an identical requirement for ADE to enhance viral replication, the source and purity of DENV preparations greatly impact the induction of IL-1β secretion by primary human monocytes. DENV propagation in Vero cells produced not only high titers of infectious virus, but also inflammatory moieties that induced IL-1β secretion. Initial experiments utilized debris-cleared supernatant derived from DENV-infected Vero cells, a common method of viral preparation to analyze the immune response to DENV (223-227). This Vero-derived supernatant displayed a strong IL-1β-inducing activity that was
unaltered by ADE, despite concurrent enhancement of infection by antibody. Crude supernatant harvested from a second, independent line of DENV-infected Vero cells similarly induced ADE-independent IL-1β secretion. Importantly, using supernatant from uninfected Vero cells as a mock condition did not strongly induce IL-1β, indicating that the IL-1β-inducing moiety is only produced upon DENV infection of Vero cells.

Once DENV virions generated in Vero cells were purified and separated from other supernatant components, a different pattern emerged. Purified DENV from Vero cells induced significantly more IL-1β secretion by monocytes in an ADE-dependent fashion. ADE similarly enhanced DENV-induced IL-1β secretion when using crude supernatant from C6/36 mosquito cells infected with DENV. This indicates that the inflammatory moiety in Vero-derived supernatant that caused IL-1β secretion is not produced in mosquito cells. As inoculation with crude supernatant from DENV-infected Vero cells is commonly used in the field, precaution should be exercised in using such a preparation to study the DENV-induced inflammatory response in immune cells.

Inoculation of primary mobilized monocytes with crude supernatant from DENV-infected Vero cells rapidly induced the expression of IL1B and pro-IL-1β. Thus, a key step leading to elevated IL-1β secretion after inoculation with this supernatant appears to be the enhancement of pro-IL-1β expression. Although bioinformatic analysis with BioGPS shows that CASP1 and NLRP3 transcripts are constitutively expressed by human monocytes, inhibition of caspase-1 with a pharmacologic inhibitor or NLRP3 with RNA interference reduced the secretion of IL-1β, indicating involvement of the NLRP3 inflammasome.

Although much effort was devoted to identifying the IL-1β-inducing moiety or moieties in DIV crude supernatant, the nature of the inducer(s) remained elusive. Could it be a cell-culture
artifact that needs to be eliminated from conventional crude viral preparations? Or is this a
physiologically relevant component (either viral- or host-derived) that merits further studying?
Our data suggests that the factor is larger in size than many key cytokines, is heat stable at 56°C,
and is not RNA, DNA, or LPS contamination. Further, immunodepletion studies confirm that
NS1 is not responsible for the IL-1β induction. Of note, one study has shown that Vero cells
infected with DENV undergo more apoptosis than infected C6/36 mosquito cells (228). It is
possible that by-products of increased cell death may alter the inflammatory phenotype of crude
supernatant from DENV-infected Vero cells.

The IL-1β-inducing factor present in crude supernatant from DENV-infected Vero cells
likely masked the response induced by DENV and anti-DENV antibody in the current system.
This is an important point to consider, as it can generate misleading results. Importantly,
successful achievement of ADE-induced cytokines by other groups using crude supernatant
derived from DENV-infected Vero cells indicates that this inflammatory phenotype is not
universally produced by all Vero cells or in all systems (224,226). However, a masking
component should be considered when disjointed results between infection and cytokine
production are found, particularly under ADE conditions. Our study also demonstrates that crude
supernatant from DENV-infected C6/36 cells does not exhibit these confounding issues and
represents a more straightforward system to study the ADE-induced inflammatory response in
human immune cells. Finally, though the use of purified virus effectively removed the
inflammatory moiety from DIV crude supernatant, this approach is much more labor-intensive
and unlikely to be used routinely.
3.4 MATERIALS AND METHODS

Ethics Statement

Non-mobilized PBMCs were isolated from fresh blood drawn from a de-identified healthy donor under Study #13-2115 approved by the University of North Carolina at Chapel Hill Institutional Review Board and Office of Human Research Ethics, with written informed consent provided. Mobilized peripheral blood mononuclear cells (PBMCs) were isolated from the blood of leukapheresed patients enrolled in Study #05-2860 approved by University of North Carolina at Chapel Hill Institutional Review Board and Office of Human Research Ethics after providing written informed consent. Samples were anonymized and provided as de-identified samples prior to use in the described studies. The University of North Carolina at Chapel Hill Office of Human Research Ethics determined that the use of the de-identified samples does not constitute human subjects research as defined under federal regulations [45 CFR 46.102 (d or f) and 21 CFR 56.102(c)(e)(l)] and does not require further Institutional Review Board approval.

PBMC isolation and cell culture

Due to the large number of cells required for this study, we mostly employed cryopreserved, primary mobilized PBMCs from two separate patients injected with G-CSF, which greatly increases the number of circulating leukocytes, for further studies (229). This established a large stock of monocytes isolated in one day that was capable of supplying months of experiments, reducing the inherent variability of human studies. We then verified key phenotypes with PBMCs from freshly-isolated, non-mobilized blood.

PBMCs were isolated from the blood using a 1.073 g/ml Ficoll-Hypaque Premium gradient (GE Healthcare) to enhance monocyte isolation. The manufacturer’s suggested protocol
was followed for buffy coat isolation. Negative isolation was done using the Dynabeads Untouched Human Monocytes kit (Invitrogen) by following manufacturer’s protocol. Antibodies against CD3, CD7, CD16 (specific for CD16a and CD16b), CD19, CD56, CDw123, and CD235a depleted cells expressing these markers. Mixed PBMCs and purified monocytes were cryopreserved in 90% heat-inactivated FBS (FBS-HI) with 10% DMSO. On experimental days, cells were thawed, washed twice, and placed at 37°C for 2 hours in PBMC cell culture media consisting of RPMI with 10% FBS-HI, 1% L-glutamine, 1% NEAA, 1% penicillin/streptomycin, and 30 units/ml DNase to prevent cell clumping from the release of DNA by dying granulocytes. Cells were counted and resuspended in PBMC medium without DNase for use in experiments.

The majority of experiments used DENV propagated in the Vero 76 cell line (ATCC CRL-1587). Confirmation of the inflammatory phenotype with an independent line of Vero cells was done using DENV propagated in the original Vero cell line, acquired from the UNC Lineberger Comprehensive Cancer Center Tissue Culture Facility (ATCC CCL-81). The Aedes albopictus C6/36 cell line was acquired from ATCC (CRL-1660).

**DENV-specific antibodies**

Crude supernatant of mAb D14G2 (4G2), a pan-flavivirus E-protein specific mouse IgG2a mAb, for use in IHC viral titrations was kindly provided by Dr. Mariano Garcia-Blanco of Duke University. Purified mAb 4G2 was generated by the UNC Antibody Core Facility. The isolation and purification of human mAbs 5G22 (α-prM used for enhancement and depletion), 2D22 (α-DENV-2 used for enhancement and neutralization), 6B22 (α-EDI/II used for depletion), 1C17 (α-EDIII used for enhancement and depletion), and 2H21 (α-prM used for immunoblots) were described in detail previously (207).
Virus stock growth

All experiments used DENV-2 strain 16681, kindly provided by Dr. Robert Tesh of UTMB-Galveston. Vero cells were cultured at 37°C with 5% CO₂ in MEM + 6% FBS-HI, 1% penicillin/streptomycin, and 20 mM HEPES buffer. To generate large stocks for experiments, near-confluent Vero cell culture monolayers were inoculated with DENV at an MOI of 0.5 in low-volume, low-serum conditions, placed in the incubator, and rocked every 15 minutes. At 2 hpi, culture medium was added. For viral stocks grown for purification, 1% FBS-HI was used so as not to clog the centrifugal filters during the concentration step. At days 3, 7, and 10, medium was collected from the flasks and centrifuged at 4,000 RPM for 10 minutes to clarify the supernatants. Infectious crude supernatants were then aliquoted into tubes for freezing at -70°C. Fresh medium replaced the collected supernatants. C6/36 cells were cultured at 29.5°C with 5% CO₂ in MEM + 10% FBS-HI, 1% NEAA, 1% penicillin/streptomycin, and 20 mM HEPES. Propagation of DENV in C6/36 cells was done as with Vero cells except for listed differences in medium composition.

We modified a previously described protocol to quantitate the infectious titer of viral stocks (206). Briefly, near-confluent Vero cell monolayers in flat-bottom, 96-well plates were inoculated with 50 μl of sequential 10-fold dilutions of DENV stocks. At 2 hpi, an overlay of 150 μl of 1.6% carboxymethylcellulose (diluted 1:1 in 2X MEM and supplemented with 1% FBS-HI, 10 mM HEPES and 1X antibiotics) was added to limit spread of virus. At 72 hpi, cells were fixed using a 1:1 mixture of acetone and methanol. Fixed cells were blocked with 2% normal horse serum in PBS and subsequently stained for 1 hour using mAb 4G2 as the primary antibody (1:500 dilution of crude supernatant into blocking solution) and 1:1000 goat anti-mouse
IgG-HRP (KPL) as the secondary antibody. Viral foci were visualized using Vector VIP Peroxidase Substrate kit (Vector Laboratories, Inc.) and counted under a dissecting microscope, with titers calculated as Vero focus forming units per ml (ffu/ml).

**Inoculation of PBMCs or monocytes**

One hour pre-inoculation, dilutions of enhancing antibodies or control medium were plated into 96-well, round-bottom, non-tissue-culture treated plates. Crude infectious supernatants or purified viral preparations of DENV-2 strain 16681 were diluted to the appropriate multiplicity of infection (MOI) and mixed with antibodies or control medium. DENV and antibody mixtures were incubated for 1 hour at 37°C with 5% CO₂ to allow immune complex formation. For mock infection wells, spent culture medium from uninfected Vero cell or C6/36 cultures was used instead of infectious supernatant. MEM with 1% FBS-HI, 1% penicillin/streptomycin, and 20 mM HEPES was used as the mock condition for purified virus. After the 1 hour incubation for complex formation, mixed PBMCs or purified primary monocytes were added. At 1-2 hours post-inoculation (hpi), cells were washed at least 2 times with PBS and resuspended in fresh culture medium. At collection, cells were resuspended and pelleted by centrifugation. After two washes with 1X PBS (four for genome copy studies), cells were fixed with Cytofix/Cytoperm (BD Biosciences) for 20 minutes at 4°C for flow cytometry, lysed with RLT Lysis Buffer (Qiagen) for RNA analysis, or lysed with 1X RIPA buffer (Boston BioProducts) with protease inhibitors (Roche) for western blot analysis. Monocyte supernatants were centrifuged to clear cells and debris, recollected, and stored at 4°C until analysis by ELISA. Unless otherwise noted, all monocyte inoculations were done with an MOI of 50.
Flow cytometry

Purified mAb 4G2 was conjugated to Alexa Fluor 647 using the Alexa 647 Protein Labeling Kit (Life Technologies) according to manufacturer’s instructions. After labeling, the labeled antibody was titrated by staining known DENV-positive monocytes to identify the best dilution for detection of DENV E protein without high levels of non-specific background in mock-inoculated cells.

For detection of DENV E protein, cells were washed twice with Perm/Wash buffer (BD Biosciences) after fixation and re-suspended with 1:400 final dilution of mAb 4G2 conjugated to Alexa Fluor 647 in Perm/Wash buffer for 30 minutes at 4°C. Cells were then washed twice with Perm/Wash buffer and finally re-suspended in 200 µl of Perm/Wash buffer for analysis on a Cyan ADP flow cytometer (Dako). Cells were initially gated on FSC-area vs. SSC-area, with single cells positively selected for by gating cells on the diagonal of FSC-height vs. FSC-area. Cells positive for DENV E-protein were detected on the APC channel, with positive gates set based on the mock-inoculated controls.

For staining of surface CD14 expression in conjunction with intracellular DENV E-protein expression, cells were washed twice with PBS and incubated at 4°C with Human Fc Receptor Binding Inhibitor in eFluor NC Flow Cytometry Staining Buffer (eBiosciences) supplemented with 2% FBS-HI for 15 minutes at 1 test per well. Cells were then stained with anti-human CD14 eFluor 605NC mouse mAb (eBiosciences) at 1 test size per well in 25 µl 1X staining buffer and incubated for 30 minutes in the dark at 4°C. Cells were washed twice with staining buffer, fixed, permeabilized, and stained with mAb 4G2 conjugated to Alexa Fluor 647, as described above. An unstained control and single-stained controls were set up for gating analysis. For compensation, antibodies were added to anti-mouse BD CompBeads at same
dilution as sample staining. Cells and beads were run on a LSRII flow cytometer (Becton Dickinson), using the APC channel for the anti-DENV antibody and QDot605 for the anti-CD14 antibody. Compensation calculations and analysis were done using FlowJo.

**Real-time PCR**

After lysis with RLT buffer, cell lysates were passed through QIAshredder columns (QIAGEN) to homogenize lysates. Homogenized lysates were then added to RNA isolation columns from RNeasy Mini kits (QIAGEN), and all steps were followed as detailed in the manufacturer’s protocol. RNA was eluted in a volume of 30 µl RNase/DNase free water. To generate cDNA, 1 µl Random Primers (3 µg/µl; Invitrogen) and 1 µl dNTP Mix (10 mM; Invitrogen) was added to 10 µl of eluted RNA and heated at 65°C for 5 minutes in a PTC-225 Peltier Thermal Cycler (MJ Research). Tubes were chilled on ice and 4 µl Invitrogen 5X First Strand Buffer, 2 µl Invitrogen 0.1M DTT, and 1 µl Promega RNasin RNase Inhibitor (40 units/µl) were added to each tube and incubated at 37°C for 2 minutes. Then 1 µl Invitrogen M-MLV Reverse Transcriptase (200 units/µl) was added per tube, and tubes were incubated at 25°C for 10 minutes and 37°C for 50 minutes, with an inactivation step of 70°C for 15 minutes.

For real-time PCR analysis, 9 µl cDNA was added to 1 µl 20X TaqMan Gene Expression Assay mix (Applied Biosystems [ABI]) and 10 µl of ABI 2X TaqMan Universal PCR Master Mix in a 384-well plate. Samples were pipetted in triplicate. Plates were run on an ABI 7900 HT Fast Real-Time PCR machine using the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, and 40 repeats of 95°C for 15 seconds followed by 60°C for 1 minute. Fold change values were calculated using the ΔΔCt method, normalized to a control value set at 1. Where applicable, all biological replicates (including their corresponding pipetting triplicates) were
combined and outliers greater than 2 standard deviations away from the mean were excluded. For all samples, 18s rRNA was used as the housekeeping-gene control.

The following ABI TaqMan Gene Expression Assays were used for real-time PCR analysis of gene expression: assay Hs01555410_m1 was used for IL1B, Hs00918082_m1 was used for NLRP3, Hs00368367_m1 was used for NLRC4, and Hs03928985_g1 was used for RN18S1. For DENV genome copies, a custom TaqMan Gene Expression Assay was designed using nucleotides 10635 – 10708, a portion of the 3’ UTR of DENV conserved between all 4 serotypes, identified and described by Gurukumar, et al. (230).

**Caspase-1 inhibition**

Z-WEHD-FMK Caspase-1 Inhibitor (R&D Systems) was diluted in sterile DMSO and cells were pre-treated with 80 μM Z-WEHD-FMK or DMSO vehicle 30 minutes prior to inoculation with DENV. Dilutions were made into cell culture medium.

**Bioinformatic analysis**

The publicly available BioGPS expression database was used for all bioinformatic analysis (231). The U133A, gcrma dataset was used for analysis of the following genes: CASP1 (probeset 206011_at), NLRP3 (probeset 207075_at), and IL1B (probeset 205067_at) (232).

**Gene knockdown**

Knockdown of NLRP3 was achieved using QIAGEN’s HiPerFect Transfection Reagent and FlexiTube GeneSolution GS114548 for NLRP3. QIAGEN’s AllStars Negative Control siRNA served as a negative control for NLRP3 knockdown. Briefly, based upon manufacturer’s
instructions, concentrations of both transfection reagent and siRNAs were optimized in a pilot experiment. Knockdown was analyzed by decrease of NLRP3 expression as analyzed by real-time PCR compared to control. For the experimental knockdown, 4 x 10^6 cells per condition were plated in 7 ml of growth medium in a 150 mm dish. 8 μl of each 10 mM stock of NLRP3 siRNAs was added to 1 total ml of serum-free RPMI (16 μl of 20 mM negative control siRNA). Then, 20 μl of HiPerFect transfection reagent was added to each tube, vortexed, and allowed to incubate at room temperature. After 10 minutes, complexes were dripped onto the cells, plates were swirled, and allowed to incubate for 24 hours. At 24 hours post-transfection, cells were collected, counted, and immediately used for infection assays.

**SDS-PAGE and immunoblots**

For protein studies to be analyzed by immunoblot, primary monocytes were lysed in the 96-well plates using RIPA Buffer (Boston BioProducts) containing 1X Complete Protease Inhibitor (Roche). Plates were rocked at 4°C for 20 minutes. Lysates were collected and centrifuged at 13,000 RPM for 10 minutes to clear debris. Lysates were recollected into fresh tubes. 4X NuPAGE LDS Sample Buffer (Life Technologies), containing 20 mg/ml DTT reducing reagent (Roche), was added to a final concentration of 1X. Samples were mixed and heated at 97°C for 5 minutes.

For viral protein analysis by immunoblot, NuPAGE LDS Sample Buffer without DTT was added directly to DENV-containing supernatant to a final concentration of 1X. Samples were vortexed and heated at 97°C for 10 minutes. DTT was added to samples at above concentrations for detection of DENV NS1 by immunoblot.
Samples were loaded in NuPAGE 4-12% Bis-Tris pre-cast gels (Life Technologies) and separated by SDS-PAGE using 1X NuPAGE MES SDS running buffer (Life Technologies). Gels were transferred onto 0.2 μm nitrocellulose membranes (Bio-Rad) under wet transfer conditions using 1X Transfer Blotting Buffer (Boston BioProducts) with 30% methanol for 45 minutes at a constant 100V. Membranes were blocked in a 10% milk solution in TBS-T for 1 hour at room temperature. Primary antibodies were incubated overnight at 4°C with rotation at the following dilutions in blocking buffer: 1:1,000 rabbit polyclonal antibody anti-IL-1β (Santa Cruz sc-7884), 1 μg/ml purified mouse mAb 4G2 to detect DENV E protein, 1 μg/ml human mAb 2H21 to detect DENV prM, 1:1000 goat anti-human Fc conjugated to HRP (Bethyl Laboratories) to detect human IgG Fc, or 1:1000 rabbit anti-DENV NS1 polyclonal antibody (Genetex GTX103346). After 4 washes with TBS-T, the membranes were incubated with the following secondary antibodies diluted into blocking buffer for 2 hours at room temperature: 1:5000 goat anti-rabbit HRP-conjugate (Santa Cruz) for IL-1β and NS1, 1:5000 goat anti-mouse HRP-conjugate (Santa Cruz) for DENV E protein, or 1:5000 goat anti-human HRP conjugate (Bethyl) for DENV prM. Human IgG in depletion studies was detected directly after primary antibody incubation because of HRP conjugate. Membranes were then washed 4-5 times with TBS-T and subsequently developed after 5 minute incubations with Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate.

**Depletion of antibody-bound virions and NS1**

Mock and DIV crude supernatants were incubated with indicated concentrations of antibodies (or equal volume of PBS control) for 1 hour on ice. Subsequently, either 50 μl of pre-washed Dynabeads Protein G beads (Life Technologies) or equal volume of PBS were added to
samples, and placed on a rotator at 4°C for 1 hour. Tubes were then placed onto a DynaMag-2 magnet for 1 minute, and bead-free supernatants were collected and transferred to a new tube. Placement on the magnet with collection of the supernatant was repeated two more times to ensure complete removal of beads. The bead-free supernatants were considered the residual fractions, while the resuspended beads (for protein analysis only) were considered the bead-bound fractions. Tubes that had PBS added in lieu of beads were considered the control fractions, as equal volumes were maintained but no antigens were depleted. Depletion of NS1 was done identically by using mouse anti-NS1 monoclonal antibody clone DN1 (Abcam #ab41490) to immunoprecipitate NS1 with protein G beads. Monoclonal antibody DN1 was washed through an Amicon filter to remove preservatives, resuspended in PBS at a 6-fold increase in concentration, and then syringe filtered to purify. This was then used at a final dilution of 1:2 for successful depletion of NS1.

**DNase and RNase treatment of viral supernatant**

Mock and DIV crude supernatants were incubated with 30 units/ml of amplification grade DNase I (Life Technologies), 6 units/ml RiboShredder RNase Blend (Epicentre), or an equal volume of PBS. Samples were incubated for 20 minutes at room temperature for DNase activity, 20 minutes at 37°C for RNase activity, and finally 12 minutes at 65°C followed by 2 minutes at 97°C to ensure inactivation of the enzymes prior to inoculation onto cells.

**Virus purification**

To purify virus, a previously described protocol was used (221). Briefly, crude supernatants from DENV-infected Vero cells were concentrated by centrifugation at 1500 x g for
20 – 25 minutes in 15 ml Millipore Amicon Centrifugal Filter Units with a 100-kDa cutoff, allowing components < 100 kDa to pass through but not virus. The final concentrated volume was approximately 2 ml, which was gently layered on top of an 8-ml 10% – 40% continuous sucrose gradient in an ultracentrifuge tube. The gradient was centrifuged in a Beckman Coulter Optima L-90K Ultracentrifuge using rotor SW40TI for 2.5 hours at 35,000 RPM and 4°C with no brake. Sequential 0.5 ml fractions were collected from the bottom of the ultracentrifuge tube into microfuge tubes. Fractions 4 – 8 were pooled and washed to remove sucrose using another 15 ml Amicon tube. After 2 washes, the virus was resuspended in MEM with 1% FBS-HI, 20 mM HEPES, and 1% penicillin/streptomycin. The pooled fractions were then titered as described earlier.

**Virus inactivation**

DIV crude supernatant was inactivated using formalin or shortwave UV exposure. For formalin inactivation, 0.25% formalin (final concentration) was added to an aliquot of virus stock and allowed to incubate at room temperature for 2.5 hours. For shortwave UV inactivation, 100 μl/well of virus stock was added to each well of a 24-well plate, placed on ice, and exposed to shortwave UVB (254 nm) irradiation for 2 min at a distance of approximately 5 cm. After inactivation, the inactivated supernatants, and an equal volume of live virus, were added into separate 15 ml Amicon tubes and washed twice using fresh medium to remove formalin (or as control for sample loss for supernatants not treated with formalin). After washing, the supernatants were resuspended in fresh culture medium to equal volumes and inoculated onto monocytes.
Removal of DENV by filtration

Whatman Anotop 10 syringe filters (0.02 μm) were attached to 5-ml Luer-Lok syringes that had plungers already removed. DIV crude supernatant grown in 1% FBS-HI conditions was carefully pipetted into the top of the syringe in 0.4-ml aliquots at a time (due to filter clogging). The plunger was re-inserted and care was taken to apply slow, steady pressure to the plunger, not allowing the plunger to move in the reverse direction. Supernatants were filtered 4 total times.

Cytokine detection by ELISA

Detection of human IL-1β in the supernatant was done using BD Biosciences’ BD OptEIA Human IL-1beta ELISA Set II. The kit was followed as per manufacturer’s instructions, and the absorbance at 450 nm was read on a PerkinElmer EnSpire Multimode Reader 2300. Wavelength correction at 570 nm was used.

Illustration

Schematic illustration of immunodepletion strategy was generated by modifying images purchased in the PPT Drawing Toolkits - BIOLOGY Bundle from Motifolio, Inc.

Data presentation and statistical analysis

Graphs are presented as mean ± SEM of 3 or more biological replicates, unless otherwise noted. All statistical analyses were done with 3 or more replicate values per group. Student’s unpaired, two-tailed t tests were used when making comparison between only 2 means. One-way ANOVAs were employed to compare the means of 3 or more groups within one independent variable. Two-way ANOVAs were used for comparisons between two independent variables.
(e.g. viral treatment vs. antibody treatment). Asterisks for comparisons done by One-Way or Two-Way ANOVA represent the multiplicity-adjusted p values from multiple comparisons tests. Multiple comparisons tests were selected as follows: Dunnett’s post-test for comparing all means to a single control value, Bonferroni’s post-test for comparing only means within one independent variable, and Tukey’s post-test for comparing all means for all independent variables. Choice of post-test was dependent on desired comparisons and is indicated in figure legends. Analyses were done using GraphPad Prism 6.0. Statistical outliers (p < 0.05) were excluded from graphs and analysis using Grubbs’ test on the GraphPad website. For all figures, statistical significance was defined as p < 0.05 (*), but all comparisons were also tested for p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****). “n.s.” = not significant.
FIGURE 3.1 – DIV crude supernatant induces IL-1β secretion independent of ADE
A. Flow-cytometric histogram overlay comparing CD14 expression levels in cells negative or positive for intracellular DENV E protein at 24 hpi. B. Cumulative percentages of mobilized monocytes positive for intracellular DENV E protein at 24 hpi with DIV crude supernatant in the presence of increasing concentrations of mAb 5G22. C. Measurement of secreted IL-1β by ELISA using 24-hpi supernatants from 1B. D. Repeat of 1B using fresh, non-mobilized monocytes (one value per point). E. Measurement of secreted IL-1β using supernatants from 1D (one value per point). F. Measurement of DENV E-protein expression in mobilized monocytes at 24 hpi. Cells were inoculated with increasing doses of DIV crude supernatant with or without 1 μg/ml mAb 5G22. G. Measurement of secreted IL-1β using supernatants from 1F. H. Left: Flow-cytometric histograms of 24-hpi DENV E-protein expression in mobilized monocytes after inoculation with mock supernatant, DIV crude supernatant alone, or DIV crude supernatant

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that was pre-incubated with 0.1 μg/ml mAb 2D22 (enhancing), 2 μg/ml mAb 2D22 (neutralizing), or 2 μg/ml mAb 1C17 (enhancing). Right: Measurement of secreted IL-1β at 24 hpi for corresponding samples. I. Measurement of intracellular DENV E-protein at 24 hpi in mobilized monocytes that were incubated with PBS or Fc-receptor binding inhibitor prior to inoculation with DIV crude supernatant. J. Secreted IL-1β at 24 hpi using supernatants from I. K. Secreted IL-1β by mobilized monocytes at 24 hpi with mock supernatant or DIV crude supernatant derived from a second line of Vero cells. For all figures: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001. Tests used: One-Way ANOVA (within DENV treatment) with Tukey’s post-test (H), Two-Way ANOVA with Bonferroni’s post-test (I and J), Two-Way ANOVA with Tukey’s post-test (K). “n.s.” = not significant (p > 0.05).
FIGURE 3.2 – DIV crude supernatant induces rapid IL-1β secretion independent of viral replication

A–D. Time course of mobilized monocytes inoculated with mock supernatant or DIV crude supernatant with mAb 5G22. Cells were washed at 1 hpi and resuspended in fresh medium. Samples were collected at 2, 8, 16, and 24 hpi. A. Secreted IL-1β. B. Intracellular DENV E-protein expression. C. Infectious virus present in the supernatant, as measured by immunoassay on Vero cells. D. Relative expression of DENV genome copies in mobilized monocytes, measured by real-time PCR. Values are normalized to 2 hpi sample. For A–D, data are pooled from two independent experiments. E. Secreted IL-1β by mobilized monocytes at 4 hpi. F. Secreted IL-1β by fresh, non-mobilized monocytes at 4 hpi. G. Intracellular DENV E-protein expression at 24 hpi with live DIV crude supernatant or DIV crude supernatant inactivated with formalin or UV exposure, all in the presence of mAb 5G22. H. Secreted IL-1β at 24 hpi using supernatants from 2G. Tests used: Two-Way ANOVA with Bonferroni’s post-test (A, B), One-Way ANOVA with Dunnett’s post-test (C, D, G, H), Two-Way ANOVA with Tukey’s post-test (E, F).
FIGURE 3.3 – Pro-IL-1β expression induced by DIV crude supernatant requires NLRP3 and caspase-1 for secretion

A. Bioinformatic analysis of CASP1, NLRP3, and IL1B gene expression in various human immune cells using BioGPS datasets. Data is mean ± SD (2 values each) of the measurements for each cell type. Dashed line represents mean of all measured tissues for probe sets listed in Materials and Methods.

B. Real-time PCR measurement of relative IL1B expression by mobilized monocytes that were lysed at indicated time points after inoculation with DIV crude supernatant. Data (mean ± SD of 2 values per condition) are normalized to 2 hpi mock samples.

C. Immunoblot assessing intracellular pro-IL-1β (31 kDa) and mature IL-1β (17 kDa) in 3 hpi lysates of mobilized monocytes after inoculation with mock supernatant or DIV crude supernatant.

D. Secreted IL-1β by mobilized monocytes at 5 hpi with DIV crude supernatant. Cells were pre-treated with DMSO vehicle or 80 μM of Z-WEHD-FMK caspase-1 inhibitor for 30 minutes prior to inoculation.

E. Assessment of NLRP3 (target) and NLRC4 (control) expression by real-time PCR, 24 hours after mobilized monocytes were transfected with a control siRNA or an siRNA targeting NLRP3.

F. Secreted IL-1β by knockdown and control cells described in 3E at 6 hpi with mock supernatant or DIV crude supernatant in the presence of 1 μg/ml 5G22. Tests used: Two-Way ANOVA with Tukey’s post-test (D), Two-Way ANOVA with Bonferroni’s post-test (F).
FIGURE 3.4 – Inflammatory component produced by Vero cells during DENV propagation is responsible for IL-1β induction

A. Schematic depicting the different depletion and control fractions for depleting antibody-bound virions from DIV crude supernatant by using magnetic protein G beads. B. Immunoblot for human IgG Fc, and two DENV structural proteins, E protein and prM, to assess efficiency of antibody-mediated DENV depletion from DIV crude supernatant. “***” denotes all 3 antibodies were used in combination at 1 μg/ml each. 3 μg/ml of each antibody was used for individual antibody conditions. C. Control supernatants (Lane C) and residual supernatants (Lane R) from panel B were inoculated onto mobilized monocytes. Monocyte supernatants were collected at 24 hpi, and secreted IL-1β was measured by ELISA. D. Mock and DIV crude supernatants were incubated with PBS or anti-DENV NS1 antibody prior to addition of protein G beads and subsequent depletion. Top: Residual supernatants (R) and bead-bound fractions (B) were assessed for efficiency of NS1 depletion by immunoblot. Bottom: Residual supernatants were inoculated onto mobilized monocytes. At 4 hpi, monocyte supernatants were collected and assessed for secreted IL-1β. E. Mock and DIV crude supernatants were incubated with PBS, DNase, or Riboshredder prior to inoculation onto mobilized monocytes. At 4 hpi, monocyte
supernatants were collected and assessed for secreted IL-1β. F. Mobilized monocytes were inoculated with mock supernatant, live DIV crude supernatant, or DIV crude supernatant that had been incubated for 30 minutes at 56°C to heat inactivate (HI) it. At 24 hpi, monocyte supernatants were collected and assessed for secreted IL-1β. G. DIV crude supernatant was centrifuged in an Amicon centrifugal filtration unit with a 100-kDa molecular-weight cutoff to generate fractions smaller and larger than 100 kDa. Fractions were brought to equal volumes and inoculated onto mobilized monocytes. At 24 hpi, monocyte supernatants were collected, and IL-1β secretion was measured. Tests used: Two-Way ANOVA with Bonferroni’s post-test (D), One-Way ANOVA with Dunnett’s post-test (F).
FIGURE 3.5 – ADE elevates IL-1β secretion induced by purified Vero-derived DENV and crude infectious C6/36 supernatant

A. Mobilized monocytes were inoculated with mock conditions or purified DENV with or without the addition of mAb 5G22. Intracellular DENV E-protein expression was measured at 24 hpi. B. Secreted IL-1β detected in 24-hpi monocyte supernatants from 5A. C. DIV crude supernatants and purified Vero-derived DENV preparations were passed through a 0.2-μm syringe filter or 4 times through a 0.02-μm syringe filter. Filtrates were assessed by immunoblot for DENV E protein (top) and immunoassay on Vero cells for infectious virus (bottom, bars aligned with immunoblot). ND = no detection. D. Filtrates were incubated with 1 μg/ml mAb 5G22 and inoculated onto mobilized monocytes. At 24 hpi, cells were fixed and analyzed for intracellular DENV E-protein expression. E. Secreted IL-1β detected in 24-hpi monocyte supernatants from 5D. F. Mobilized monocytes were inoculated with control supernatant from uninfected C6/36 cells or infectious supernatant derived from DENV-infected C6/36 cells, with or without 1 μg/ml mAb 5G22. Intracellular DENV E-protein expression was analyzed at 24 hpi. G. Secreted IL-1β detected in 24-hpi monocyte supernatants from 5F. Tests used: Two-Way ANOVA with Bonferroni’s post-test (B, G), Individual two-tailed t tests (E).
CHAPTER 4: DISCUSSION AND FURTHER DIRECTIONS

4.1 INTRODUCTION

This chapter will briefly summarize the major findings and discuss the future directions for the two projects described in this work. Chapter 2 will be discussed in sections 4.2 and 4.3. Chapter 3 will be discussed in sections 4.4 and 4.5.

4.2 SUMMARY OF FINDINGS – SYK MEDIATES ADE-INDUCED CYTOKINE EXPRESSION

In my studies, we have identified that ADE of DENV infection induces proinflammatory cytokine expression and IL-1β secretion within 4 hours of replication. Interestingly, this was independent of the enhanced replication induced by ADE. Instead, DENV immune complexes activate a pathway previously identified in other immune complex studies (Fig. 4.1) (193-197). This involved the activation of Syk and ERK1/2, and the importance of both kinases for the pathway was confirmed by pharmacologic inhibition.

Inhibition of Syk did not completely ablate inflammatory cytokine induction by DENV immune complexes. Instead, cytokine induction decreased to levels nearly equivalent with those induced by free DENV. This suggests that antibody-bound virions maintain signaling capabilities independent of the FcγRs. Additionally, inhibition of Syk did not reduce the number of cells positive for viral replication, indicating that Syk is dispensable for endocytosis and fusion of DENV immune complexes. Thus, Syk inhibition may be a promising target to interfere with ADE-induced cytokine production in primary monocytes, but interfering with Syk alone cannot
prevent ADE-mediated entry of DENV or eliminate inflammation induced by free DENV. Syk inhibition may prove to be most beneficial in conjunction with another therapeutic.

4.3 FURTHER QUESTIONS – SYK MEDIATES ADE-INDUCED CYTOKINE EXPRESSION

Does the choice of anti-DENV antibody used to enhance infection impact Syk activation?

Expanding the study of Syk activation by using additional enhancing antibodies will be important to gain more insight into stimulation of this pathway by ADE. Anti-DENV antibodies can target specific epitopes on prM, EDI/II, EDIII, or even epitopes that are only present on an intact virion (12). Some virions have no prM because of complete cleavage, while many virions maintain some or all of their prM expression (22). This alone indicates that immune complexes can be formed with a varying number of antibodies bound depending on the availability of each specific epitope. As recently identified, the signaling phenotype after FcγR cross-linking is dramatically impacted by the extent of cross-linking and receptor aggregation (146). Could enhancing antibodies that target epitopes with low availability contribute to intrinsic ADE? The lower number of antibodies bound could lead to less extensive receptor aggregation than enhancing antibodies bound to more highly-expressed epitopes.

Additionally, a consideration should be given to the subclass of IgG used. IgG1 is the most abundant antibody in human serum (233), but humans can form anti-DENV antibodies of all 4 IgG subclasses (234). As each FcγR has varying affinity for the different IgG subclasses, could IgG subclass impact the strength of Syk activation induced by ADE? Low-affinity IgG-FcγR interactions by DENV immune complexes may induce differential Syk activation compared to high-affinity IgG-FcγR interactions. An interesting way to address this question
would be to make a chimeric antibody that only varied the Fc regions. Thus, ADE using the same epitope target could be assessed with IgG1, IgG2, IgG3, and IgG4 Fc regions.

**Does antibody-mediated neutralization of DENV activate the same signaling pathway as ADE?**

In theory, neutralization of DENV with antibody should activate Syk in a manner similar to ADE, as it is the cross-linking of the FcγRs that activates Syk. However, the situation is likely to be more complex. A virion bound for neutralization will be thoroughly coated with antibody, while a virion bound for enhancement has fewer antibodies attached. This could impact the degree of FcγR aggregation, thereby altering signaling.

It will be interesting to determine how cytokine expression changes when DENV is coated with neutralizing antibodies at a concentration that leads to neutralization. What could be the consequences of monocytes neutralizing virus but still producing cytokines in response? Is elevated inflammatory cytokine expression by monocytes only detrimental when they are also producing virus?

**How does ADE impact inflammasome activation at later time points?**

Coming into these studies, we needed to compare an interesting dichotomy. DENV reportedly activates the inflammasome in primary human monocytes 4 days after infection (98). However, it has been known for years that monocytes secrete IL-1β within 4 hours of infection (95). How could this be? Thus, we focused at early time points to assess both pro-IL-1β synthesis and processing. In the early hours following infection, ADE was only able to induce elevated pro-IL-1β synthesis and not activate caspase-1. However, as described for monocytes
(169-173), a small portion of pro-IL-1β was processed without detectable increases in caspase-1 activation.

It will be interesting to extend caspase-1 assessments to later time points. Based on the reports, caspase-1 activation should occur at some point. At that time point, an important question will be to see how ADE modulates the caspase-1 activation. Will Syk activation increase inflammasome activation? Or will the proposed phenomena of intrinsic ADE and ITAMi suppress caspase-1 activation?

**How does Syk activation impact intrinsic ADE?**

There is increasing evidence that ADE suppresses the antiviral response to increase DENV replication (106,129,235). This includes dephosphorylation of STAT1 to impair IFN signaling. SHP-1, a phosphatase known to be activated by Syk, dephosphorylates STAT1 to inhibit its activation and is implicated in ITAMi signaling downstream of FcγR activation (142,143,146,149). If some of the phenotypes described in intrinsic ADE studies can be replicated in the system described here, Syk inhibition should be tested to see if intrinsic ADE can be reversed. There are Syk inhibitors currently in use in clinical trials for cancers and autoimmune conditions. Through a collaboration with a pharmaceutical company, we will be able to test Syk inhibition under ADE conditions using an inhibitor approved for clinical trials in humans.

An extension of this question is the need to reconcile the contrasting roles described for Syk in ADE. It has been reported that DENV immune complexes must inhibit Syk through co-ligation of the inhibitory receptor LILRB1 in order to induce ADE (204). However, much of the evidence implicating Syk in this pathway is based upon inhibition of Syk with piceatannol, an
inhibitor that is not very selective for Syk (236). Additionally, in the Chan, et al., study, all of the mechanistic findings were elucidated using THP-1 cells and not primary monocytes (204). Further investigation is clearly required, as inhibition of Syk in my studies with BAY 61-3606, a highly selective Syk inhibitor at the concentrations used, did not impact viral replication (188).

4.4 SUMMARY OF FINDINGS – VERO CELLS PRODUCE INFLAMMATORY MOIETY

Additionally, we were surprised to find strikingly different phenotypes depending upon the host cell used to generate stocks of DENV for experimentation. Many different cell lines are highly susceptible to DENV infection, both mammalian and insect. Often, the choice of cell line for DENV propagation may be based on convenience rather than scientific reasoning, such as availability of the cell line in the lab or incubator settings amenable to other cells in use. Vero cells were initially employed to propagate DENV in my studies because no 30°C incubator was available for mosquito cells. Further, the use of infectious supernatant harvested from infected cells as an inoculum is also common practice, as purification of virus stocks is labor intensive and decreases virus yield.

It was unexpected that inoculating monocytes with infectious supernatants from Vero cells would yield such a different phenotype than infectious supernatants from mosquito cells. It took much experimentation to realize that Vero cells were producing an inflammatory factor during DENV propagation. The striking differences in DENV replication in primary monocytes, virtually no replication with DENV alone versus high infection with DENV complexed to enhancing antibody, were key. Without ADE, it would have been very easy to assume that the cytokine induction induced by infectious Vero supernatant was DENV-specific.
The fact that ADE so drastically increased replication without altering cytokine production seemed unlikely and set us on a course that identified the masking phenotype. Purification of DENV derived from Vero cells eliminated this masking phenotype and confirmed that ADE does induce elevated IL-1β secretion in primary human monocytes. In stark contrast, infectious supernatant derived from mosquito cells had no such masking phenotype and could be used without purification. This is troubling, identifying that the host cell can produce factors that induce inflammatory cytokine expression but that this is cell-type specific. Thus, such a masking phenotype should be suspected in the face of unexpected results, and should perhaps be universally tested to be certain.

4.5 FURTHER QUESTIONS – VERO CELLS PRODUCE INFLAMMATORY MOIETY

What component of infectious Vero supernatant induces this masking phenotype?

Still unclear to this stage is whether the inflammatory moiety present in infectious supernatants derived from Vero cells is an artifact of Vero-cell infection or a physiologically relevant molecule. We tested many likely possibilities, such as free nucleic acids, secreted NS1, cytokines, and other components smaller than 100 kDa. However, we were only able to eliminate possibilities, not positively identify the factor.

It is known that Vero cells undergo a higher level of apoptosis than mosquito cells after DENV infection (228). Thus, supernatant components derived from increased apoptosis could induce higher cytokine production. Though apoptosis is an orderly cell death process in which the cell membrane remains intact, apoptotic cells eventually undergo secondary necrosis (237). During secondary necrosis, cell membrane viability is lost and many intracellular contents are
released. Could this be the cause of the masking phenotype induced by infectious Vero supernatants? And, if so, why is this not universally seen in the literature?

One potential explanation could be the wide varieties of DENV strains used, even within each serotype. The genetic differences between strains may change induction of cell death. More importantly, perhaps, is the fact that DENV mutates and evolves with serial passaging (238,239). My studies utilize DENV-2 16681, a clinical isolate from the serum of a severe patient in 1964 (240). Many other studies also utilize DENV-2 16681. However, without publishing sequence information for each passage, it would be impossible to know how genetically different the strains are between labs. Based on total passage numbers, and the cell-type used for passaging, DENV-2 16681 in one lab could be quite different from DENV-2 16681 in another lab.

A potential solution would be to take a lab-passaged strain from the parental isolate and compare its phenotype with the infectious clone. If adaptations from serial passaging differentially modulate the phenotypes induced by the virus, this would confound comparisons of studies between labs. Such a finding would be concerning but would be a critical detail to know.

What is the best way to eliminate the inflammatory moiety?

Ideally, studies assessing the immune response to DENV would utilize only purified preparations of virus in lieu of infectious crude supernatants. However, this is not universally feasible. For labs without access to an ultracentrifuge, purifying the virus in a sucrose gradient would be impossible, as the maximum speeds of a standard centrifuge are far too slow. Even with access to an ultracentrifuge, the process is time consuming, labor intensive, decreases total virus yield, and is technically challenging. These factors would likely yield varying degrees of success across different labs.
An important caveat to purification done in this way is that the preparation is not truly pure. The collected fractions are greatly enriched for DENV, but the co-isolation of subcellular membranous vesicles cannot be excluded. In density gradient ultracentrifugation, within a given set of experimental methods, the velocity travelled by any membranous vesicle through the gradient will be determined primarily by radius and density (241). A particle with a large diameter and high density travels fastest through the gradient but comes to rest upon reaching a gradient density equal to its own. Thus, centrifugation time is a critical factor. Given enough time, a small particle with a given density will eventually reach the same fraction as a larger particle with an equivalent density.

Exosomes are membranous particles released from cells that are often similar in size and density to virus particles, making it difficult to separate virions and exosomes with sucrose gradients (242,243). Though other gradients may provide greater discrimination, this further goes to prove that gradient centrifugation is not purification. Additionally, pelleting virus by ultracentrifugation would be confounded by similar challenges.

Thus, a true purification of DENV virions would be best achieved through affinity purification. As shown in my studies, DENV can be specifically depleted from infectious supernatants using anti-DENV antibodies and protein G beads. This procedure is not technically challenging and takes only 2–3 hours from start to finish. For my purposes, the immunoprecipitated virus was only needed for measurements of viral protein. Thus, it was not important to elute the virions from the antibodies in a way that would preserve virion structure and viability.

However, could such a procedure be adapted? Could the immunoprecipitated virions be eluted from the beads and antibodies without harming the virions? Many commercial
immunoprecipitation kits use low pH (less than 3) conditions to elute antigens from antibodies. However, exposure of a chimeric flavivirus to pH 5.8 for just 10 minutes at room temperature completely inactivated it due to structural rearrangements (244). Thus, acidic conditions are completely incompatible with flavivirus viability.

Eluting with high ionic strength may work. Physiological NaCl concentration is 150 mM, and West Nile virus infectivity was maintained at 1 M NaCl (245). Antibodies can be covalently cross-linked to many beads used for immunoprecipitation. Thus, a titration experiment could be done to determine if an ionic strength can be achieved that can dissociate DENV from the antibody without affecting virion antigenicity and viability. In such a scenario, only DENV virions would be eluted from the complex, and the virus could then be buffer-exchanged into an isotonic solution. The resulting virus stock would be highly pure, but the procedure would not be technically challenging once optimal conditions were identified.

4.6 CONCLUDING REMARKS

My dissertation research originally sought to focus specifically on the impact of ADE on inflammasome activation in primary human monocytes. However, this eventually evolved into the identification of an important pathway that may have far greater consequences than solely IL-1β production, as severe dengue patients suffer from a dysregulation of a large number of cytokines. Thus, my dissertation studies took both an interesting and important direction. Though there are no antivirals for DENV, the signaling pathway involving Syk and ERK should be investigated as a potential target in severe dengue disease. Additionally, Syk may be the common link between ITAMi studies and intrinsic ADE, placing increased importance on further investigating the actions of Syk during ADE.
In the second part of my studies, I believe that the identification of an inflammatory supernatant component that can mask the DENV-specific immune response is important. It serves as a cautionary tale that often is not considered. A major drawback to the study of viruses is the requirement for host cells for viral propagation. It can be easy to forget that these living cells view infection as an insult and produce far more than virions. More troubling, as many viruses infect a wide variety of cells, the components produced in conjunction with the virions could vary substantially. This is in stark contrast to the growth of bacteria in broths or the use of purified immune agonists that are reconstituted in the suitable buffer of choice. Viral immunologists must not lose sight of the inherent complications of working with virus.
FIGURE 4.1 – Working model of ADE-induced pro-inflammatory cytokine production in primary human monocytes

Inoculation of primary human monocytes with unbound DENV activates NF-κB through an undetermined pathway. Inoculation of primary human monocytes with DENV immune complexes activates Syk, which is known to be phosphorylated after its recruitment to phosphorylated FcγR ITAMs. ERK is activated through phosphorylation downstream of Syk, and ERK has been shown to mediate NF-κB activation induced by immune complexes. Both pathways appear to converge at NF-κB, as Syk inhibition diminishes elevated NF-κB activation induced by ADE. Both infection routes induce elevated expression of pro-inflammatory cytokine genes *TNF*, *IL1B*, and *IL6*. Correspondingly, both infection routes induce elevated synthesis of pro-IL-1β (signal 1) by 2 hpi. However, neither infection route induces elevated caspase-1 activation (signal 2) in the early hours post-inoculation. DENV-induced pro-IL-1β is inefficiently processed by a low level of caspase-1 activity that is dependent upon Syk through an
undetermined mechanism. IL-1β processing can be significantly enhanced by providing an exogenous signal 2 to activate the NLRP3 inflammasome, such as ATP. Recent reports indicate that Syk is required to license the NLRP3 inflammasome by phosphorylating ASC, but how this relates to the current system is undetermined.
REFERENCES


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