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Dengue virus infection of erythroid precursor cells is modulated by both thalassemia trait status and virus adaptation

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ABSTRACT

Dengue is the most significant arthropod borne viral disease worldwide, and infection with the dengue virus causes a wide range of symptoms in humans, including bone marrow suppression. While the target cells of the virus remain poorly characterized, cells of the myeloid lineage have been shown to be important mediators of the disease. This study sought to determine whether erythroid precursor cells were susceptible to dengue virus infection, and whether erythroid cells from thalassemia trait carriers showed any protection against infection. Infection with a laboratory adapted high passage DENV-2 resulted in high levels of infection during certain stages of differentiation, and cells derived from thalassemia trait carriers showed significantly reduced susceptibility to dengue virus infection. Infection with low passage isolates resulted in only scattered cells showing evidence of infection, but high bystander apoptosis that was reduced by both a caspase 8 inhibitor and anti-tumor necrosis factor 1 receptor antibodies.

Introduction

Dengue virus (DENV) is recognized as the most common arthropod borne human viral pathogen and is endemic in many tropical and subtropical countries. The virus belongs to family *Flaviviridae*, genus *Flavivirus* and possesses a positive sense single stranded RNA genome. Four serotypes of DENV have been classified, namely DENV-1, DENV-2, DENV-3 and DENV-4. Human infection with the virus occurs after the bite of an infected female mosquito and results in a broad range of clinical manifestations including fever, rash and headache as well as muscle and joint pain (Gubler, 1998). Early bone marrow suppression, thrombocytopenia and leukopenia are clinical hallmarks in dengue patients, but the etiology remains unclear (Bierman and Nelson, 1965; Srichaikul and Nimmannitya, 2000). Infection of the bone marrow has been suggested as a contributing factor as this compartment is the major site for hematopoiesis and bone marrow infection was found in *in vivo* studies of non-human primates, and it was proposed that megakaryocytes are the major target for DENV in this compartment (Noisakran et al., 2012).

Pluripotent hematopoietic stem cells (HSCs) located in the bone marrow give rise to lymphoid (natural killer (NK), T and B cells) and myeloid (granulocyte, monocyte, dendritic, erythrocyte and megakaryocyte) cells (Gunsilius et al., 2001), and several cells of the myeloid lineage including dendritic cells (Wu et al., 2000), monocytes (Scott et al., 1980) and megakaryocytes (Noisakran et al., 2012) have been shown to be susceptible to dengue virus infection. Mature erythroid cells arise from hematopoietic stem cells which give rise to early erythroid committed progenitor cells (burst forming units-erythroid or BFU-E) which subsequently give rise to late erythroid progenitor cells (colony forming units-erythroid or CFU-E) which further differentiate to erythroid committed precursors which consist of a series of erythroblastic cells (proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts and reticulocytes) which finally develop into mature erythrocytes (Gunsilius et al., 2001). While previous *in vitro* studies have shown that hematopoietic progenitor cells are susceptible to DENV infection and inhibition of cell proliferation has been observed (Murgue et al., 1997; Nakao et al., 1989), no study has to date investigated the susceptibility to DENV infection of the more differentiated erythroid precursor cells.

Erythroid cells have been well characterized as a target for mosquito borne protozoan parasite *Plasmodium* spp., which are responsible for the disease malaria (Garcia, 2010) and the protective effects of hemoglobin variants or globin gene deletions has been well

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established (Taylor et al., 2012). For example heterozygous sickle cell traits have been well characterized as providing a protective advantage against malaria in endemic areas (Aidoo et al., 2002; Allison, 1954; Piel et al., 2010; Williams et al., 2005). Similarly, α^+-thalassemia traits have been proposed as another determinant that may provide protection against severe malaria (Enevold et al., 2007; Mockenhaupt et al., 2004; Wambua et al., 2006). These studies support the malaria hypothesis which proposes that it is the selective pressure provided by the malaria parasite that maintains the sickle cell and thalassemia traits in populations (Clegg and Weatherall, 1999). In some areas of the world, and particularly in Southeast Asia, thalassemia traits are found in the population at extremely high levels (Fucharoen and Winichagoon, 1992). In parts of Thailand the hemoglobin variant Hb E is found in up to 50% of the population and other traits show similarly high penetrance (Fucharoen and Winichagoon, 1992). It is possible therefore that other mechanisms besides malaria are providing selective pressure on maintaining these traits in the population. This study sought to determine whether erythroid precursor cells were susceptible to DENV infection, and whether thalassemia traits resulted in any modulation of DENV infectivity.

Materials and methods

Cells and virus

LLC-MK_2 cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithesburg, MD) supplemented with 5% heat inactivated fetal bovine serum (FBS; Gibco BRL) and 100 units/mL of penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria). The human erythroleukemia cell line K562 was grown in RPMI-1640 (Gibco BRL) supplemented with 10% FBS and 100 units/mL of penicillin/streptomycin. Both cell lines were incubated at 37 °C with 5% CO_2. Dengue virus serotype 2 (DENV-2; strain 16681; GenBank accession number M84727.1), Dengue virus serotype 2 (DENV-2 DHF; DENV-2/THAI/NS1-141/2006; GenBank Accession number KM519587) and Dengue virus serotype 4 (DENV-4 DF; DENV-4/THAI/NS1-058/2006; GenBank accession number KM519591) were used in this study. DENV-2/THAI/NS1-141/2006 and DENV-4/THAI/NS1-058/2006 were passaged 3 times through C6/36 cells prior to use in this study. All dengue viruses were propagated in C6/36 cells (ATCC CRL-1660) and chikungunya virus (CHIKV) ECSA 226V genotype was propagated in Vero (ATCC: CCL-81) cells as described elsewhere (Wikan et al., 2012). The supernatants were partially purified by centrifugation and stored at −80 °C as viral stock. All virus titers were determined by standard plaque assay on LLC-MK_2 cells or Vero cells as previously described (Sithisarn et al., 2003).

Sample collection and erythroid cell culture

The study was performed in accordance with the Helsinki Declaration and was conducted after approval by the Ethical Committee, Mahidol University Institutional Review Board. Written informed consent was obtained from all subjects. Complete blood count (CBC) indices, hemoglobin typing and α-globin gene genotyping were used to classify thalassemia carriers and normal controls. Fifty milliliter of peripheral blood was taken from at least three individuals per group which were classified either as normal subject, β-thalassemia trait, Hb E trait or α-thalassemia 1 trait. CD34+ hematopoietic stem cells (HSCs) were isolated from peripheral blood samples and cultured under conditions that produced differentiation in the erythroid lineage as described in previously (Lithanatudom et al., 2010; Wannatung et al., 2009) and see more detail in Supplemental materials and methods. Under these growth conditions more than 95% of cells show erythroblast morphology as established previously (Wannatung et al., 2009). Cell numbers were established by trypan blue exclusion assay and counting using a cell counting chamber.

Virus infection

Cells (K562 cells or erythroid committed progenitor or precursor cells) were cultured in 6-well culture plates or 12-well culture plates under standard conditions and were infected with DENV-2 strain 16681 at various multiplicity of infection (m.o.i.) and DENV-2 DHF and DENV-4 DF at m.o.i. 50. Some normal erythroid committed precursor cells were infected with CHIKV at m.o.i. 0.1, 1 and 5. Cells and virus were incubated for 2 h in normal medium without serum albumin with occasional agitation after which complete medium was added and cells were incubated under standard conditions until required. In some experiments DENV-2 was pre-incubated for 1 h at 4 °C with a 1:100 dilution of a mouse monoclonal anti-DENV E protein antibody produced by hybridoma HB114 (Henchal et al., 1982) before addition to cells as described elsewhere (Klomporn et al., 2011). For viral production measurement, after 2 h of incubation, unbound virus was washed 4 times with IMDM medium without serum albumin. Supernatant was collected at several time points and virus titer was measured.

Flow cytometry, indirect immunofluorescence assay and immunocytochemical staining

Flow cytometry and indirect immunofluorescence were undertaken essentially as described elsewhere (Leecharoenkiat et al., 2011; Lithanatudom et al., 2010; Panyasrivanit et al., 2009). Immunocytochemical staining was performed using a Vectastain ABC kit following the manufacturer's protocol and counterstained with Wright–Giemsa staining. For full details, see file Supplemental materials and methods.

Western blot analysis

Supernatant was collected from DENV-2 infected cells. Total protein was extracted from mock infected and infected cells. Proteins were subjected to western blot analysis exactly as described previously (Leecharoenkiat et al., 2011). For further details, see file Supplemental materials and methods.

Caspase 8 inhibition

Day 7 erythroid precursor cells were pretreated with or without 10 μM of Caspase 8 inhibitor (Z-Ile-Glu(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone; Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C, 5% CO_2 with agitation for 2 h. Incubated cells were infected with DENV-4 DF with or without 10 μM of Caspase 8 inhibitor. After 2 h of viral incubation, complete IMDM medium with or without a final concentration of 10 μM of Caspase 8 inhibitor was added and cells were subsequently incubated at 37 °C with 5% CO_2 for 48 h.

TNF-R1 receptor blocking

Erythroid precursor cells were infected with DENV-2 DHF under the standard protocol and at 6 h post infection the medium was supplemented an anti-TNF-R1 (sc-7895; Santa Cruz Biotechnology Inc., Dallas, TX) antibody to final concentrations of 5 μg/mL. An identical amount of antibody was added at 24 h post infection. Treated and untreated cells were incubated under standard conditions.