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Maturation of dengue virus nonstructural protein 4B in monocytes enhances production of dengue hemorrhagic fever-associated chemokines and cytokines

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ABSTRACT

High levels of viremia and chemokines and cytokines underlie the progression of severe dengue disease. Dengue virus (DENV) preferentially infects peripheral blood monocytes, which secrete elevated levels of immunomediators in patients with severe disease. Further, DENV nonstructural proteins (NS) are capable of modifying intracellular signaling, including interferon inhibition. We demonstrate that peak secretions of immunomediators such as IL-6, IL-8, IP-10, TNFα or IFNγ in DENV-infected monocytes correlate with maximum virus production and NS4B and NS5 are primarily responsible for the induction of immunomediators. Furthermore, we demonstrate that sequential NS4AB processing initiated by the viral protease NS2B3 (pro) and via the intermediate 2KNS4B significantly enhances immunomediator induction. While the 2K-signal peptide is not essential for immunomediator induction, it plays a synergistic role with NS4B. These data suggest that NS4B maturation is important during innate immune signaling in DENV-infected monocytes. Given similar NS4B topologies and polyprotein processing across flaviviruses, NS4B may be an attractive target for developing *Flavivirus*-wide therapeutic interventions.

Introduction

Dengue virus (DENV) causes considerable risk to human health worldwide infecting an estimated 50–100 million people annually and causing explosive outbreaks with infection rates as high as 80–90% among individuals previously unexposed to the virus (World Health Organization, 1997). The incidence of dengue diseases has dramatically increased during the past two decades as the result of an expanding geographical distribution of the *Aedes* mosquito vector and increased human travel (Gubler, 2002; Gubler, 2006; World Health Organization, 1997). Over 90% of DENV infections are asymptomatic or result in self-limiting dengue fever (DF) cases that resolve without complications (Guzman and Isturiz, 2010; Guzman and Kouri, 2002). However, a subset of infected patients progresses to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), resulting in over a half-million hospitalizations each year worldwide (Gubler, 1998; World Health Organization, 2009). No definitive mechanisms explain the progression of DHF/DSS, which can be defined in part as bleeding and increased plasma leakage into the pleural cavities and peripheral tissue without morphological damage to the capillary endothelium (Chang et al., 1990; Srikiatkhachorn et al., 2007). Clinical studies indicate that patients who progress to severe disease demonstrate elevated viremia (Libraty et al., 2002; Murgue et al., 2000; Vaughn et al., 2000) and high levels of interleukin (IL)-6, IL-8 and tumor necrosis factor alpha (TNFα) in the bloodstream (Hober et al., 1993; Huang et al., 2000; Juffrie et al., 2001; Nguyen et al., 2004; Priyadarshini et al., 2010; Raghupathy et al., 1998). Similarly, peripheral blood monocytes from patients with DHF/DSS display elevated DENV antigen and increased expression of activation markers and production of immunomediators (Durbin et al., 2008; Halstead, 1989; Kou et al., 2008), implicating monocytes as important cells during infection and severe disease pathogenesis. Moreover, DENV-infected primary monocytes secrete DHF/DSS-associated immunomediators (Bosch et al., 2002; Chen and Wang, 2002).

DENV belongs to the family Flaviviridae and consists of four genetically distinct serotypes having a positive-sense, single-stranded RNA genome of approximately 11 kilobases (kb) in length. The RNA encodes for a polyprotein precursor that is co- and post-translationally processed into three structural (C, PrM and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Bell et al., 1985; Chambers et al., 1990). The NS are responsible for various enzymatic activities during replication, including the NS5 RNA-dependent RNA polymerase (RdRP) and methyltransferase activity required for viral RNA capping (Chung et al., 2010; Dong et al., 2010; Geiss et al., 2009; Selisko et al., 2010; Zou et al., 2011), the NS3 helicase and the NS2B3 protease (NS2B3pro) (Cahour et al., 1992; Falgout et al.,

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1991; Padmanabhan et al., 2006). Several NS, such as NS3, NS4B and NS5, interact as part of the viral replication complex facilitating transcription and translation of the viral genome (Harris et al., 2006; Khromykh et al., 1999a; Khromykh et al., 1999b; Mackenzie et al., 2007; Roosendaal et al., 2006; Westaway et al., 1997a; Westaway et al., 1997b). Further, accumulating evidence suggests that intrinsic DENV genetic characteristics within NS4B and NS5 are associated with severe disease outcomes (Leitmeyer et al., 1999).

DENV NS5 induces IL-8 transcription and protein secretion in human embryonic kidney cells, (Medin et al., 2005) and inhibits the interferon alpha (IFNα) response through binding and degradation of STAT2 (Ashour et al., 2009; Mazzon et al., 2009). Also, NS4B strongly inhibits the IFN transduction cascade by interfering with STAT1 phosphorylation (Munoz-Jordan et al., 2003); and sequential processing of NS4AB by viral and host proteases is required to initiate an IFN antagonistic function (Munoz-Jordan et al., 2005). Nonstructural protein-induced subversion of the host IFN response and induction of immunomediators may simultaneously promote DENV survival while increasing the risk of severe disease outcomes. Based on the aforementioned data, to further understand the role of NS in dengue immunopathogenesis, we hypothesized that NS5 and maturation of NS4B expressed in monocytes would induce DHF-associated immunomediators. In this report, we demonstrate that both NS5 and NS4B induce immunomediators and that NS4B maturation via cleavage of the NS4AB polypeptide, in a 2KNS4B-dependent manner, significantly enhanced immunomediator production in monocytes.

Results

Elevated secretion of immunomediators from DENV-infected THP-1 monocytes corresponds with peak viral titers and copy numbers

To establish whether DENV-infected monocytes secrete DHF-associated immunomediators, we infected THP-1 cells with DENV-2 New Guinea (NGC) strain and collected cells and culture supernatants each day for five consecutive days. Plaque assay and quantitative real-time polymerase chain reaction (qRT-PCR) data demonstrated that peak viral titers and copy numbers for both MOI-0.1 and -1 occurred on day 3 after infection; however, infection with MOI-1 resulted in approximately log 1.4 higher titer and log 0.5 higher viral RNA transcripts than infection with an MOI-0.1 on day 3 after infection (Fig. 1A). We determined by qRT-PCR that the peak induction of IL-8 and TNFα transcripts occurred at day 3 after infection (Supplemental Fig. 1); therefore, we assayed the THP-1 supernatants for immunomediators at days 1 and 3 after infection. The Luminex® multiplex data corresponded with transcript data in that the THP-1 cells infected with an MOI-1 induced peak secretion of IL-6, IL-8, interferon gamma-induced protein 10 (IP-10) and TNFα at day 3 after infection (Fig. 1B). Levels of IL-6, IL-8, IP-10, and TNFα peaked at 52, 2000, 40 and 65 pg/mL, respectively. Also slight overall increases were observed in VEGF and IFNγ levels at day 3 after infection. THP-1 cells incubated with lipopolysaccharide (LPS) significantly produced all of the immunomediators tested both at the transcript and protein levels (Supplemental Fig. 1 and Fig. 1B) whereas THP-1 cells mock-infected with UV-treated virus produced low expression levels similar to those observed in untreated THP-1 cells (Fig. 1B). Overall, these results demonstrated that DENV-2

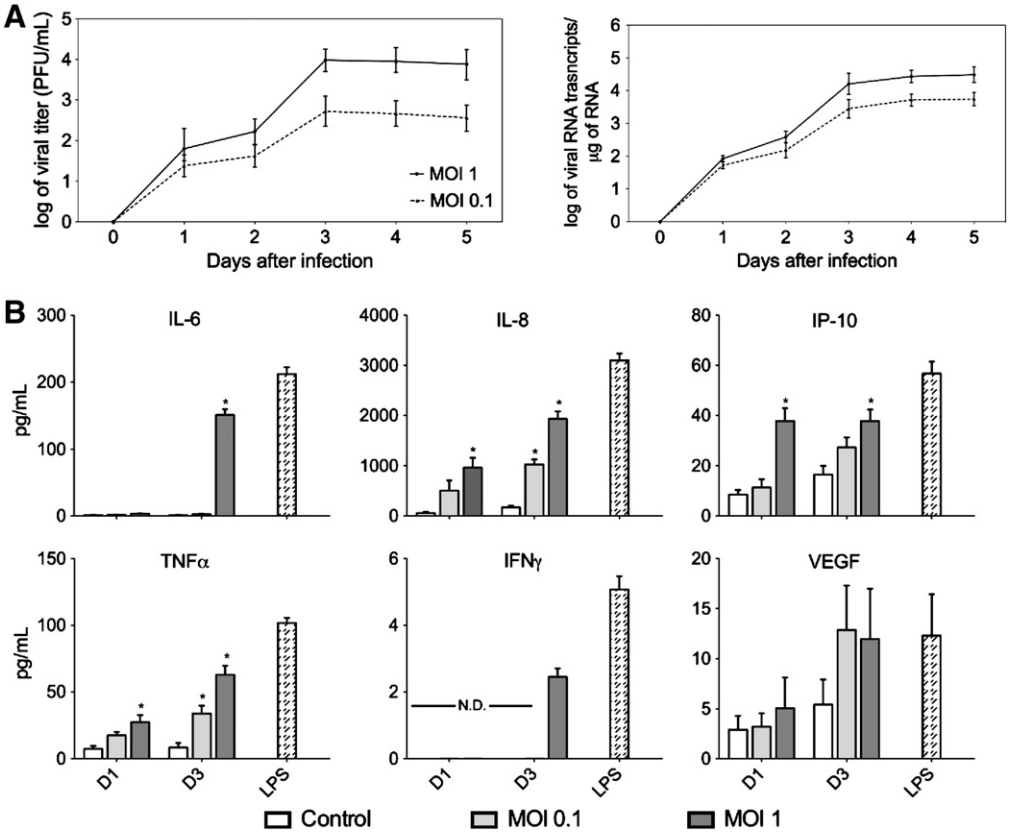

Fig. 1. Kinetics of chemokine and cytokine secretion in DENV-infected THP-1 cells. THP-1 cells were infected with DENV-2 NGC strain at MOI-0.1 and -1 and cells and supernatants were collected every 24 h after infection for five consecutive days. (A) Viral titers and RNA copy numbers were determined by plaque assay and qRT-PCR, respectively, on samples collected each day after infection (MOI-1, solid line; MOI-0.1, dotted line). (B) Chemokine and cytokine levels in mock- and DENV-infected THP-1 cell culture supernatants collected at days 1 and 3 after infection (mock-infected, white bars; DENV-infected MOI-0.1, light gray bars; DENV-infected MOI-1, dark gray bars; and LPS treatment, striped bars). Data points and bars represent the mean ± SD of three independent experiments conducted in duplicate. (*) indicates statistical significance at $p<0.05$ as compared to same day controls; N.D., not detected.