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The Role of CD1a in Dengue Infection

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Abstract

Dengue is one the most prevalent tropical infectious diseases. It causes a heavy healthcare burden globally, while treatment and prevention approaches are limited. Like other virus-induced diseases, T cells are considered to play an important role in the immunity against dengue virus infection. However, compared to conventional MHC-activated T cells, the role of CD1a-restricted, lipid-specific T cells in dengue infection has not been studied.

The aim of this project was to investigate the role of CD1a in the immunity of dengue virus infection.

Human monocyte-derived dendritic cells (moDCs) were used as models to study the regulation of surface CD1a expression in the context of dengue virus infection. Components in sera derived from acute phase dengue patients were found to downregulate CD1a expression by moDCs which may in part be explained by IL-10. To investigate the effect of dengue infection on the function of CD1a-reactive T cells, polyclonal blood T cells from patients and controls, and CD1a-autoreactive T cells clone/lines were utilised. The production of IFN-γ and GM-CSF by CD1a-autoreactive T cells was reduced when co-cultured with dengue-infected presenting cells. Receptors for dengue viral entry are incompletely understood. Langerhans cells represent an early

target cell for dengue virus, and express langerin, a C-type lectin which supports CD1a antigen presentation. Here, we define langerin as a novel target for dengue NS1 and E proteins *in vitro*, and provide data supporting an anti-viral effect of langerin engagement.

In summary, this thesis describes the first study of the role of CD1a in dengue virus infection. The data show that the expression of CD1a and the activity of CD1a-reactive T cells are regulated by dengue virus infection, and that langerin is a novel dengue receptor. Collectively the data implicate the CD1a pathway in dengue pathogenesis and provide insights into future therapeutic and vaccine development.

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Abbreviations

ADE	Antibody dependent enhancement
AP	Adaptor protein complex
APC	Antigen-presenting cell
BSA	Bovine serum albumin
C protein	Capsid protein
CD	Cluster of Differentiation
cDNA	Complementary DNA
CLA	Cutaneous lymphocyte-associated antigen
CLR	C-type lectin receptor
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocyte
DAG	Diacylglycerol
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DDM	Didehydroxymycobactin

DENV, DV	Dengue virus
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DSS	Dengue shock syndrome
E protein	Envelope protein
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
ER	Endoplasmic reticulum
EV	Empty vector
FACS	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FFU	Foci-forming unit
FMO	Fluorescence minus one

GA	Glutaraldehyde
GM-CSF	Granulocyte-macrophage colony stimulating factor
HC	Healthy control
HDM	House dust mite
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HRV	Human rhinoviruses
HS	Human serum
IFN-γ	Gamma-interferon
Ig	Immunoglobulin
IL	Interleukin
ILC2	Group 2 innate lymphoid cell
ILT4	Immunoglobulin-like transcript 4
iNKT	Invariant natural killer T cell
ISG	Interferon-stimulated gene

LC	Langerhans cells
LPC	Lyso-phosphatidylcholine
M protein	Membrane-associated protein
mAb	Monoclonal antibody
MACS	Magnetic cell separation (Miltenyi Biotec)
MBL	Mannose binding lectin
MDA5	Melanoma differentiation-associated gene 5
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
moDC	Monocyte-derived dendritic cell
MOI	Multiplicity of infection
moLC	Monocyte-derived Langerhans cell
NEAA	Non-essential amino acids
NKT	Natural killer T cell
NS1	Non-structural protein 1
PAF	Platelet activating factor
PAMPs	Pathogen-associated molecular patterns

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PFA	Paraformaldehyde
PG	Phosphatidylglycerol
pH	Potential of Hydrogen
PI	Phosphatidylinositol
PLA2	Phospholipase A2
PNGase F	Peptide:N-glycosidase F
PrM	Precursor membrane-associated protein
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RIG-I	Retinoic acid inducible gene I
rLangerin	Recombinant langerin
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction

SM	Sphingomyelin
SNP	Single nucleotide polymorphism
TAG	Triacylglyceride
TCR	T cell receptor
Tfh	T follicular helper cell
TGF-β1	Transforming growth factor beta 1
TGN	Trans-Golgi network
TLR	Toll-like receptors
TMB	3,3’,5,5’-tetramethylbenzidine
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
β2m	β-2-Microglobulin

1 Introduction

1.1 Dengue

1.1.1 Dengue pathology

Dengue is one of the most prevalent mosquito-borne diseases, caused by infection with one of at least 4 serotypes of dengue virus (DENV 1-4). Dengue virus belongs to the flavivirus genus, which also includes zika virus, yellow fever virus, West Nile virus, Japanese encephalitis virus and several other viruses that can cause human disease (Rey, 2013).

In addition to the well-studied 4 serotypes, the fifty serotype of dengue virus (DENV-5) was reported in 2013. DENV-5 was detected in a patient sample collected in Sarawak state of Malaysia in 2007 and was considered as a cross-species transmission of sylvatic form of dengue virus (Mustafa, Rasotgi, Jain, & Gupta, 2015). The discovery of DENV-5 indicated that the re-emergence of sylvatic stains into human transmission circle is a realistic problem. Ecological study on dengue strains in non-human primates is essential.

Dengue infections cause a broad spectrum of disease, from asymptomatic to potentially fatal severe dengue syndromes. As of yet there is no specific treatment/cure for dengue, and the current treatment requires careful management of fluids by medical professionals. Early detection of warning signs for severe dengue and access to proper supportive care is critical in lowering the fatality rates of severe dengue.

Epidemiology and aetiology

Dengue infections cause a heavy burden to public healthcare every year. Approximately 3.9 billion people are at risk of dengue virus infection worldwide in the tropical and sub-tropical regions of 128 countries (Jentes et al., 2016). A report published in 2013 estimated a 390 million annual dengue infections globally (95% CI: 285-528 million), among which 96 million cases (95% CI: 67-136 million) were symptomatic (Bhatt et al., 2013). Furthermore, an estimated 500,000 cases require hospitalisation and about 20,000 deaths are caused by dengue annually ("Dengue vaccine: WHO position paper, September 2018 - Recommendations," 2019). The incidence of dengue infection has increased rapidly in recent years, from 1990 to 2013, each decade more than doubling the number of symptomatic cases (Stanaway et al., 2016).

Dengue virus (DENV) is transmitted to humans through the bite of a dengue-infected female mosquito. *Aedes aegypti* is the major vector for this disease. Other species from the Aedes genus, such as *Aedes albopictus*, can also contribute to the dengue transmission, but less efficiently (Ferreira-de-Lima & Lima-Camara, 2018). *A. aegypti* are most active during the day, with peak biting periods distributed early in the morning and in the hours before sunset (Trpis, 1973). *A. aegypti* are highly adapted to urban habitats and breed easily in man-made water containers. *A. albopictus* is the secondary dengue vector in Asia. Due to its ability to survive in temperate climates, it has spread to North America and parts of Europe. However, the number of dengue outbreaks caused by *A.*

albopictus in temperate regions is limited, as *A. albopictus* is a less efficient vector of dengue virus (Ferreira-de-Lima & Lima-Camara, 2018).

Dengue infections are mainly maintained in a human-mosquito-human cycle. Mosquitoes can be infected after feeding on viremic dengue positive individuals. The virus first replicates in the mosquito midgut, then infects secondary tissues, such as salivary glands. After infection of salivary glands, the mosquito is capable of transmitting DENV for life (Nguyet et al., 2013). It is of note that dengue virus can also be transmitted vertically, from pregnant mother to her baby. Although the rates of vertical transmission appear low, it may lead to complications including pre-term birth, fetal distress and low birthweight (Pouliot et al., 2010). In rare cases, dengue has been seen to be spread through infected blood, including blood transfusion, organ transplant, or needle stick injury (Teo, Ng, & Lam, 2009). Furthermore, non-human primates act as reservoir host in western Africa and south-east Asia, through a monkey-mosquito-monkey cycle. However, the transmission from non-human primates to humans is rare (Valentine, Murdock, & Kelly, 2019).

Clinical presentation

Dengue infections cause a wide spectrum of clinical presentations, while in most cases the disease is self-limiting and mild, occasionally, patients progress to severe disease, characterised by plasma leakage. No chronic infection or carriage state of dengue virus has been found ("Dengue vaccine: WHO position paper, September 2018 -

Recommendations," 2019). Mild and asymptomatic infections represent approximately 75% of dengue infections. Symptomatic cases are grouped into three categories: dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), where DHF and DSS are both classified as severe dengue and are characterised by plasma leakage and are potentially lethal (World Health Organization. Regional Office for South-East Asia., 2011). Dengue fever is a relatively mild from of the disease with symptoms such as acute fever, headache, retro-orbital pain, myalgia, arthralgia, abdominal pain and nausea.

The clinical features of DF are age dependent. Undifferentiated fever with a maculopapular rash is frequently seen in infants and young children. While in older children, adolescents and adults, common symptoms include acute fever (sometimes biphasic), headache, retro-orbital eye pain, myalgia, arthralgia and rashes. DF patients may also show leucopenia and thrombocytopenia. Recovery from DF can be accompanied by depression and prolonged fatigue, which is more common in adults (World Health Organization., 1997). In practice, DF patients are further split into two subgroups: patients with or without warning signs of developing severe disease. Warning signs of severe dengue include: abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy, restlessness, liver enlargement >2 cm and an increase in haematocrit with a rapid decrease in platelet count. However, some patients with no apparent warning signs may still develop severe dengue (Morra et al., 2018).

Four major clinical symptoms are associated with DHF: high fever, haemorrhage, liver enlargement and circulatory failure (World Health Organization., 1997). Moderate to significant thrombocytopenia with concurrent haemoconcentration is the major laboratory determinant of DHF. Indeed plasma leakage is the defining characteristic of DHF (World Health Organization., 1997).

Hypovolemic shock may be induced if the loss of plasma is significant. DSS is characterized by symptoms including: a rapid and weak pulse with narrowing of the pulse pressure, or hypotension. Cold and clammy skin, restlessness and delayed capillary refill (>3 seconds) are the signs of reduced tissue perfusion (World Health Organization. Regional Office for South-East Asia., 2011). With timely and appropriate volume-replacement therapy, the shock is reversible and the duration is short. However, if no proper treatment is given, patients with DSS may die within 12-24 hours. Complicated conditions can be induced by uncorrected shock, including: metabolic acidosis, severe bleeding and multi-organ failure, especially hepatic and renal failure (World Health Organization. Regional Office for South-East Asia., 2011).

Dengue is a dynamic disease. For symptomatic cases, the incubation period ranges from 3 to 14 days (most in 4-7 days). Following the onset of symptoms, the development of disease can be divided into three phases: febrile phase, critical phase and recovery phase (Rigau-Perez, 1997). The acute febrile phase usually lasts 2-7 days and is characterised by high-grade fever (40˚C). The critical phase starts between day 3 and day 7 of illness, when the body temperature drops to 37.5-38˚C or less. This is the period of significant

plasma leakage in the severe cases and usually last 24-48 hours. The patients who improve after defervescence are considered non-severe dengue patients. After the critical phase, patients start to recover and general well-being improves. In severe dengue, the extravascular accumulated fluid caused by plasma leakage is reabsorbed within 48-72 hours (World Health Organization. Regional Office for South-East Asia., 2011).

Risk factors of severe dengue

There are a number of known risk factors for severe dengue, including both viral characteristics and host attributes.

There is evidence to suggest that the different strains and serotypes of dengue virus diverge in pathogenicity. Several studies indicate that infection with DENV-2 and DENV-3 may confer greater risk of developing severe disease, while DENV-4 is associated with milder illness. (Nisalak et al., 2003; Passos et al., 2004; Vaughn, 2000). However, the precise nature of the association between certain clinical characteristics and viral serotype remains elusive (Balmaseda et al., 2006).

There are many host conditions that influence the outcome of dengue infection. Secondary dengue infection is one of the most significant risk factors of developing severe dengue (Katzelnick et al., 2017; St John & Rathore, 2019). Other individual risk factors include age (infants and young children are at greater risk) (Sangkawibha et al.,

1984), ethnicity and chronic medical conditions (including obesity (Pichainarong, Mongkalangoon, Kalayanarooj, & Chaveepojnkamjorn, 2006), asthma (Pang, Hsu, Yeo, Leo, & Lye, 2017), diabetes (Pang et al., 2017) and sickle cell anaemic (Wilder-Smith & Leong, 2019)).

Primary dengue infection could provide life-long protection against the same serotype of dengue virus and a temporary cross-protection to other serotypes, which lasts up to 2 years (Reich et al., 2013). However, after the period of cross-protection, an infection with a secondary serotype confers a higher risk of developing severe dengue. Antibody dependent enhancement (ADE) is the major hypothesis to explain increasing disease severity upon secondary dengue infection. In the ADE model, after the cross-reactive antibodies wane and can no longer neutralise the secondary infection, these antibodies instead facilitate the viral infection in an Fc-receptor- dependent manner. Resulting in a greater cellular infection rate and a higher viral burden, contributing to increased disease severity (Halstead, 2014).

However, after recovery from secondary dengue infection, a broad immune response can provide better protection against further infections caused by other viral serotypes. Importantly severe illness is rare during a third or fourth dengue infection (Reich et al., 2013).

Dengue diagnosis

A number of methods are employed to diagnose dengue infection, including virological tests to detect viral elements directly, and serological tests to detect anti-dengue antibodies. The choice of diagnostic test depends on the time since onset of illness.

During the febrile phase, before day 4-5 of illness, dengue virus and non-structural protein 1, or NS1 viral antigen can be detected in patient blood samples. Samples collected during this period can be tested by reverse transcriptase-polymerase chain reaction (RT-PCR) to determine the viral load and serotype, and can be tested by rapid diagnostic tests or enzyme-linked immunosorbent assay (ELISA) for the presence and/or concentration of circulating NS1 antigens (Special Programme for Research and Training in Tropical Diseases. & World Health Organization., 2009).

Dengue virus and antigens are undetectable in patient blood after 4-5 days of illness, at this time the concentration of anti-dengue antibodies starts to increase. However, the rate of antibody production depends on whether the patients have been infected with dengue (or other flavivirus) previously. For patients without a previous flavivirus infection, dengue IgM antibodies can be detected approximately 1 week after the onset of illness, with the level peaking after 2 weeks and remaining detectable for about 2-3 months. The development of IgG is slower than IgM, which usually appears from day 10 of illness, but is thought to persist for life. In patients who have had previous flavivirus infections, IgG can be detected within the first week of illness ("Dengue vaccine: WHO position paper, September 2018 - Recommendations," 2019). Due to the diverging kinetics of IgM and

IgG, performing ELISA for anti-dengue antibodies can provide information on the timing of infection; the presence of IgM indicating a recent DENV infection, while the detection of IgG indicates a past infection. Detection of IgG in acute infections also suggests a secondary dengue infection ("Dengue vaccine: WHO position paper, September 2018 - Recommendations," 2019).

Treatment

There is currently no specific antiviral therapeutic for dengue infection. Symptoms of fever and aches can be ameliorated by fever reducers and pain killers, such as acetaminophen or paracetamol. Non-steroidal anti-inflammatory drugs should be avoided due to their role in thinning the blood, which may exacerbate the prognosis in patients with risk of haemorrhage.

Whilst for most individuals, dengue is self-limiting, not requiring hospital admission, it is critical that severe dengue patients receive appropriate management based on supportive therapy and careful control of the intravascular volume replacement. In this way the mortality rate of hospitalised cases can be limited to less than 1%. (Special Programme for Research and Training in Tropical Diseases. & World Health Organization., 2009). Therefore, case classification is important for clinical triage, especially during outbreaks, where the health care system needs to cope with a sudden surge in demand (Special Programme for Research and Training in Tropical Diseases. & World Health Organization., 2009).

Prevention

Dengvaxia (CYD-TDV) developed by Sanofi Pasteur is the first and so far only approved vaccine for Dengue Virus. It is a live attenuated, recombinant tetravalent vaccine, based on an attenuated yellow fever virus 17D strain substituted with dengue PrM and E proteins (Guy et al., 2011). Dengvaxia was first registered in Mexico in December 2015 and is currently approved in 19 dengue-endemic countries. The vaccine is registered for use in individuals aged 9 - 45 years in most of these counties ("Dengue vaccine: WHO position paper, September 2018 - Recommendations," 2019).

However, although currently the only available dengue vaccine, CYD-TDV shows limited preventative effects. CYD-TDV displays imbalanced protection against different serotypes and actually increases the risk of severe dengue for some dengue naive individuals. In a trial assessing the efficacy of CYD-TDV vaccine against DENV 1-4, dengue virus protection was measured to be 58.4% (95% CI: 47.7–66.9%), 47.1% (95% CI: 31.3–59.2%), 73.6% (95% CI: 64.4–80.4%) and 83.2% (95% CI: 76.2–88.2%) respectively (Hadinegoro et al., 2015). CYD-TDV afforded limited protection against DENV-2, less than 50%- the lowest among all serotypes. Importantly DENV-2 is associated with greater risk of severe disease, as discussed previously.

In a separate long-term clinical trial, the risk of severe dengue over 5 years was found to depend on whether vaccinees had dengue infection prior to vaccination. Seropositive individuals showed reduced incidence of severe dengue upon vaccination, from 4.8 per

1000 to 1 per 1000. While seronegative individuals showed increased incidence of severe dengue, from 1.7 per 1000 in control group to 4.0 per 1000 in vaccinated group (Sridhar et al., 2018). Based on these data, WHO only recommends CYD-TDV vaccination for confirmed seropositive individuals ("Dengue vaccine: WHO position paper, September 2018 - Recommendations," 2019).

Due to the limitations of the current dengue vaccination strategy, combating the mosquito vectors is still the predominant approach to control and prevent the spread of dengue virus. The main strategies include: environmental interventions to prevent mosquito breeding (removing man-made mosquito habitats and proper management of domestic water containers) and personal protection from mosquito bites (such as using window screens, mosquito repellents and insecticide) ("Dengue vaccine: WHO position paper, September 2018 - Recommendations," 2019).

In addition to the abovementioned strategies, many alternative strategies have been developed and tested in recently years (Achee et al., 2019):

- Novel larvicide approasches, such as insect growth regulators, microbial insecticides (*Bacillus thuringiensis israelenisis*) and entomopathogenic fungi, especially *Metarhizium anisopliae* and *Beauveria bassiana*.
- Attractant toxic sugar baits (ATSBs): toxic sugar meals used in bait stations or sprayed on plants to reduce mosquito populations.

- Sterile insect technique (SIT): male insects has been radiated sterile and released into the environment to sterilize females.
- Release of insects with dominant lethality (RIDL): release of transgenic insects with dominant lethal construct led to eliminate female progeny production.
- *Wolbachia*: *Wolbachia* is an intracellular bacterial symbiont which can alter reproduction of its host. *Wolbachia* is present in the female germline and is transmitted maternally. It can induce cytoplasmic incompatibly and cause the eggs of infected males failed to develop. Therefore, regular releases of male mosquitoes infected with a novel *Wolbachia* strain could reduce the viability of eggs and suppress the mosquito population.
- Gene drives: gene drives strategy use CRISPR/Cas9-based transgenic constructs that can invade target populations even regardless of fitness cost. The gene-drive strategy can be used to achieve population replacement or population suppression. In population replacement strategy, drive construct is designed to confer mosquito resistance to a given pathogen. In population suppression strategy, a drive element is designed to inactivate a sex-specific fertility gene.

1.1.2 Dengue Virology

Dengue virus (DENV) belongs to family *Flaviviridae*, genus *Flavivirus*. Human disease-causing members of the *Flavivirus* genus also include Yellow fever virus, West Nile virus, Japanese Encephalitis virus and Tick borne encephalitis virus. Flaviviruses are enveloped

viruses with positive-sense, single-stranded RNA genomes. The genome of DENV is 10.7 kb in length and encodes for ten proteins, including 3 structural proteins (Capsid protein, C; Membrane-associated protein, PrM, M; Envelop protein, E) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The non-structural proteins are responsible for viral replication and assembly but do not form part of the virion structure (Diamond & Pierson, 2015).

The four dengue virus serotypes, DENV 1-4, share around 60%-75% amino acid sequence identity (Guzman & Harris, 2015). The serotypes are classified according to their antigenic properties. Dengue virus serotyping can be achieved by 1) identifying serotype-specific nucleotide sequences or antigenic determinants of the isolated virus or virus in acute phase patient serum samples; 2) analysing serotype-specific IgM and/or IgG antibodies in acute and convalescent phase patient serum samples (E/M serotype-specific IgM and/or NS1 serotype-specific IgG) (Shu et al., 2004).

Virion morphology

DENVs have spherical structure with a diameter of approximately 50nm or 60nm, for the mature and immature viral particles respectively. The DENV particle consists of an envelope (host-derived lipid bilayer covered with viral PrM/M and E proteins) and an RNA-protein core (genomic RNA capped with C proteins). The conformation of PrM/M and E proteins is different in the mature and immature virus. The protein shell of immature viral particles, is made up of PrM and E proteins, forming 90 heterodimers

which extend to form 60 trimeric spike structures. During the secretion and maturation of viral particles, the conformation of E proteins is changed by the low pH of the trans-Golgi network (TGN) and the PrM proteins are cleaved into 'Pr' peptides and M proteins by host protease, furin. In contrast with the spike structure of immature virions, mature viral particles have 'smooth' morphology with 90 homodimers of E proteins in the outer shell overlying the M proteins that span the membrane (R. Perera & Kuhn, 2008). The cleavage of PrM during virion secretion is often incomplete, thus viral particles can be either mature or immature or partially mature, exhibiting only partial PrM cleavage. The ratio of virion maturity depends on the cell type that has been infected. PrM cleavage is critical for dengue virus to become infectious. Viruses displaying a large amount of PrM are not infectious, and PrM cleavage during virus maturation increases infectivity. However, viruses bearing a high ratio of PrM:Pr can be infectious via antibody dependent enhancement (ADE) (Dejnirattisai et al., 2015).

Infection and replication

When DENV infects host cells, the virions first bind to cell surface receptors and attachment factors. Viral entry is achieved by clathrin mediated internalization. Viral particles are then trafficked to the late endosome, where the confirmation of E proteins is altered by low pH, promoting the fusion of viral and host membranes and release of viral RNA to the cytoplasm of the host cell (Diamond & Pierson, 2015). A single polyprotein is translated from the viral RNA, which is then directed to the ER membrane.

Signal sequences within the polyprotein direct the viral proteins into position around the ER membrane: with NS1 and E on the luminal side, C, NS3 and NS5 on the cytoplasmic side, and PrM, NS2A/B and NS4A/B spanning the membrane (the ectodomains of PrM are on the luminal side). The cleavage of this polyprotein is carried out by host proteases in the ER lumen and the viral NS3 protein with its co-factor NS2B in the cytoplasm (R. Perera & Kuhn, 2008). Viral genomic RNA (negative strands and positive strands) is then synthesised associated with a membranous network induced by virus. The positive-stranded RNA is then packaged into immature viral particles, which bud into the ER lumen. The viral particles are then trafficked following the secretory pathway through the Golgi apparatus and released from the infected cell by exocytosis (Diamond & Pierson, 2015). Maturation of viral particles happens during this secretion process in the TGN as previously discussed.

Cell surface receptors

To enter target cells the virus needs to bind to the plasma membrane via viral surface proteins. Host cell surface attachment factors play important roles in the virus-cell first contact and concentrate the virus on the cell surface, which facilitates virus binding to specific receptors that promote viral entry, dependent on clathrin. Specific receptors for dengue virus have not been identified to date, however a number of cell surface molecules that facilitate viral binding and infection have emerged (Cruz-Oliveira et al., 2015). These diverse molecules include C-type lectins (details are discussed in the

introduction of Chapter 6), glycosaminoglycans (such as heparan sulfate) and stress-induced proteins (such as the ER chaperonin GRP78 and the heat-shock protein 70 and 90). The usage of cell surface attachment molecules differs between target cell types. Indeed, the ability to infect a variety of cell types and organs in both mammals and mosquitoes is critical for successful spread of dengue virus. Therefore, it is not surprising that the virus has evolved to infect diverse target cells through different invasion mechanisms (Cruz-Oliveira et al., 2015).

Dengue glycoproteins

Glycoproteins exhibit complex oligosaccharide structures enzymatically attached to the protein peptide backbone. Protein glycosylation can be classified based on the site of oligosaccharide attachment: N-linked glycosylation and O-linked glycosylation, where the glycans are linked to the nitrogen of asparagine or the oxygen of serine or threonine residues respectively (Yap, Nguyen-Khuong, Rudd, & Alonso, 2017). Glycosylation occurs in the ER-Golgi complex through a highly organised network of glycotransferases and glycosidases that synthesise and trim down the oligosaccharide structure. Viruses do not have their own glycosylation machinery, instead they depend on the host cell to glycosylate the viral proteins. Dengue virus has two major glycoproteins: envelope protein (E) and non-structural protein 1 (NS1).

DENV E protein is glycosylated in the ER at two asparagine residues, N67 and N153. No O-linked glycan has yet been detected (Johnson, Guirakhoo, & Roehrig, 1994). The N67 glycosylation has been suggested to directly interact with one of the DENV host receptor DC-SIGN (Pokidysheva et al., 2006). In mammalian cells, DENV E protein is modified by complex glycans. While in mosquito cells, the dominant glycoforms are high mannose due to the different glycosylation enzymes in insect cells (Yap et al., 2017).

DENV NS1 is glycosylated at two asparagines, N130 and N207 (Yap et al., 2017). DENV NS1 is a multifunctional protein that presents as both a membrane-bound dimer and soluble hexamer structure. The hexameric NS1 can be secreted from DENV-infected host cells. The circulation hexameric NS1 has been reported to contribute to disease pathogenesis of severe dengue disease (Beatty et al., 2015; Modhiran et al., 2015). Intracellular and extracellular DENV NS1 bear different types of N-glycans. Intracellular dimeric NS1 proteins have high mannose N-linked glycosylation at both sites. While for extracellular hexameric NS1, the N130-glycans are complex oligosaccharides, contrasting the high mannose N207-glycans (Yap et al., 2017).

1.1.3 Dengue Immunity

The host immune response is usually divided into an innate immune response and an adaptive immune response based on the speed and specificity of reaction. Innate

immunity usually provides immediate host defence using broadly applicable germline encoded mechanisms, such as the complement system, receptors of pathogen-associated molecular patterns (PAMPs) and innate immune cells (Janeway & Medzhitov, 2002). Unlike the innate system, adaptive immunity recognises non-self cells or molecules with exquisite specificity. The effector cells of adaptive immunity are B and T lymphocytes. The antigen specificity of these lymphocytes is based on their unique immunoreceptors: B cell immunoglobulins (Ig) and T cell receptors (TCR) (Laird, De Tomaso, Cooper, & Weissman, 2000).

Both innate and adaptive immune responses are critical to limit Dengue virus infection and provide protection from reinfection. However, host immunity may also contribute the pathogenesis of the dengue infection and influence disease severity (Murphy & Whitehead, 2011).

Innate immune response

Upon DENV infection of host cells a variety of host innate immune responses will be immediately activated through pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), melanoma differentiation-associated gene 5 (MDA5) and retinoic acid inducible gene I (RIG-I) (Harapan, Michie, Sasmono, & Imrie, 2020). Engagement of these PRRs leads to the activation of several transcription factors, such as NF-κB and interferon regulatory factors, which then induce the production of interferons and other inflammatory cytokines. Among these responses, the type I interferon system is

activated within hours of DENV infection and is central to the early-stage host defence against DENV infection (Ngono & Shresta, 2018). Type I interferons consist of IFN-α and IFN-β, and can be detected or secreted by almost all nucleated cells. By binding to the specific receptors on target cells, type I interferons can trigger a series of transcriptional programs and induce expression of hundreds of interferon-stimulated genes (ISGs). ISGs influence various immunity-related cellular processes, limit virus infection and replication, and also enhance the lateral adaptive immune response (Schneider, Chevillotte, & Rice, 2014).

Many studies have suggested that type I interferon signalling plays critical roles in immune protection from DENV infection. The concentration of type I interferon in the blood of patients with dengue infection was observed to be higher than in healthy donors and correlated with viral load. Furthermore, decreased expression of type I interferon correlated with more severe clinical outcome, such as DHF and DSS (Upasani et al., 2020), suggestive of a protective role for type I interferon. An *in vitro* study using human primary fibroblasts and immortal cell lines also found that type I interferon protects against DENV infection (M. S. Diamond et al., 2000). A systemic study of wild-type mice and mice lacking interferon receptors or T cells and B cells revealed that type I interferon is essential for immune resistance at the early stage of infection, while T- and B-cell dependent immunity was less critical at this stage (S. Shresta et al., 2004).

However, as a pathogenic flavivirus causing human disease, DENV has evolved multiple strategies to circumvent the activation of the type I interferon pathway. Several DENV proteins, including NS2A, NS4A, NS4B, NS2B3 as well as subgenomic flavivirus RNA are reported to inhibit type I interferon induction and downstream signalling (Ngono & Shresta, 2018).

Humoral response

The role of humoral immunity in DENV infection is complex. As in other infections, pathogen specific antibodies play critical roles in infection limitation and pathogen removal. However, in the context of antibody dependent enhancement (ADE), virus-specific antibodies may facilitate viral infection and lead to severe dengue disease, in the context of secondary dengue infection (St John & Rathore, 2019). Therefore, the contribution of DENV-specific antibody in DENV immunity and pathogenesis in primary and secondary infection needs to be discussed separately.

After a primary DENV infection, antibodies are generated against DENV structural proteins as well as non-structural proteins. E proteins are the main targets of neutralising antibodies, while antibodies against C, PrM, NS1, NS3, NS4B and NS5 proteins have also been detected in patient serum (Hurtado-Monzon et al., 2020). However, due to the high sequence divergence between different DENV serotypes, cross-reactive antibodies generated in the first infection may not neutralise the virus upon secondary infection. Instead, these non-neutralising antibodies may facilitate viral

entry into host cells via Fc receptor binding (Halstead & O'Rourke, 1977). An analysis of DENV-specific antibodies separated from patient blood demonstrated that the anti-PrM response was dominant, compared with the anti-E response, and displayed limited dengue virus neutralisation. The partial cleavage of prM, reducing available antigen, and significant cross-reaction between serotypes contribute to making the anti-prM response especially vulnerable to enhancement (Dejnirattisai et al., 2010). Pre-existing anti-DENV antibody concentration, irrespective of protein target, was found to correlate with disease severity. In a study of a pediatric cohort in Nicaragua, the highest risk of severe dengue disease was found to associated with a narrow range of pre-existing antibody titre. Lower titres did not affect disease severity and higher titres were found to be protective (Katzelnick et al., 2017). Unlike antibodies against DENV structural antigens, NS1-specfic antibodies do not contribute to ADE. However, this group of antibodies was found to play pathogenic roles in severe dengue. NS1 antibodies titres were found to be significantly higher in patients with DHF compared with DF, and antibodies binding specific epitope regions could predict disease severity (Jayathilaka et al., 2018). Potential mechanisms by with such anti-NS1 antibodies could contribute to severe disease pathogenesis include: 1) antibody binding to membrane bound NS1 activates cellular signalling pathways and results in proinflammatory cytokine release (Lin et al., 2005), 2) anti-NS1 antibodies may display cross-reactivity to endothelial autoantigens and therefore contribute to the endothelial dysfunction characteristic of severe dengue (Falconar, 1997).

T cell responses

Studies investigating the T cell response to dengue infection and the serum cytokine profile following immunisation with a live-attenuated DENV vaccine candidate (Gwinn, Sun, Innis, Caudill, & King, 2003) revealed that the T cell response against DENV is predominantly T helper 1 (Th1) polarised. A study using mouse models lacking CD4+ T cells and/or IFN-γ indicated that T cell-derived IFN-γ is critical for the host immune response against DENV infection (Wakil, Wang, Ryan, Fowell, & Locksley, 1998). Analysis of the PBMC transcriptional response of individuals with asymptomatic DENV infection revealed that control of infection correlates with increases CD4+ T cell activation (Simon-Loriere et al., 2017). In addition, a CD4-restricted HLA, HLA-DRB1 has been found to associate with less severe clinical outcomes, suggesting a protective role for CD4+ T cells (Weiskopf et al., 2016).

In addition to Th1 cells, other CD4+ T cell subsets are expanded during DENV infection, such as regulatory T cells (Treg) and T follicular helper cells (Tfh). Increased Treg:effector T cells ratio was found to associate with milder clinical outcome (Luhn et al., 2007). However, in a different cohort, Treg frequency was not found to be associated with disease severity or viraemia (Jayaratne et al., 2018). Tfh cells were found to be expanded in acute DENV infection, especially in secondary infection

(Haltaufderhyde et al., 2018; Saron et al., 2018). The frequency of activated Tfh cells was also found to correlate with the frequency of plasmablasts (Haltaufderhyde et al., 2018).

CD8+ T cells usually play important roles in the adaptive immune response during viral infection to kill infected host cells. In the context of DENV infection, CD8+ T cells were found to display a skin-homing phenotype with expression of skin-homing markers like CXCR3, CCR5 and cutaneous lymphocyte-associated antigen (CLA) (L. Rivino et al., 2015). Another human study revealed that although CD8+ cells were expanded in the acute phase of DENV infection and expressed cytotoxic markers, few cells produced IFN-γ when stimulated with DENV peptides *ex vivo* (Chandele et al., 2016). An interesting study comparing the DENV genome with closely related viruses that show a broader host species range (such as West Nile Virus) revealed the evolutionary loss of parts of genome that were predicted to encode CD8+ T cell epitopes in DENV strains, which indicated that the CD8+ T cell response is protective and drives population-level selections of DENV (Hughes, 2001).

1.2 CD1 family

The CD1 (cluster of differentiation 1) family is a group of glycoproteins, that are structurally related to MHC class I proteins and associate with β-2-Microglobulin (β2m). Importantly CD1 molecules diverge from MHC in the structure of the antigen binding

grooves, which are capable of presenting lipid, lipopeptide and glycolipid antigens to T cells. CD1a, CD1b, CD1c, CD1d and CD1e are the five isoforms of the CD1 family, and are categorised into three groups based on sequence analysis: CD1a, CD1b and CD1c in group 1, CD1d group 2, and CD1e group 3. (Brigl & Brenner, 2004) CD1d is the only CD1 isoform expressed by mice, and as such more studies have focused on CD1d-restricted T cells.

1.2.1 Evolutionary biology

CD1 antigen presentation is conserved throughout mammalian evolution, suggesting lipid antigen presentation is complementary to the MHC peptide-antigen presenting system and indispensable in the immune system. However, the number and combination of CD1 isoforms diverge between mammalian species. Six to thirteen group 1 CD1 genes are harboured by various mammalian species, such as horses, cattle, pigs, dogs and rabbits (Dascher, 2007). Humans express all five CD1 isoforms, whereas mice express only CD1d, and ruminants express all CD1 proteins apart from CD1c (Kaczmarek, Pasciak, Szymczak-Kulus, & Czerwinski, 2017). The diversity in CD1 isoforms among species may reflect differing selective pressures within the individual immune systems (Van Rhijn, Godfrey, Rossjohn, & Moody, 2015). The homology shared by the same isoform across species is larger than the homology within different isoforms of the same animal, indicating the early divergence of the CD1 isoforms

during evolution and suggesting functional non-redundancy of isoforms (Dascher, 2007).

In addition to mammals, two CD1 genes are found in birds and reptiles. Non-mammalian CD1 genes are distinct to the mammalian CD1 isoforms, which indicates the emergence of CD1 genes before the divergence of mammals and birds or reptiles, between 300 to 500 million years ago. Additionally it can be inferred that the gene duplications that resulted in isoforms CD1a-e occurred following this divergence (Dascher, 2007).

1.2.2 Human CD1

Human CD1 isoforms differ in tissue and cellular distribution, recycling pattern, ligand binding pocket structure, and target cell receptors, which affords CD1 molecules diverse potential to sample lipid species at various locations, and suggests a lack of redundancy.

Tissue/cellular distribution

Although similar to MHC class I in association with B_2M, the tissue and cellular distribution of CD1 proteins is more similar to MHC class II, which is restricted to thymocytes and professional antigen-presenting cells (APCs). CD1d is an exception among CD1 isoforms, and can be expressed by diverse non-hematopoietic cells,

including hepatocytes, Schwann cells, biliary and vascular endothelium, gut epithelium and keratinocytes (Dellabona, Consonni, de Lalla, & Casorati, 2015).

The expression pattern of CD1s by APCs varies. CD1a is predominantly expressed on epidermal Langerhans cells (LCs) and subsets of dermal dendritic cells (DCs) and is inducible on group 2 innate lymphoid cells (Hardman et al., 2017). CD1b and CD1c are found in dermal DCs and lymph node interdigitating DCs (Dougan, Kaser, & Blumberg, 2007), and CD1c is additionally expressed on subsets of B cells (Dougan et al., 2007). CD1d is the only CD1 molecule constitutively expressed by most monocytes and macrophages. Unlike cell surface CD1a-d, the DC expression of CD1e is intracellular, mainly localised to the Golgi apparatus. (Angenieux et al., 2005). CD1e is thought to be involved in the processing and loading of lipid antigens for CD1b, CD1c and CD1d presentation (Angenieux et al., 2005; Facciotti et al., 2011).

Recycling pattern

After synthesis, group 1 and 2 CD1 proteins follow the secretory pathway to traffic to the cell surface. The CD1 heavy chains are assembled and glycosylated in the ER and bind to chaperones (calnexin and calreticuin) (D. C. Barral & Brenner, 2007). Some endogenous lipids (e.g. phosphatidylinositol) are considered to be loaded onto CD1 proteins in the ER (D. C. Barral & Brenner, 2007). Binding to β2m facilitates CD1 exit from the ER and trafficking through the secretory pathway (Bauer et al., 1997), allowing

the CD1 complexes to be transferred to the plasma membrane through the Golgi apparatus.

Following egress to the cell surface, the mechanisms of lipid antigen capture and recycling diverge for the CD1 isoforms. CD1a and CD1c can capture lipid ligands at the cell surface, while CD1b and CD1d depend on internalisation and trafficking through the endocytic system (D. Branch Moody & Suliman, 2017). CD1b, CD1c and CD1d contain tyrosine residues in their cytoplasmic tails which bind adaptor protein complexes (AP) and guide trafficking towards endosomes. CD1b and mouse CD1d bind to both AP2 and AP3, where AP2 initialises protein internalisation and AP3 guide the CD1 molecules to the late endosomes and lysosomes (D. C. Barral & Brenner, 2007). CD1c and human CD1d bind to AP2 but not AP3, thus recycle in endosomes but not lysosomes (D. C. Barral & Brenner, 2007). Analysis of AP3-deficient human cells revealed the functional importance of CD1 intracellular trafficking; AP3-deficient cells displayed defective CD1b-restricted antigen presentation but normal function of other CD1 isoforms (Sugita et al., 2002). In contrast to CD1b, CD1c and CD1d, CD1a proteins lack cytoplasmic tail sorting motifs and predominantly stay on the cell surface; however, there is evidence to suggest CD1a can be internalised and undergo a shallow recycling pathway by yet unknown mechanisms (D. Branch Moody & Suliman, 2017).

Antigen binding groove structure

The heavy chains of CD1 proteins consist of three extracellular domains (α1, α2, α3), a transmembrane segment and a short cytoplasmic tail. Similar to MHC class I, the antigen binding region consists of α1 and α2 domains, while the binding clefts of CD1 molecules are deeper, and the total groove volumes larger than MHC class I. CD1 clefts are lined with hydrophobic amino-acid residues, reflecting the specialisation towards lipid antigen binding (D. C. Barral & Brenner, 2007). All CD1 isoforms have binding pockets A' and F', named after MHC Class I A and F pockets by analogous location (D. B. Moody, Zajonc, & Wilson, 2005).

The size of binding grooves of CD1 isoforms is diverse, 1350 A^3 for CD1a, 2200 A^3 for CD1b, 1780 A^3 for CD1c, and 1650 A^3 for CD1d (Dellabona et al., 2015). When lipid antigens are anchored within the CD1 proteins, the hydrophobic alkyl chains of the lipids are buried inside the A' and F' pockets, while polar head groups, if present, are exposed at the surface of CD1-lipid complex (Kaczmarek et al., 2017). CD1a has the smallest binding groove (1350A^3) among the CD1 isoforms. The A' channel of CD1a has a closed end thus limiting the length of alkyl chains to be inserted, as such the CD1a A' channel is considered likely to act as a 'molecular ruler' selecting the lipid antigens with hydrophobic tails 18-23 carbon atoms in length (Zajonc, Elsliger, Teyton, & Wilson, 2003). In contrast to other CD1 isoforms, CD1b has two additional channels (C' and T') in the antigen binding region, and the cumulative size of the CD1b binding groove (~2200A^3) is the largest of the CD1 proteins. The large volume and complexity of the

CD1b pockets allow binding of lipid antigens with long alkyl chains. (D. C. Barral & Brenner, 2007) Interestingly, to aid presentation of smaller lipid antigens, CD1b was found to contain endogenous lipids which act as scaffolds to stabilise the protein structure (Van Rhijn et al., 2015).

Responder cell receptors

CD1 molecules present lipid ligands to T cells which bear cognate T cell receptors (TCRs), T cells that respond to different CD1 isoforms have defining TCR characteristics. Natural killer T (NKT) cells are well studied CD1d-restricted T cells, among which invariant NKT (iNKT) cells harbour the invariant TCR α chain (TRAV10 with TRAJ18) and a biased TCR β chain usage (TRBV25-1), while the type II NKT cells display a diverse TCR repertoire (Ogg, Cerundolo, & McMichael, 2019). In contrast, analysis of group 1 CD1-reactive T cells reveals a variety of TCR usage (Van Rhijn et al., 2015). However, invariant TCRs have been identified for certain CD1-lipid complexes. Two invariant T cell subsets are restricted to CD1b presenting mycobacterial glucose monomycolate or GMM: GEM T cells (invariant TCR α chain: TRAV1-2 with TRAJ9, biased TCR β chain: TRBV6-2) and LDN5-like T cells (biased TCR α chain: TRAV17, biased TCR β chain: TRBV4-1) (Van Rhijn et al., 2015).

Interestingly, in addition to TCRs, CD1 proteins can bind other cell surface receptors. Immunoglobulin-like transcript 4 (ILT4), expressed on myeloid cells, is found to bind to CD1d inhibiting recognition by NKT cells. Furthermore, the binding of ILT4 to CD1c was

considered to compete with CD1d thus fine tuning the activation of NKT cells (Li et al., 2012).

In summary, the heterogeneity of CD1 isoforms in tissue distribution, cellular localisation, lipid recycling and binding capacity emphasise their functional specificity and non-redundant roles in immune surveillance.

1.3 CD1a and CD1a restricted T cells

1.3.1 CD1a discovery

CD1a was originally discovered during efforts to identify markers of thymic T cell developmental stages. The discovery was facilitated by the hybridoma antibody generation technique developed by Milstein and colleagues in the 1970s (McMichael et al., 1979). The first anti-CD1a monoclonal antibody (mAb), clone NA1/34, was produced in this way by immunising a BALB/c mouse with human thymocytes. At the time the antigen recognised by this mAb was named Human Thymus Antigen 1 (HTA1). HTA1 was found to be present on 85% of thymocytes, but not on T cells. HTA1 was also found to be expressed by the human thymic leukaemia cell line MOLT4 (McMichael et al., 1979). Using a similar strategy and technique, Schlossman and colleagues independently identified HTA1/CD1a with mAb OKT6 (Reinherz, Kung, Goldstein, Levey, & Schlossman, 1980). With the help of these mAbs, expression of CD1a was also found in thymic acute lymphoblastic leukemia (Bradstock, Janossy, Bollum, & Milstein, 1980; Reinherz et al., 1980) and Langerhans cells (Fithian et al., 1981).

The unified nomenclature of cluster of differentiation 1 (CD1) was established in the First International Workshop on Human Leucocytes Differentiation Antigens in 1984 (Gelin, Boumsell, Dausset, & Bernard, 1984). By this time, researchers had identified and investigated two isoforms of CD1 (van de Rijn, Lerch, Knowles, & Terhorst, 1983). However, the designation of isoforms CD1a, CD1b, CD1c (the third CD1 molecule

identified in 1986 by Amiot and colleagues) was not established until 1987 (Amiot, Dastot, Schmid, Bernard, & Boumsell, 1987).

1.3.2 Molecular cloning

In 1986, cDNA of CD1a (also known as HTA1 or T6) was cloned by Calabi and Milstein from a CD1a high expression mutant of MOLT-4 (Calabi & Milstein, 1986). Using a CD1a cDNA probe, all five CD1 isoform genes were identified within the human genome, and mapped to chromosome 1 (Martin, Calabi, & Milstein, 1986). Additionally, cloning of CD1 cDNA expedited the transgenesis and overexpression of CD1 in immortal cell lines, and has facilitated numerous functional studies.

1.3.3 CD1 antigen-presenting function

By the end of the 1980s researchers understood that the newly identified CD1 molecules shared structural homology with MHC molecules, associated with β2m and were mainly expressed by professional APCs, highly suggestive of antigen presentation functionality.

The first functional study of CD1 interaction with T cells utilised a $CD4^{-}CD8^{-}$ γδ T cell line, IDP2. IDP2 was found to lyse MOLT4 cells in an MHC-independent manner, additionally a CD3 blocking antibody partially abolished the cytolytic effect (Brenner et

al., 1987). This finding led to further efforts in screening $CD4^-CD8^-$ T cell lines for MOLT4 cytolytic potential (Porcelli et al., 1989). From 21 $CD4^-CD8^-$ peripheral-blood T cell lines, one MOLT4-cytolytic cell line (BK6, $\alpha\beta TCR^+$) was identified (Porcelli et al., 1989). MOLT4 cells were at this time known to express CD1a, CD1b and CD1c (McMichael et al., 1979), and CD1 molecules were proposed as the potential mediators of cytolytic IDP2 and BK6 activity. Use of blocking antibodies against CD1a, CD1b and CD1c revealed the cytolytic function of IDP2 and BK6 T cells was dependent on CD1c and CD1a respectively. In addition, the blockade of cell lysis upon CD3 neutralisation suggested a role for the CD3-TCR complex in CD1 recognition. The activation of IDP2 and BK6 cells by CD1 molecules was considered autoreactivity due to the lack of foreign antigens present in the experimental system. However, whether these T cell lines were autoreactive to unmodified CD1 proteins or recognised self-antigens bound to CD1a was still an open question (Porcelli et al., 1989).

The first direct evidence of functional antigen presentation by CD1 was published in 1992 (Porcelli, Morita, & Brenner, 1992). In this study, DN1, a $CD4^-CD8^-$ $\alpha\beta TCR^+$ T cell line, was isolated and demonstrated to respond to CD1 expressing cells treated with antigens from mycobacteria, *Mycobacterium tuberculosis* and *Mycobacterium leprae*. The activation of DN1 was found to be restricted to CD1b, through experimental analysis of C1R cells transfected with different CD1 isoforms (Porcelli et al., 1992). The antigen recognised by DN1 was soon identified as mycolic acid by systematic

fractionation and screening of mycobacterial components (Beckman et al., 1994). Since all the antigens known at the time were peptide antigens presented by MHC proteins, this finding was surprising as mycolic acid is a lipid molecule. The study profoundly extended the understanding of the chemical nature of antigens recognised by the immune system.

In the following decades, more CD1 lipid antigens were identified, including both self-lipids and foreign antigens. CD1 antigens were found to include mycolates, glycosphingolipids and phospholipids. However, the majority of lipid antigens identified during this period were CD1b antigens derived from mycobacteria, which may reflect the focus of CD1 research at this time. Additionally this may highlight a specific function for CD1b in microbial infections among the CD1 isoforms (Matsuda & Kronenberg, 2001). The surprising frequency of human CD1a-autoreactive T cells in peripheral blood primed the study of CD1a self-antigens and autoreactivity, which yielded the identification of numerous CD1a lipid antigens.

1.3.4 Ligands of CD1a and CD1a-TCR recognition

Sulfatide, a self-glycolipid, was the first identified CD1a antigen (Shamshiev et al., 2002), and the sulfatide complex crystal structure was the first atomic structure solved of CD1a protein (Zajonc et al., 2003). The structural analysis revealed that the two alkyl chains of

sulfatide were buried within the A' pocket and F' pocket separately and the headgroup protrudes from the groove for TCR recognition. It was in this study that the authors proposed the concept of a "molecular ruler" to describe the end-closed A' pocket of CD1a, suggesting selection of lipid antigens based on alkyl chain length (Zajonc et al., 2003).

The first foreign CD1a antigen was isolated from *Mycobacterium tuberculosis* (D. B. Moody et al., 2004). Similar to the strategy for identifying CD1b antigens (Beckman et al., 1994), the first step was to separate a T cell line (CD8-2) responding to mycobacterium-treated CD1a-expressing APCs. CD8-2 is a $CD8^{+}$ $\alpha\beta TCR^{+}$ T cell line capable of lysing *M. tuberculosis* infected macrophages (Rosat et al., 1999; Stenger et al., 1997). By separation, purification and functional screening of fractionated *M. tuberculosis*, the CD1a antigen recognised by the CD8-2 TCR was identified as the lipopeptide didehydroxymycobactin (DDM) (D. B. Moody et al., 2004). The molecular structure of CD1a co-crystallised with DDM revealed that the hydrophobic alkyl chain of the antigen was inserted into the A' pocket of CD1a, and the peptidic branches protruded for TCR recognition (Zajonc et al., 2005).

Despite the high frequency of CD1a-autoreactive T cells in peripheral blood suggested in 1989 (Porcelli et al., 1989), few studies were published regarding CD1a-autoreactive

T cells until 2010. This may have been due to the lack of known cell surface markers or tracking techniques for CD1a-restricted T cells. Using K562 cells (a human myelogenous leukaemia cell line with low to absent levels of HLA) transfected with CD1a, de Jong and colleagues overcame the problem of HLA alloreactive responses allowing measurement of CD1 autoreactivity in T cells from different donors.

CD1a-autoreactive T cells were found to be a normal component of human peripheral blood. In contrast, few blood T cells displayed autoreactivity against CD1b, CD1c and CD1d (de Jong et al., 2010). The high frequency of CD1a-autoreactive T cells was also confirmed by de Lalla and colleagues with the limiting dilution method of culturing T cell clones. It was estimated that CD1a-autoreactive cells represent 0.1%~10% of total blood T cells (de Lalla et al., 2011). As such, understanding the nature of the self-lipids presented by CD1a was and is of great importance. Advancements of lipidomics and development of APC-free T cell functional assays were key to characterising CD1a self-ligands. Lipidomic analysis of CD1a eluate revealed a large number of lipid ligands, and APC-free T cell functional assays helped to identify which lipid antigens were recognised by CD1a-autoreactive T cells. Using purified CD1a protein to present antigens to T cells in an APC-free system, researchers avoided the baseline activity caused by autoantigens presented by the APCs that masked responses to candidate lipids.

De Jong and colleagues reported the first lipidomic analysis of CD1a bound autoantigens. Analysis of eluents from purified CD1a protein revealed more than 100 distinct lipid species belonging to several classes, including sphingomyelin (SM), triacylglyceride (TAG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS) and phosphatidylcholine (PC). To identify antigenic ligands of CD1a, de Jong and colleagues developed a CD1a plate bound assay. In this assay, purified CD1a was coated to the bottom of a plate, stripped with acid wash to remove the endogenously bound self-lipids, then pulsed with candidate lipids. CD1a-autoreactive T cells were then cultured in the CD1a bound plate, and antigenicity of the candidate ligands was then assessed by the activation level of T cells. Using this method, the researchers identified several lipid antigens recognised by a CD1a-autoreactive T cell clone, BC2. Surprisingly, in contrast to the previously identified CD1a ligands with large polar head groups, BC2 cells were found to respond to diverse headless skin oils, including wax esters, squalene, TAGs and free fatty acids. Notably, DDM and sulfatide inhibited the activation of BC2 cells (A. de Jong et al., 2014). These observations lead to the development of a hypothesis that a section of the CD1a-responsive TCR repertoire follows an on-until-off model, where the TCR recognises the surface of CD1a protein and this recognition can be disrupted by the binding of lipid ligands with large polar headgroups.

The study of CD1a-autoreactive T cells was greatly advanced by analysis of the BK6 T cell clone (R. W. Birkinshaw et al., 2015; Porcelli et al., 1989). To identify the ligands recognised by BK6 TCR, Birkinshaw and colleagues mixed purified TCR protein with CD1a protein presenting endogenous ligands and isolated the resulting CD1a-BK6 TCR complex and the non BK6 TCR-bound CD1a. By comparing the lipidomic analysis of these two fractions, it was found that self-ligands of CD1a could be characterised as permissive or non-permissive ligands, which either promoted or disrupted BK6 TCR-CD1a recognition respectively. Some phospholipids (including PG, PI and PC) were characterised as BK6-permissive ligands, whereas SM was identified as a non-permissive ligand. A Jurkat cell line transfected with BK6 TCR (BK6 Jurkat) and CD1a-tetramers were developed to confirm these findings. To further characterise CD1a ligands BK6 Jurkat cells were stained with differentially loaded CD1a-tetramer. Tetramer staining intensity acts as a surrogate for the avidity of CD1a-lipid complex to BK6 TCR. Using this strategy, PC and Lyso-PC (LPC) were confirmed as permissive ligands for BK6, whereas SM was confirmed to inhibit binding of CD1a to BK6 TCR. Importantly, the authors also solved two TCR-lipid-CD1a ternary structures: BK6 TCR-CD1a-LPC and BK6 TCR-CD1a-endo, which are the first and only TCR-CD1a complex structures solved to date. These two complex structures showed that BK6 TCR recognises the A' roof of CD1a and had no direct interaction with the lipid ligands. Comparing the binary structure of CD1a bound with either permissive ligands (e.g. LPC) or nonpermissive ligands (DDM, SM, sulfatide), it was observed that the structure of the A' roof was

disrupted with nonpermissive ligands which emphasized the importance of A' roof in BK6 TCR-CD1a recognition (R. W. Birkinshaw et al., 2015).

Recently, BC2 T cells were reported to similarly recognise CD1a bound with foreign small molecules, such as benzyl benzoate and benzyl cinnamate derived from balsam of Peru, farnesol and coenzyme Q2, which were structurally related to the balsam lipids. The CD1a-farnesol complex crystal structure revealed that farnesol is deeply inserted within CD1a. Farnesol displaced self-lipids and unmasked the CD1a surface for recognition by autoreactive T cells (S. Nicolai et al., 2020).

1.3.5 Function of CD1a reactive T cells

In addition to the variety of CD1a ligands recognised, CD1a-reactive T cells harbour large phenotypic diversity. With regards to co-receptor expression, CD1-restricted T cells were initially considered to be limited to the CD4-CD8- population. However, in the late 1990s, two publications reported CD4+ and CD8+ T cells could recognise CD1 molecules in the study of mycobacterial antigens (Rosat et al., 1999; Sieling, 2000). In the following decade, with systematic study of CD1a-autoreactive T cells (de Jong et al., 2010; de Lalla et al., 2011), we gained insight into the phenotype of CD1a-restricted T cells. CD1a-autoreactive T cells were found to be CD4+, CD8+ or double negative with

a diverse TCR repertoire. Furthermore, CD1a-autoreactive T cells were found in both the naïve and memory T cell populations, defined by surface marker expression (CD45RA+ and CD45RO+ respectively). CD45RO+ CD1a-autoreative memory T cells were increased in adult blood comparing to cord blood samples (de Lalla et al., 2011). De Jong and colleagues also found CD1a-autoreative T cells were enriched for expression of the skin homing marker, CLA.

Many studies had revealed the important functions of CD1a-autoreactive T cells in multiple skin diseases, such as allergy to bee and wasp venom(Bourgeois et al., 2015), atopic dermatitis(Betts et al., 2017; Jarrett et al., 2016; S. Nicolai et al., 2020), and psoriasis(Cheung et al., 2016; Kim et al., 2016).

In the context of bee and wasp venom allergy, a proportion of T cells were found to be activated via CD1a proteins. However, the analysis of different chromatographic separation fractions of venoms showed that the stimulatory factors were in the protein rather than lipid-containing fractions. Subsequent analyses found that venom-derived phospholipase A2 (PLA2) was the protein that activated T cells via generation of neolipid antigens, such as lysophospholipids and free fatty acids, from phosphodiacylglycerides. This study revealed a new pathway of CD1a ligand generation *in vivo* by phospholipases (Bourgeois et al., 2015). A similar pathway of neoantigen

generation was confirmed in the context of atopic dermatitis (Jarrett et al., 2016) and psoriasis (Cheung et al., 2016), in which the house dust mite (HDM) PLA2 and endogenous PLA2G4D were found to be responsible for CD1a-reactive T cell activation respectively.

Jarrett et. al. found that in individuals with atopic dermatitis, the frequency of HDM-responsive CD1a-reactive blood T cells was higher than in healthy donors (Jarrett et al., 2016). In addition, the authors also discovered that filaggrin, a skin barrier protein, inhibited PLA2 activity and decreased PLA2-dependent CD1a-reactive T cell activation. Insufficiency of filaggrin is known to associated with atopic skin disease. Despite the PLA2 dependent mechanism, several small molecules found in botanical extracts and commercial skin care products were also confirmed to be contact dermatitis allergens by serving directly as CD1a ligands (S. Nicolai et al., 2020). CD1a-autoreactive T cell clones were used to screen clinically important allergens in skin patch testing kits and found balsam of Peru (a tree oil commonly used in cosmetics) was stimulatory. Further analysis identified benzyl cinnamate and benzyl benzoate within balsam of Peru, as well as structurally related compounds farnesol and coenzyme Q2, were able to bind to CD1a molecules and displace self-lipids. Binding of balsam of Peru lipids unmasked the CD1a surface and therefore enabled the formation of TCR-CD1a complex and downstream T cell activation (S. Nicolai et al., 2020). In another study of contact sensitizers of allergic contact dermatitis, dinitrochlorobenzene, 1,3-benzoquinone,

resorcinol, isoeugenol and cinnamaldehyde were also found able to activate CD1a and CD1d dependent T cell responses. These studies cumulatively supported the pivotal role of CD1a in the immunopathogenesis of dermatitis.

In the context of psoriasis, CD1a was also found to play critical roles in the disease pathogenesis. Using a CD1a-trangenic mouse model, plant-derived lipid urushiol was found to trigger skin inflammation via CD1a-dependent Th17 cell (CD4+ helper T cells that produce IL-17 and IL-22) activation. In addition, treatment with CD1a blocking antibodies alleviated skin inflammation in the CD1a-transgenic mouse with induced psoriasis (Kim et al., 2016). As mentioned above, PLA2-dependent neolipid antigen generation was also found in psoriasis (Cheung et al., 2016). A cytoplasmic PLA2 (PLA2G4D) was found to be expressed by psoriatic mast cells and released by IFN-α-induced mast cell exosome release. The exosomes transferred PLA2G4D to neighbouring CD1a-expressing cells and promoted the neolipid antigen generation for CD1a presentation and T cell activation. A higher frequency of PLA2G4D-responsive CD1a-restricted T cells was also found in both blood and skin samples of psoriasis patients compared to healthy controls (Cheung et al., 2016).

In addition to T cell activation via CD1a expressed by Langerhans cells, CD1a was also surprisingly found to be expressed by skin group 2 innate lymphoid cells (ILC2s)

(Hardman et al., 2017). CD1a expression on ILC2 was shown to be upregulated by TSLP and other alarmins at levels observed in the skin of individuals with atopic dermatitis. The CD1a-restricted T cell response was also found to depend on the activity of another endogenous PLA2, PLA2G4A. ILC2 exposed to heat-killed *Staphylococcus aureus* were found to further activate T cells in a CD1a dependent manner, suggesting a broad role of CD1a-dependent T cell activation in skin inflammation responses (Hardman et al., 2017).

Further to the role of CD1a in skin inflammatory diseases, a study has suggested a pathogenic role for CD1a in autoimmune diseases, such as Graves' disease (Roura-Mir et al., 2005). CD1a was found to be expressed by some dendritic cells in inflamed thyroid tissues. Thyroid-derived lymphocytes were found to recognise and lyse cells in a CD1a- and CD1c-dependent manner, which suggested a potential function for CD1-reactive T cells in tissue destruction in Graves disease.

2 Aims and Objectives

As discussed in the introduction, CD1a-autoreactive T cells are found to be common component of human T cell repertoire (R. W. Birkinshaw et al., 2015; A. de Jong et al., 2014; de Jong et al., 2010). These T cells are also indicated to play important roles in the pathology of several skin inflammation diseases, such atopic dermatitis (Betts et al., 2017; Hardman et al., 2017; Jarrett et al., 2016; Sarah Nicolai et al., 2020), psoriasis (Cheung et al., 2016; Kim et al., 2016), and allergy(Bourgeois et al., 2015; Sarah Nicolai et al., 2020) as well as some autoimmune disease, such as Graves' disease(Roura-Mir et al., 2005). However, the role of CD1a in the immune response against infection disease is not clear yet, especially in the context of virus infection.

Dengue virus as well as several other flavivirus are found to be able to hijack lipid metabolism of infected host cells to facilitate viral proliferation and therefore alter the lipid component of infected cells (Cloherty, Olmstead, Ribeiro, & Jean, 2020; Melo et al., 2018; Rushika Perera et al., 2012), which may affect the antigen presentation of CD1a. In addition, Langerhans cells, featured with high CD1a and langerin expression, also serves as primary target of dengue virus infection (Wu et al., 2000). Therefore, it is of interest for us to investigate whether CD1a plays any roles in the early immune response caused by dengue virus infection, and if the function of CD1a-autoreative T cells is affected in dengue infection. In addition, langerin, a C-type lectin highly expressed on the surface of Langerhans cells, has been reported to support CD1a

antigen presentation (Hunger et al., 2004), while many members of C-type lectin family (such as DC-SIGN and mannose receptor) are found to facilitate dengue virus binding and evading host cells (Miller et al., 2008; Tassaneetrithep et al., 2003). This also poses the question that if langerin, like DC-SIGN and mannose receptor, may bind to dengue virus and play some roles in the virus infection.

These hypotheses were tested by investigating the following aims:

Chapter 4 Investigation of if CD1a expression is affected by dengue virus infection.

Chapter 5 Characterisation of CD1a-autoreactive T cells in response to dengue virus infected CD1a-expressed APCs.

Chapter 6 Investigation of the role of langerin in dengue virus infection.

3 Materials and Methods

3.1 Reagents

Blocking antibodies:

Anti-CD1a: OKT6 (in house), HI149 (BioLegend), SK9 (BioLegend)

Anti-CD207: 10E2 (VWR)

Recombinant proteins:

Native Antigens: DENV2 NS1, DENV2 E

R&D system: recombinant human langerin (CD207)

Flow Cytometry reagents (dilutions):

Invitrogen: fixable live/dead Aqua (1:1000)

Thermo Fisher Scientific: CellTrace Violet (1:1000), CellTrace CFSE (1:1000)

BioLegend: CD1a BV421 (1:100), CD1a APC (1:100), CD1a PE (1:100), CD1a AF700 (1:100), CD3 APC (1:100), CD3 BV711 (1:100), CD4 PE/Cy7 (1:100), CD4 BV711 (1:100), CD8 PerCP/Cy5.5 (1:100), CD8 FITC (1:100), CD25 BV421 (1:100), CD38 PE/Cy7 (1:100), CD69 APC/Cy7 (1:100), CD69 AF700 (1:100), CD207 PE (1:100), CD209 AF647 (1:100), CD206 AF488 (1:100), CD80 Pacific Blue (1:100), CD86 PerCP/Cy5.5 (1:100), HLA-A,B,C AF700 (1:100), HLA-DR APC/Cy7 (1:100)

Novus: 4G2 PE (1:200), 4G2 AF488 (1:200)

3.2 Media and buffers

RHEPES media: RPMI supplemented with 10 mM HEPES (Life Technologies).

R10 media: RPMI supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, 10 mM HEPES and 10% foetal calf serum (FCS).

Complete media: RPMI supplemented with 10% FCS, 100-fold dilution of 100× non-essential amino acids (NEAA), 10 mM HEPES, 5 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine

MACS buffer: PBS with 0.5% bovine serum albumin (BSA) and 0.2 mM EDTA.

FACS buffer: PBS with 3% FCS and 0.2 mM EDTA.

T cell media: RPMI supplemented with 5% heat-inactivated human serum, 100-fold dilution of 100× NEAA, 10 mM HEPES, 5mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 100 IU/ml IL-2.

C6/36 culturing media: L15 media supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

C6/36 inoculating media: L15 media supplemented with 2% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

Vero culturing media: DMEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

Vero inoculating media: DMEM supplemented with 2% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

3.3 Cell line culture

K562 cells: K562 cells transfected with CD1a-expression vector or empty vector (gift form Dr. Branch Moody, Harvard Medical School) were cultured in complete medium supplemented with geneticin G418 as selection reagent for stable expression of transfected proteins. Cells were maintained at 0.4~1.0×106/ml in T-75 cell culture flask, 1:2 split conducted every other day.

C6/36 cells: C6/36 cells were cultured in T-75 closed-cap flasks with C6/36 culturing media at 28℃. The cells were sub-cultured when the confluence achieved 90%. For subculturing, C6/36 cells were removed from the flask bottom by vigorous pipetting and added into the new flasks at 1:4-1:10 dilution.

Vero cells: Vero cells were cultured in T-75 vented-cap flasks with Vero culturing media at 37℃ with 5% CO2. The cells were passaged to the new flasks when the confluence achieved 90%. Trypsin was used for subculture: 1) old media was removed and the cells were washed with 6 ml/flask cold PBS; 2) 3 ml/flask trypsin was then added into flasks and incubated at 37℃ for around 5 minutes to dissociated the cells from the flasks; 3) 10 ml/ flask Vero culturing media was added and cell suspension collected and centrifuged at 500g for 5 minutes; 4) the cells were resuspended in fresh Vero culturing media and added into new flasks (1:5-1:10 splitting).

3.4 PBMC isolation from blood

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood donated by healthy donors (National Health Service (NHS) National Research Ethics Service (NRES) research ethics committee 14/SC/0106). Density gradient centrifugation by using Lymphoprep (STEMCELL Technologies) was used for PBMC preparation: 1) blood was diluted 1:1 with RHEPES media and layered over 15ml Lymphoprep in 50ml falcon tubes; 2) tubes were centrifuged for 20 minutes at 800g with brake switched off; 3) the buffy coat layer was aspirated and washed twice with RHEPES media, centrifuged at 720g for 10 minutes and 600g for 10 minutes; 4) the PBMCs were finally resuspended in R10 media.

3.5 Isolation of T cells from PBMCs

Human primary T cells were separated by MACS CD3 magnetic bead separation (Miltenyi Biotec) from PBMCs: 1) PBMCs were resuspended in MACS buffer (80 µl MACS buffer per 10 million cells) and mixed with CD3 MACS beads (20 µl beads suspension per 10 million cells); 2) The mixture was incubated for 15 minutes at 2-8℃; 3) cells were then washed with MACS buffer (2 ml buffer per 10 million cells) and centrifuged at 300g for 5min; 4) the cell pellets were resuspended in MACS buffer (500 µl MACS buffer per 10 million cells) and were passed through the LS column previously equilibrated with 3ml MACS buffer; 5) the column was then washed with 3 ml MACS buffer 3 times; 6) the CD3 enriched cells were flushed out from the column upon removal from the magnet by adding 5 ml MACS buffer and pushing the plunger into the column. The CD3 enriched cells were then washed and resuspended in T cell media (1 ml media per 1 million cells).

3.6 Monocyte-derived dendritic cell (moDC) culture

moDCs were differentiated from CD14+ monocytes *in vitro* with IL-4 and GM-CSF. Human CD14+ monocytes were separated from PBMCs using CD14 MACS microbeads (Miltenyi Biotech) according to the manufacturer's instruction (akin to the T cell separation process described above). The cells were then cultured with complete media containing 100 ng/ml GM-CSF and 10 ng/ml IL-4 in 6-well plate at 0.5 x 106 cells/ml. After 4-6 days, the expression of CD1a and other surface markers were confirmed by Flow Cytometry.

In the experiments investing the effect of dengue patient serum on moDC CD1a expression. The monocytes were cultured in media supplemented with 5% heat-inactivated serum (from dengue patient or healthy donor) instead of 10% FCS in the complete media, the other supplements for the media were kept the same as described above.

In the experiments investigating the effect of various reagents on moDC CD1a expression. The reagents (including platelet activating factor, recombinant dengue 2 NS1, IL-10) were added at day 1 of moDC differentiation.

3.7 Monocyte-derived Langerhans like cell culture

moDCs were differentiated from CD14+ monocytes *in vitro* with GM-CSF, IL-4 and TGF-β1. Human CD14+ cells were separated from PBMCs and cultured with complete media in the presence of 100 ng/ml GM-CSF, 10 ng/ml IL-4 and 10 ng/ml TGF-β1. To achieve the optimal Langerin expression, the culture media was changed to complete media

supplemented with only 100 ng/ml GM-CSF and 10 ng/ml TGF-β1 after 2 days. At day 4, the cells were stained to confirm the expression of CD1a, Langerin and other surface markers.

3.8 Dengue virus culture

Dengue virus were propagated using Aedes albopictus Larva cell line C6/36. C6/36 cells were replated into T-75 flasks 1 day before virus inoculation to achieve 80% confluence when infected with dengue virus. Multiplicity of infection (MOI) 0.005 was used to inoculate C6/36. The inoculant was diluted in C6/36 inoculation media (5ml per flask) and added into the flasks after removing the culture media. The flasks were then incubated at 28℃ for 4 hours and topped up with 10ml per flask C6/36 inoculation media. The inoculated C6/36 cells were then cultured at 28℃ for 4 days and cytopathogenic effect (CPE) was checked by observing the cells morphology every day. At day 4, if 80-90% CPE was observed, then the supernatant was collected and centrifuged at 1000g for 10min to remove any cell debris. The supernatant might be concentrated by 100 kDa cut-off spin filter unit if necessary. After concentration, the supernatant was aliquoted and kept at -70℃.

3.9 Foci-forming assay

Foci-forming assay was conducted to determine the dengue virus titer in the virus stock. Vero cells were plated in 98-well flat-bottom plate ($1x10^4$ cells/well) and incubated for 2 days at 37℃. At Day 0 of foci-forming assay, virus stock samples were diluted serially (10^{-1} to 10^{-6}) in duplicate or triplicate with DMEM media with 2% FCS. 70µl of medium was

removed from each well of vero cell plate and 50μl of diluted virus suspension was added into the appropriate wells immediately. The plate was then incubated at 37℃ for 2 hours. After incubation, 100μl of overlay (DMEM media containing 2% FCS and 1.5% carboxymethulcellulose) was added into each well. The plate was then incubated at 37℃ for 3 days. At Day 3 of foci-forming assay, media and overlay were discarded from the plate. The plate was washed 5 times with 200μl/well PBS, rinsed once with 100μl/well 4% FA and fixed with another 100μl 4% FA for 10 minutes at room temperature. The plate was then washed with 200μl/well PBS 3 times and incubated with diluted primary anti-envelope antibody (4G2) for 1 hour at 37℃. Plates were then washed with PBS for 2 times and incubated with diluted secondary Ab (goat anti-mouse HRP) for another 1 hour at 37℃. The plate was then washed with PBS twice and incubated with TrueBlue substrate for foci development. ddH2O was added to stop the reaction after foci appeared. The plates were left to dry and the foci in the wells were images and counted with ELISpot reader (Autimmun Diagnostika gmbh ELISpot Reader Classic, Germany).

3.10 Dengue virus inoculation of human cells

To inoculate K562 cells or moDCs with dengue virus, MOI 1 of dengue virus was used. Inoculation was done in 96-well U bottom plates. $1x10^5$ cells were added into each well in the plate and the media removed before 50μl/well inoculant was added into the plate. Cells were resuspended in the inoculant and incubated at 37℃ for 2 hours. After 2 hours, the inoculant was removed with centrifugation at 500g for 5min. Cells were then washed once with pre-warmed culture media to remove the unabsorbed virions and

resuspended in 200µl/well culture media and kept at 37℃ with 5% CO_2. After 2 days, the cells were collected and some cells were sampled to determine the surface markers expression and dengue virus infection rate by flow cytometry.

For FACS staining, the cells were firstly stained with a surface antibody cocktail in FACS buffer for 15 minutes at 4℃. The cells were then washed with FACS buffer twice and treated with fix/perm buffer (BD) for 20 minutes at 4℃. The cells were then washed with 1x perm/wash buffer (BD) and stained with AF488 or PE conjugated 4G2 antibody (anti-flavivirus envelope protein) diluted in perm/wash buffer at 37℃ for 1 hour. After intracellular staining, the cells were then washed 2 times with perm/wash buffer and resuspended in PBS for analysis by flow cytometry.

3.11 IFNγ secretion assay

To discriminate T cells and K562 cells by flow cytometry, K562 cells were stained with CellTracer (Invitrogen) at least one day before co-culture. T cells were co-cultured for 4-6 hours with mock- or dengue-infected EV- or CD1a-K562 cells. IFNγ secretion was performed using the IFNγ secretion assay kit (Miltenyi Biotec) following the manufacture's instruction. Briefly, after the co-incubation, the cells were washed in cold MACS buffer before being resuspended in cold R10 media. Then, the cells were stained with anti-IFNγ mAb conjugated to anti-CD45 mAb and following the addition of 10 ml of pre-warmed R10 media were incubated for 45 minutes to capture cytokine release at 37℃ with slow rotation every 5 minutes to keep the cells suspended. The cells were then

washed with cold MACS buffer and stained with PE-conjugated IFNγ detection antibody as well as fixable live/dead dye and other antibodies for surface markers of interest. Cells were then washed with MACS buffer. If the aim of the assay was to measure frequency of IFNγ secreting T cells, the cells were then fixed with 4% PFA for 10 minutes at room temperature and data were acquired with Fortessa (BD) or Attune NxT (Thermo Fisher Scientific) flow cytometers. If the aim of the assay was to separate CD1a-reactive T cells, then the cells were not fixed and sorted by flow cell sorter.

3.12 *Ex-vivo* ELISpots

Blood polyclonal T cell response to CD1a was assessed by IFN-γ and GM-CSF ELISpot (Mabtech AB, Sweden). T cells were isolated from PBMCs using CD3+ MACS beads as described above and cultured in T cell medium for 3 days to rest.

On day 1 of ELISpot assay, ELISpot plates (96-well multiscreen IP-plates Millipore Corp., MA, USA) were treated with 50 µl/well 35% ethanol for less than 1 minute to prime the membrane and were then washed with distilled water 6 times. The plates were then coated with anti-IFN-γ or anti-GM-CSF capture antibody overnight at 4℃ according to the ELISpot kit instructions. On day 2 of ELISpot assay, polyclonal T cells and mock- or DV-infected K562 cells with were washed 2 times in R-HEPES and resuspended in R10 media. The plates were washed with RPMI 6 times and blocked with 200 µl/well R10 media for 1 hour at 37℃. After plate blocking, 50,000 T cells and 25,000 K562 cells were then added into the each well of the plate in a total volume of 200 µl/well R10 media. T cells alone was included as negative control and PMA/Ionomycin stimulated T cells were

included as positive control. On day 3 of the ELISpot assay, the plates were developed. The plates were washed with PBS 6 times and incubated with 50 µl/well anti-IFN-γ or anti-GM-CSF detection antibody for 2 hours at room temperature. Plates were then washed with PBS 6 times and incubated with streptavidin-alkaline phosphatase for another 1 hour at room temperature. The plates were then washed with PBS 4 times and with distilled water 3 times. Spots were then developed by incubating plates with alkaline phosphatase conjugate substrate kit (Bio-Rad, USA). The plates were left to dry completely. The spots in each well were then imaged and counted by ELISpot reader.

3.13 Cytokine ELISA

IFNγ, GM-CSF, IL-13, IL-22 levels were measured in supernatant collected from co-culture of CD1a-autoreactive T cell clones/lines and CD1a- or EV-K562 cells by ELISA (detailed product information can be found in 3.1 Reagents) following manufacturer's instructions. Briefly, 96-well half area, high protein binding plates were incubated with 100 µl/well cytokine capture antibodies at 4℃ overnight. The plates were then washed 6 times with 0.05% PBS-Tween20, followed by blocking with 200 µl/well 1x ELISA diluent at room temperature for 1 hour and washed with PBS-T 3times. Cytokine standards and diluted samples were then added into the plates as duplicates, 50 µl/well. Diluted samples were prepared by diluting neat supernatant with 1x ELISA diluent. To ensure the cytokine concentration of the diluted samples was within in the measurement range of certain ELISA kits, different dilutions were tested, such as 1:2, 1:10, 1:50. The sample incubation was conducted at room temperature for 2 hours or at 4℃ overnight. Plates were then

washed 6 times with PBS-T and incubated with biotinylated cytokine detection antibody at room temperature for 1 hour. After 6 washes with PBS-T, 100 μ/well horseradish peroxidase (HRP) conjugated streptavidin or HRP-avidin (based on manufacturer's instructions) was added and incubated at room temperature for 0.5 hour. The plates were washed 6 times with PBS-T, and substrate solution (3,3',5,5'-Tetramethylbenzidine, or TMB) was added into the plates. The reaction was stopped by adding 50 μl/well sulphuric acid. The absorbance of at 450 nm wavelength were then measured by ELISA plate reader.

3.14 Flow cytometry

Cells were washed twice in FACS buffer and incubated for 15 minutes at 4℃ with antibodies for surface staining and fixable live/dead stain (Invitrogen) followed by x2 washes with FACS buffer. If the cells were only to be stained for surface markers, the cells were then fixed with 4% PFA at room temperature for 10 minutes or at 4℃ for up to 2 days. If intracellular staining was need, the cells were then incubated with fix/perm buffer (BD) at 4℃ for 20 minutes followed with washing with 1x perm/wash buffer (BD). Cell were then incubated with the antibodies for intracellular staining, followed by 2 washes with 1x perm/wash buffer. The list of antibodies and titrations can be found in 3.1 Reagents. Data were acquired using Fortessa (BD) or Attune NxT (Thermo Fisher Scientific) flow cytometers and analysed by FlowJo software.

3.15 Cytotoxicity assay

The cytotoxicity assay was conducted with the CellTox Green Cytotoxicity Assay kit (Promega Corporation) following manufacturer's instructions. Briefly, the CD8+ CD1a-

autoreactive T cell lines/clones were co-cultured with CellTrace violet labelled K562-EV or K562-CD1a cells with or without DV-infection at 1:1 ratio in a 96-well flat bottom plate. The CellTox Green dye was added into the media at 1:1000 dilution. The co-culture was conducted for 2-4 days. After co-culture, the cells were collected for surface maker staining (as described in 3.16 Flow Cytometry) and fixed with 4% PFA. Flow cytometers were used to analyses the CellTox staining and surface marker expression.

3.16 ELISA-like binding assay

The ELISA-like binding assays were conducted to assess binding of purified langerin and dengue glycoproteins. 96-well half area ELISA plates were coated with 50 μl/well purified dengue glycoproteins (recombinant DENV2 NS1 or DENV2 E from Native Antigens) diluted in DPBS (containing Ca2+). Each condition was performed in duplicate. The coating step was conducted at 4℃ overnight. The plates were then washed with 200 μl/well PBS + 0.05% Tween-20 (PBS-T) 6 times and blocked with SuperBlock™ (PBS) Blocking Buffer (Thermo Fisher Scientific) at room temperature for 30 minutes. After blocking, the plates were washed with PBS-T 6 times and incubated with 50 μl/well recombinant langerin diluted in DPBS (containing Ca2+) at room temperature for 2 hours. The plates were then washed 6 times with PBS-T and incubated with 50 μl/well HRP conjugated anti-FLAG or anti-langerin mAb (diluted in Ca2+ containing DPBS) for 1 hour at room temperature. The plate was then washed 6 times with PBS-T and substrate solution (3,3',5,5'-Tetramethylbenzidine, or TMB) was added into the plates. The reaction

was stopped with 50 µl/well sulphuric acid. The absorbance of at 450 nm wavelength was then measured by ELISA plate reader.

3.17 Protein deglycosylation

PNGase F (New England Biolab) was used to remove the N-linked glycosylation of DENV2 NS1 and DENV2 E protein following manufacture's instruction. In denaturing reaction conditions, dengue glycoproteins were mixed with Glycoprotein Denaturing Buffer (10x) (New England Biolab) and water in 10 µl total reaction volume and heated at 100℃ for 10 minutes. The mixture was then chilled on ice and centrifuged for 10 seconds. 2 µl GlycoBuffer 2 (10X) (New England Biolab), 2 µl 10% NP-40 (New England Biolab) and 6 µl H2O were then added into the tube to make a 20 µl total volume. 1 µl PNGase F was then added into the system and mixed gently. The reaction was conducted at 37℃ for 1 hour. In non-denaturing reaction conditions, the glycoproteins were directly mixed with GlycoBuffer 2 (10x) and water to make a 20 µl total volume. 5 µl PNGase F was then added into the system and mixed gently. Incubation were then conducted at 37℃ overnight (~16 hours). After reaction with PNGase F, the efficiency of deglycosylation was then measured by SDS-PAGE.

3.18 SDS-PAGE

The protein components in the PNGase F treated samples were separated using SDS-PAGE and detected by Coomassie Blue staining. Samples were mixed with NuPAGE LDS Sample Buffer (Thermo Fisher Scientific), NuPAGE Sample Reducing Agent (Thermo

Fisher Scientific) and water and heated at 90℃ for 10 minutes. After cooling on ice, the samples were then loaded onto 4-12% Bis-Tris protein gels (NuPAGE Novex 4-12% Bis-Tris, 1.0mm, 12-well, Thermo Fisher Scientific) and MES SDS Buffer was used as running buffer. Electrophoresis was conducted at 120V for 60 minutes. The gel was then stained with Coomassie G-250 (SimplyBlue SafeStain, Thermo Fisher Scientific) following manufacture's instruction. Briefly, after electrophoresis, the gels were rinsed with water for 5 minutes to remove SDS and buffer salts. Then the gels were incubated with SimplyBlue SafeStain at room temperature for 1 hour with gentle shaking. After staining, the gels were wash with 100 mL of water for 1 hour. 20 mL of 20% NaCl (w/v) solution was then added into the water and continued to wash for another 2 hours or overnight to maximise the sensitivity of detection.

3.19 Statistics

Statistical analyses were performed using GraphPad Prism 8. The statistical methods used for each data are recorded in the figure legends.

4 Dengue infection affects lipid antigen presentation by regulating monocyte CD1a expression

Introduction and Aims

Myeloid cells (including monocytes, macrophages and dendritic cells) are the major targets of dengue virus infection and replication in humans. Specifically, Langerhans cells in the epidermis are the primary targets of dengue virus infection after a person is bitten by an infected mosquito (Wu et al., 2000). Langerhans cells (LCs) are a unique population of epidermis-resident mononuclear phagocytes. They form a network of cells across the skin barrier and serve a function as immune sentinels (Mass et al., 2016). LCs express high level of CD1a and have been shown to be able to present lipid ligands to activate specific populations of T cells (de Jong et al., 2010).

In addition to Langerhans cells in the epidermis, there are several dengue-targeting, CD1a-expressing cell types located in the dermis as well, including CD1a+ dermal DCs and monocyte-derived dendritic cells (moDCs) (Daniela Cerny et al., 2014; Duyen et al., 2017). In a mouse model of dengue infection, subsets of monocytes were found to be recruited to the dengue-infected dermis and differentiated to moDCs. It is considered as a viral virulence mechanism to recruit monocytes and moDCs to the initial infection site to increase the number of targets for infection (Michael A. Schmid & Eva Harris, 2014).

Regarding the overlap of dengue-targeting and CD1a-expressing cells, it is reasonable to hypothesise that the infection of dengue virus in these cells might be detected by the immune system via CD1a on the cell surface. Two important questions related to this hypothesis are:

1) if dengue infection is able to affect the CD1a expression level on these cells directly (infection) or indirectly (cytokines or other soluble factors);

2) if dengue infection is able to alter the CD1a-reactive T cell response via differential antigenic lipids expressed on the surface of infected APCs.

This chapter will focus on the data related to the first question, while the experiments and results related to the second question will be discussed in Chapter 5.

CD1a expression on APCs can be affected by many factors, including cytokines, lipids, several types of drugs, as well as infectious agents and microorganism products (Aquino et al., 2011). *In vitro* and *in vivo* studies generally indicated that CD1 protein expression is upregulated by bacteria and chlamydia, and TLR-2 activation is regarded to play critical roles in this regulation (Aquino et al., 2011). However, infections with protozoa, helminths and several type of virus, such Human Herpes Virus-8, cytomegalovirus and HIV impaired the expression of CD1 proteins (Aquino et al., 2011). Despite the direct infection of the cells, cytokines induced by host immunity as well as antigens and toxins produced by the

invading microorganism could also affect the expression level of CD1 proteins (Aquino et al., 2011). Therefore, it is reasonable to hypothesise that dengue virus infection is able to alter the expression of CD1a in certain type of APCs. However, it is worth noting that although many viruses have been found to downregulate CD1 protein expression in APCs, they probably bear distinct underlying mechanisms. In this regard, it is possible that dengue virus infection might regulate CD1a expression in a different manner and through different mechanisms. This possibility is supported by the findings that unlike most viruses that downregulate human leukocyte antigen (HLA) in infected cells, dengue virus upregulates class I HLA in the infected myeloid cells (Sanchez, Hessler, Demonfort, Lang, & Guy, 2006).

Studies of the regulation of CD1a expression have mainly used the model of monocyte-derived dendritic cells. Monocytes are separated from PBMCs and cultured with IL-4 and GM-CSF for 4-6 days to differentiate into moDCs. Stimuli to be tested for an effect on CD1a expression in moDCs are added in the culture media during the differentiation or maturation stage of dendritic cells; CD1a expression is subsequently measured by FACS .

The key aims of this chapter were to test that if culturing moDCs with serum from dengue-infected patients and healthy donors could influence different CD1a expression levels, to identify the factors in the patient serum that regulate the CD1a expression, and

to test the effect of dengue virus infection on the expression of CD1a in moDCs. In addition, to achieve virus infection in moDCs with a consistent infection rate, a robust protocol of dengue virus propagation and virus inoculation of target cells also needed to be established and optimised. Under the laboratory conditions, dengue virus can be propagated in various cell types, including both the mosquito cell line (C6/36 cells) and a mammalian cell line (Vero cells) and even from monocyte-derived dendritic cells. Dengue virus derived from different cell types displayed various levels of maturation (Wanwisa Dejnirattisai et al., 2011; Slon Campos, Mongkolsapaya, & Screaton, 2018). Although dengue virus produced from C6/36 cells were reported to show higher percentage of partial maturation, C6/36 cells are still the most common cell line used for producing infective dengue virus stock. As a mosquito larval cell line, the optimal culture condition of C6/36 cells has been defined to be at 28℃ as an attached single layer with no need of CO_2 supplement. Under these conditions, C6/36 cells are known to be highly permissive to dengue virus infection and proliferation. 4-7 days after inoculation with low MOI (multiplicity of infection), high level of infective virus can be detected in the supernatant. The supernatant of the culture can be collected and stored in -70℃ for future experiments. The titre of infective virus can quantified by a foci-forming assay using Vero cells with the result presented by foci-forming unit (FFU) per millilitre of stock volume. MOI can then be calculated as FFU per target cells.

4.1 Dengue patient sera regulate CD1a expression in monocyte-derived dendritic cells

CD1a expression level can be affected by multiple factors, such as cytokines, lipid species and IgG (Aquino et al., 2011). moDCs can be derived from primary human blood monocytes by culturing in FCS containing media with IL-4 and GM-CSF; and the cells have high levels of surface group I CD1 expression (Aquino et al., 2011). moDC represent an established experimental model to study the factors affecting the regulation of the expression of CD1a and other group I CD1 proteins. It is known that adding human serum instead of FCS in the culture of moDCs inhibits the expression of CD1a due to specific lipid species (lysophosphatidic acid and cardiolipin) in human serum (Leslie et al., 2008). Dengue infection usually leads to change in status of the host with altered level of serum cytokines, lipids, and other metabolites, which can potentially regulate the expression of CD1a. Therefore, it would be important to assess if serum from healthy donors and dengue-infected patients with different severities could differentially affect the expression of CD1a and other moDC markers, which might shed light on the role of CD1a in the immunology against dengue infection.

Human primary monocytes were isolated by CD14 MACS beads from fresh PBMCs donated by healthy individuals. Monocytes were then cultured in media containing IL-4 and GM-CSF and serum from different sources. After 4 days of culture, moDCs were

stained for several surface markers (CD1a, CD14, CD1b, CD1c, HLA-DR, HLA-A,B,C, CD80, CD86) and assessed by flow cytometry.

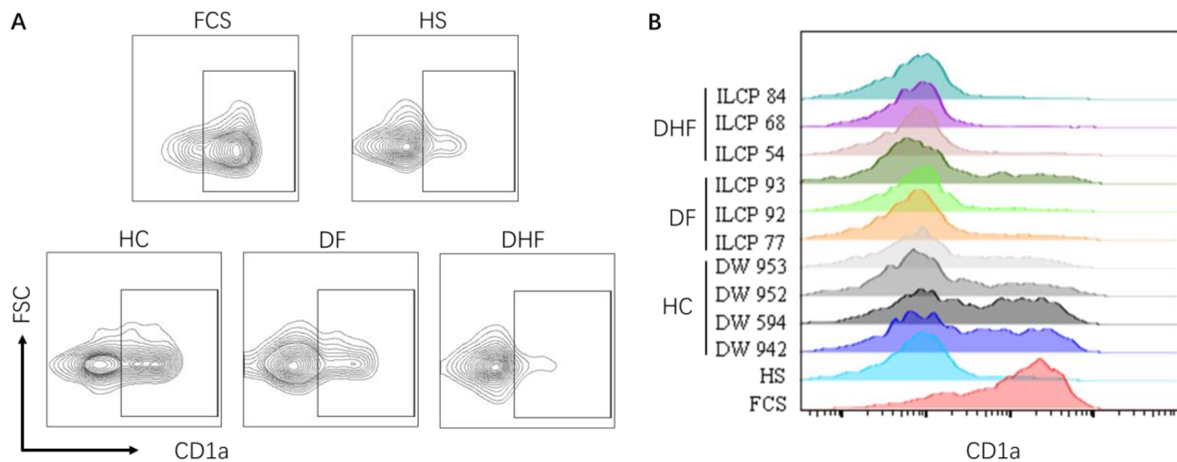

Figure 4.1. Examples of CD1a expression by moDCs cultured with different sera. Monocytes were isolated by CD14 MACS beads from healthy donor PBMC and incubated in media supplemented with IL-4, GM-CSF and different serum (FCS: fetal calf serum; HS: human serum; HC: healthy control; DF: dengue fever; DHF: dengue haemorrhagic fever) for 4 days. (A) Representative FACS plots of CD1a expression level tested by flow cytometry in the moDC derived from monocytes from donor SL1. (B) Histogram of CD1a expression in the moDC cultured with different serum samples.

It was noted that CD1a expression varied between different monocyte donors. To control for the differences caused by cell donors, monocytes from at least two healthy individuals were tested in the same time, with the same settings in each experiment. Figure 4.1 shows

examples of CD1a expression on moDCs from one monocyte donor. In this experiment, the sera samples were collected from 4 healthy control donors (HC), 3 dengue fever patients (DF) and 3 dengue haemorrhagic fever donors (DHF). Fetal calf serum (FCS) was used as positive control for CD1a expression in moDC. moDCs cultured with FCS were observed to have high level of surface CD1a expression as expected. While the CD1a+ percentage was reduced in moDC cultured with human sera, serum samples from dengue patients demonstrate higher level of inhibition of CD1a expression than serum samples from healthy controls. The level of inhibition showed to relate with the disease severity, where less CD1a+ moDCs were detected following culture with sera from DHF donors than moDCs cultured with DF sera.

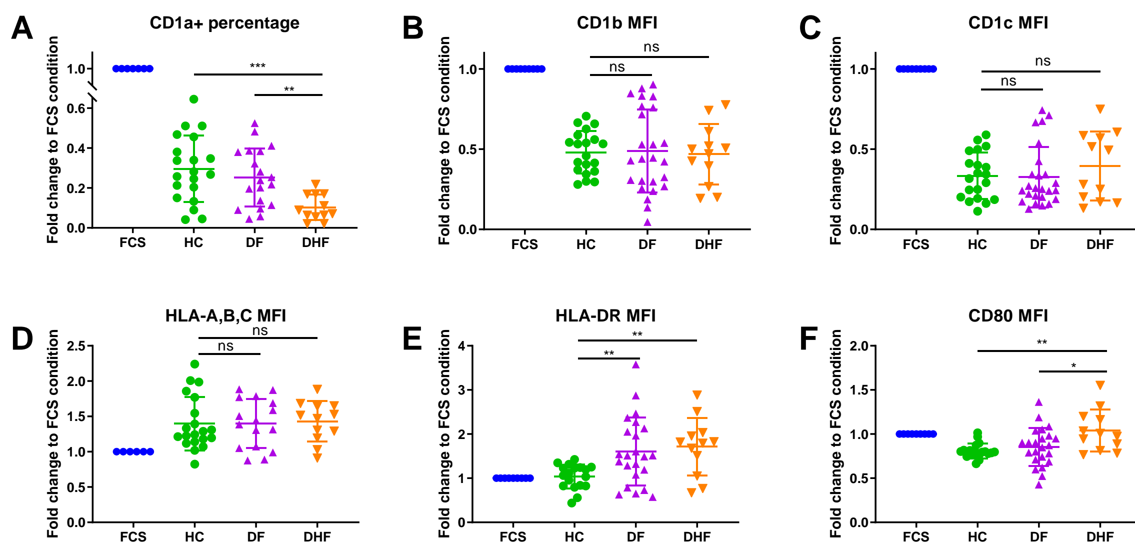

Figure 4.2 Expression of DC surface markers in the moDCs cultured with different sera. Monocytes were isolated by CD14 MACS beads from healthy donor PBMC and

incubated in media supplied with IL-4, GM-CSF and different serum. Expression levels of CD1a (A), CD1b (B), CD1c (C), HLA-A,B,C (D), HLA-DR (E), and CD80 (F) were measured by flow cytometry (The CD1a expression level were measured by percentage because of the clear separation of CD1a+ and CD1a- population (Figure 4.1, B). While the other markers did not show separation of different population, therefore mean fluorescent index (MFI) better reflected the expression level of certain markers.). To control for the variation between monocyte donors, the expression level of all surface markers were adjusted to the fold change of expression level in the FCS condition.

While CD1a expression level was observed to be different in the moDCs cultured with human sera from different donor groups (CD1a+ percentage: HC > DF > DHF), the expression levels of other group I CD1 (CD1b and CD1c) were not affected (Figure 4.2), which indicated that the regulation of CD1a was specific. In addition, there was no difference observed on the expression level of HLA-A,B,C. The expression level of HLA-DR and DC maturation marker CD80 was observed to increase with the serum from patients with severe disease (MFI: HC < DF < DHF), which indicated the higher level of maturation of the moDCs cultured with patient sera, especially the sera from patients with severe disease. This matured phenotype of moDCs might be caused by the higher level of inflammatory cytokines present in the serum from dengue patients.

4.2 CD1a expression level is associated with IL-10 level in patient sera

To determine the factors in the serum from dengue patients that affect the CD1a expression in the moDCs, several serum components which have been shown to be elevated in dengue patient sera and demonstrated to be correlated with disease severity were considered, including platelet activating factor (PAF) (Jeewandara et al., 2015), dengue NS1 protein (D. H. Libraty et al., 2002) and IL-10 (Malavige et al., 2013). The effect of these components in CD1a expression in moDCs were tested by adding the purified reagents in the culture of moDCs at day 0 and measuring the CD1a expression level by flow cytometry on day 4 of moDC culture.

Platelet activating factor (PAF) is a phospholipid mediator involved in various physiological processes. In humans, PAF can be synthesised by a variety of cell types, such as platelets, macrophages, granulocytes (neutrophils, basophils, eosinophils and mast cells) and endothelial cells (R. Lordan, Tsoupras, & Zabetakis, 2017). Although usually considered as a pro-inflammatory molecule, some studies also demonstrated an anti-inflammatory function of PAF (Ronan Lordan, Tsoupras, Zabetakis, & Demopoulos, 2019). Higher level of PAF were detected in dengue patient sera compared to its level in healthy individuals (Jeewandara et al., 2015). The increase of circulating PAF is also correlated with disease severity and shown to contribute to the dysfunction of endothelia cells and vascular leak in severe disease (Jeewandara et al., 2015).

As PAF level increased in the dengue patient sera, while CD1a level decreased in the moDC cultured with patient sera, we considered whether PAF could inhibit the expression of CD1a in moDCs. To address this hypothesis, monocytes was cultured with media suppled with IL-4, GM-CSF and different concentration of PAF (125ng/ml, 250ng/ml, 500ng/ml). Due to the possibility that PAF might need to function on moDCs with other serum components, FCS or human serum (HS) were used in addition with concentration gradient of PAF. However, no difference of CD1a expression was observed within each of the serum conditions with various concentration of PAF (Figure 4.3).

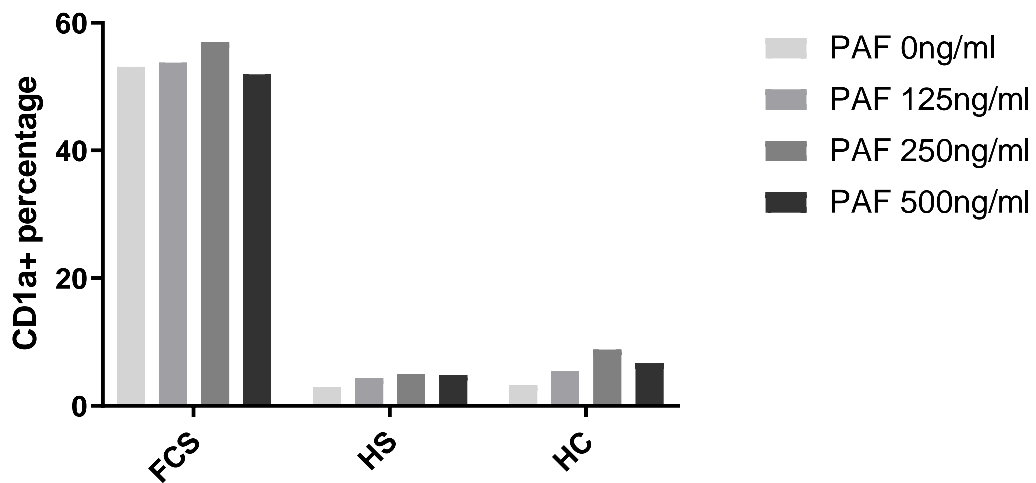

Figure 4.3 Effect of PAF on the CD1a expression in moDCs. Monocytes from one representative healthy donor are shown, isolated by CD14 MACS beads from PBMC and incubated in media supplied with IL-4, GM-CSF and different sera (FCS or HS). Concentrations of PAF are 0ng/ml, 125ng/ml, 250ng/ml and 500ng/ml in the conditions. CD1a expression level in the moDCs was measured by flow cytometry.

NS1 protein is one of the non-structural proteins encoded by the genome of dengue virus. It is present as either membrane-bound dimers in the infected cells or as soluble barrel-like hexamers secreted outside of the infected cells (Watterson, Modhiran, & Young, 2016). Interestingly, lipid moieties are carried in the centre of soluble hexamers of NS1 protein with unknown functions. Soluble forms of NS1 were also reported to contribute to the vascular leak (Watterson et al., 2016). Increasing levels of circulating dengue NS1 were found in the sera of severe dengue patients than in sera of patients with dengue fever (D. H. Libraty et al., 2002). Because NS1 was reported to bind monocytes or moDCs and activate the cells (Modhiran et al., 2015), it was possible that dengue NS1 might be one of the factors that affect the CD1a level in moDCs cultured with dengue patient sera.

To address the effect of dengue NS1 on moDC CD1a expression, 10ug/ml of recombinant dengue 2 NS1 purified from HEK293 cells (Native Antigen) was added into the moDCs culture supplied with FCS. However, no difference on surface CD1a expression level was observed in the presence or absence of recombinant dengue NS1 protein (Figure 4.4).

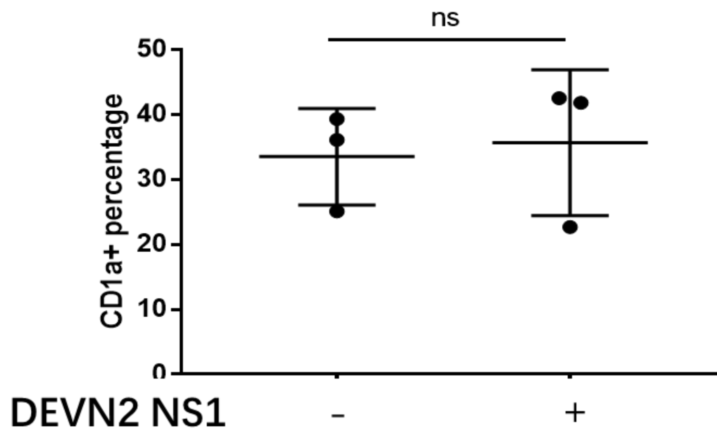

Figure 4.4 Effect of dengue NS1 on the CD1a expression in moDCs. Monocytes from 3 healthy donors were isolated by CD14 MACS beads from PBMC and incubated in media supplied with IL-4, GM-CSF and FCS. 10ug/ml of recombinant dengue 2 NS1 was added in the moDC culture on Day 0. CD1a expression level in the moDCs was measured by flow cytometry on Day 4.

IL-10 was reported to inhibit CD1a expression in moDCs (Allavena et al., 1998). Increasing levels of IL-10 were reported to exist in dengue patient blood and associated with disease severity (Malavige et al., 2013). To assess the contribution of IL-10 on the regulation of CD1a expression in moDC cultured with dengue patient sera, the IL-10 level in the sera samples used for the moDC experiments was measured by ELISA (figure 4.5, A). Consistent with published studies (Malavige et al., 2013), increasing IL-10 concentration was found in the dengue patient sera, especially in the patient with DHF, while no human IL-10 could be detected in the FCS as expected.

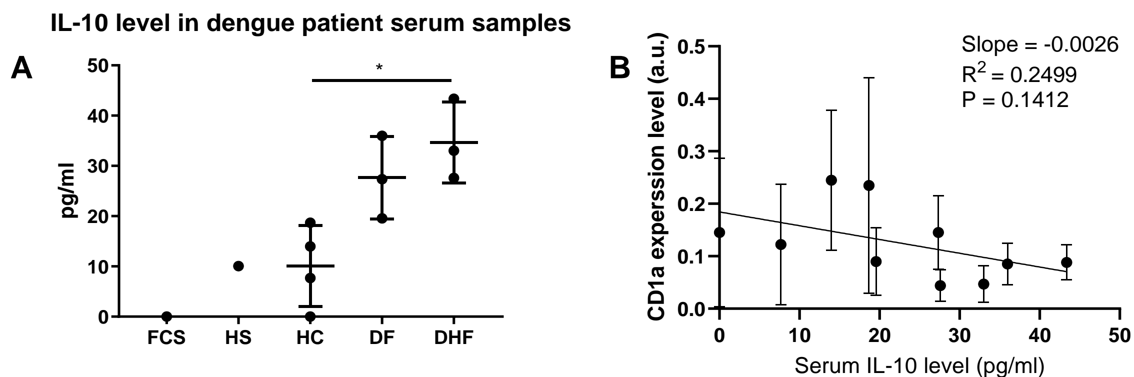

Figure 4.5 Increased level of IL-10 in dengue patient serum may contribute to the inhibition of CD1a expression. (A) IL-10 concentration in serum samples used for moDC culturing experiments was measured by ELISA. Sample tested: 4 healthy controls, 3 DF samples, 3 DHF samples. One-way ANOVA with Sidak's multiple comparisons test, *P<0.05. (B) Correlation of moDC CD1a expression level and serum IL-10 concentration associated with the same serum sample. The correlation was analysed by simple linear regression.

When the data correlation was analysed (figure 4.5, B), moDC CD1a expression level demonstrated a trend of negative correlation against serum IL-10 level, although the P value (0.1412) indicated that the linear regression slope was not significantly deviated from zero. However, this correlation was not enough to demonstrate that the serum IL-10 was among the serum component that decreased the moDC CD1a expression. To demonstrate the direct contribution of IL-10, IL-10 neutralising antibody could be used

in the future moDC CD1a expression experiments. However, since the linear regression slope of this correlation analysis was not significantly deviated from zero, IL-10 is not likely to be the only contributor of the moDC CD1a downregulation induced by dengue patient serum.

4.3 Dengue virus infection affect CD1a expression in moDCs

To investigate how dengue virus infection affects the CD1a expression in moDCs, dengue 2 virus (SL 5-17-04) was propagated and used for inoculating moDCs (cultured for 4 days). The infection rate and CD1a expression level was measured after 2 days by flow cytometry.

Dengue virus was propagated using the mosquito larva cell line, C6/36. After inoculation, dengue virus rapidly replicated in the infected cells and was released into the supernatant after assembling to infect other cells. Morphology and viability of C6/36 cells were affected by dengue virus infection. Cytopathic effect (CPE) was observed in the infected C6/36 cells several days after inoculation. Dengue virus in the cell culture will lose infectivity if insufficient live cells remain to be infected. Therefore, to achieve an optimised titre of infectious viral particle in the culture, multiple factors need to be optimised in the process, such as initial MOI, initial cell density and collecting time after inoculation. Other factors, like area/volume of culture flask may also affect the outcome.

To control the number of tested factors, Initial MOI was set at 0.005 and initial cell density was set as the cell confluency reached 80%. Supernatant of infected C6/36 cells was collected at D4 and D6 after inoculation. The supernatant was aliquoted and kept in -80℃ immediately after collection until used to determine infectious virus titre by Foci-

forming assay (FFA). To define the optimised protocol of virus propagation, two experiments were designed to compare the culture in T25 flask and T75 flask, and the virus titre at different time points after inoculation (Figure 4.6).

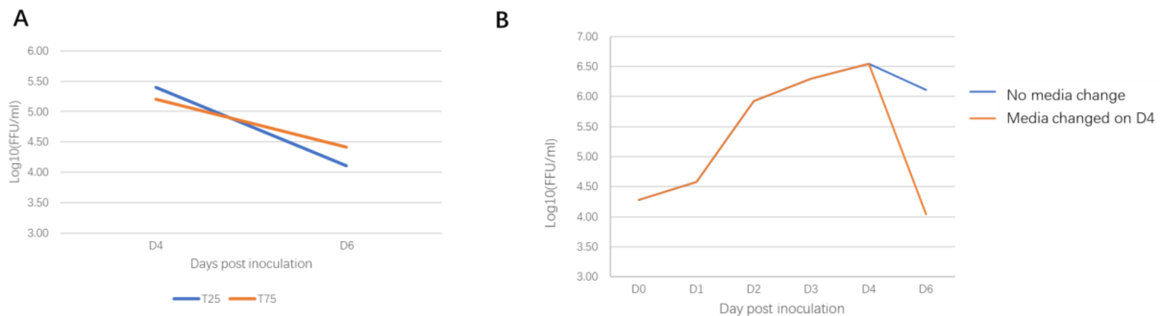

Figure 4.6 Optimisation of dengue virus propagation in C6/36 cells. C6/36 cells were inoculated with dengue 2 virus at MOI 0.005 when the cell confluency was approximately 80%. Samples of supernatant were collected at D0-D6 after inoculation and were frozen at -80 until further tests. Foci-forming assay (FFA) was used to determine the infectious virus titre. (A)C6/36 cells were cultured in T25 or T75 flasks, supernatant was collected on Day 4 and Day6 after inoculation. (B)C6/36 cells were cultured in two T75 flasks. Samples of supernatant were collected at D0-D4 and D6. Media was changed on Day 4 (Orange line), or not changed (blue line).

No significant difference of virus titre was observed in the culture using T25 flasks or T75 flasks (Figure 4.6, A). In the time-course experiments, two flasks of C6/36 cells were cultured and inoculated under the same conditions. 0.5ml of supernatant were collected

both flasks from Day 0 to Day 4 and were frozen at -80℃. At Day 4, supernatant was removed and refilled with fresh media in one flask, while the media was not changed in the other flask. At Day 6, supernatant was collected and frozen at -80℃. Virus titre was observed to peak at Day 4 in both flasks, while change in media largely reduced the amount of infectious viral particles collected on Day 6 (Figure 4.6, B). Based on these results, culturing C6/36 in T75 flask and collecting the supernatant of infected cells at Day 4 post inoculation were fixed in the following experiments.

To inoculate moDCs with dengue virus, moDCs were cultured as described previously for 4 days, dengue 2 virus with MOI 1 was used for the moDC inoculation. 2 days after inoculation, moDCs were stained for intracellular dengue E protein and surface CD1a and DC-SIGN expression. DC-SIGN is a C-type lectin expressed on dendritic cells and helps the dengue virus binding on the target cells (Tassaneetrithep et al., 2003).

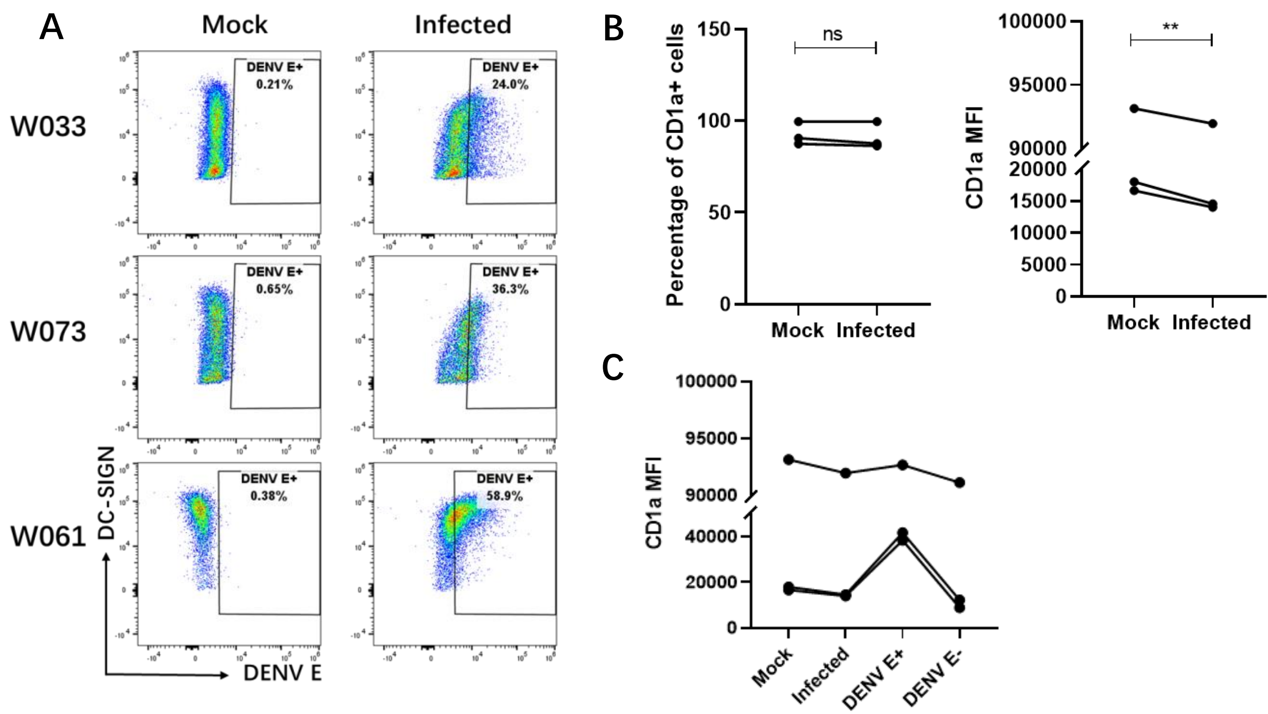

Figure 4.7 CD1a expression in moDC infected by dengue virus. moDCs were derived from monocytes, cultured with IL-4 and GM-CSF for 4 days, N=3. The moDCs were then infected with dengue virus, MOI=1. At 2 d.p.i, the cells were collected and stained for surface marker expression and intracellular dengue E antigen. (A) Dengue virus infection was detected in all the moDCs samples tested. (B) The CD1a+ percentage was not changed by dengue virus infection, while the CD1a MFI was slightly reduced in moDCs in the DV-infected condition. (C) The moDCs in the DV-infected condition was separately analysed based on the presentence of DENV E antigen: DENV E+ cells are DV-infected moDCs, DENV E- cells are bystander cells in the same culture. Compared to the average level of CD1a MFI in the mock and infected conditions, CD1a level was higher in the DENV E+ cells while lower in the DENV E- cells. Paired t test, ns: not significant, **: $p<0.01$.

In the moDC cultured derived from 3 different donors, the dengue virus infection was confirmed by intracellular DENV E staining (figure 4.7, A). The CD1a expression level was measured by CD1a+ percentage and MFI in both mock-infection conditions and DV-infection conditions. There was no significant difference between mock and DV-infected conditions, however, CD1a MFI was slightly decreased in the DV-infected samples (figure 4.7, B). Interestingly, the CD1a MFI level was higher in the DENV E+ cells in the DV-infected conditions compared to the DENV E- bystander cells in the same culture, although this was not statistically significant. Further experimental repeats are required.

Chapter 4 Summary

These data showed that the CD1a expression level in moDC was downregulated by dengue patient serum, with the CD1a level lower in the moDCs cultured with the serum from patients with severe disease – dengue haemorrhagic fever (DHF). Several components known to correlate with severity, such as PAF and dengue NS1 protein were tested in the moDC culture. However, both reagents demonstrate no effect on the moDC CD1a expression. IL-10 is known to be associated with severe dengue (Malavige et al., 2013) and inhibited CD1a expression in moDCs (Allavena et al., 1998). The IL-10 level in the serum samples used for culture moDC was tested by ELISA, and the results was correlated to the inhibition of CD1a expression in moDCs. Therefore, IL-10 is possibly one of the components in the sera of dengue patients contributing to the downregulation of moDC CD1a expression.

The direct infection of dengue virus in the moDCs affected the CD1a expression level in a different manner. Although the overall CD1a surface level was not significantly different between mock-infected moDCs and dengue-infected moDCs, the CD1a expression appeared higher in the infected cells comparing to the bystander cells in the same culture. However, whether this difference was caused by regulation of CD1a expression or by selective infection could not be distinguished. While further experimental repeats are required, one of the potential explanations of decreased CD1a expression in the bystander cells could be the effect of type I IFN, which was reported to downregulate

CD1a in both moDCs and primary DCs (Aquino et al., 2011). Higher levels of type I IFN were found to be released by DV-infected moDCs (Hamlin et al., 2017), however, the downstream signalling was blocked in the DV-infected moDCs (Castillo Ramirez & Urcuqui-Inchima, 2015), which could explain why the CD1a downregulation was only found in the DENV E- bystander cells. However, it was of interest that CD1a expression level was higher in the DENV E+ cells, even compared to the CD1a level in the mock-infected moDCs. Two possibilities could be considered: 1) CD1a could facilitate dengue virus infection, leading to infected cells being found to be associated with higher surface CD1a levels; 2) CD1a does not play any roles in the virus infection, instead, the expression of CD1a is associated with subsets of moDC which are easier to infect by dengue virus. These hypotheses will be tested in future work.

To investigate the other possible components in the serum of dengue patient that affect CD1a expression in moDCs, dengue patient serum can be pooled and fractioned by organic phase separation methods (Leslie et al., 2008). By testing the function of each fraction on regulating the moDC CD1a expression, the properties of the bioactive factors can be defined.

Schmid and Harris found that in the murine model of dengue infection, monocytes were recruited to the skin infection site and developed into moDCs, which can be infected by

dengue virus and can contribute to further viral dissemination (M. A. Schmid & E. Harris, 2014). However, the role of moDC in human dengue infection is less studied. Since the data in this chapter demonstrated that some component in the dengue patient serum reduced CD1a expression in the moDCs, it is of interest to ask that what is the biological function of this CD1a downregulation in the infection and immunity against dengue virus. Presenting lipid antigen to activate certain T cells was the main function of CD1a. Therefore, the key to answer the above question is to investigate the role of CD1a-restricted T cells in the context of dengue virus invading, which will be discussed in Chapter 5.

5 CD1a-reactive T cells response to dengue virus infected antigen presenting cells

Introduction and Aims

T cells play complex roles in the host immune response against dengue virus infection, with both protective and pathological functions having been reported(Yuan Tian, Grifoni, Sette, & Weiskopf, 2019).

Murine studies demonstrated that CD8 T cells as well as CD4 T cells can play a protective role against DENV challenge (Elong Ngono et al., 2016; Lauren E. Yauch et al., 2010; Zellweger, Eddy, Tang, Miller, & Shresta, 2014; Zellweger et al., 2015). Furthermore, studies analysing the T cell response in the population in endemic areas of dengue virus revealed that certain HLA alleles were associated with the protective role of T cells (Grifoni et al., 2017; Weiskopf et al., 2013). In addition, some T cell populations could also produce anti-viral cytokines, such as IFN-γ and GM-CSF (detailed roles of IFN-γ and GM-CSF in dengue infection will be discussed in the Summary part at the end of this Chapter).

However, the role of cytokines in the context of dengue infection is complex. Cytokine storm was suggested to be one of the most important pathological factors in severe dengue infection (Srikiatkhachorn, Mathew, & Rothman, 2017). T cells were also considered to contribute to the excessive production of inflammatory cytokines

(Srikiatkhachorn et al., 2017). Antigenic sin was one of the hypotheses related to the immunopathological roles of T cells, potentially helping to explain the observation that secondary dengue infection with altered virus strains usually shows a more severe clinical outcome (Mongkolsapaya et al., 2003). This hypothesis suggested in secondary dengue infection, pre-existing cross-reactive and low-affinity memory T cells contributed to the production of excessive level of inflammatory cytokines rather than effectively controlling the virus infection (Alan L. Rothman, 2011; Screaton, Mongkolsapaya, Yacoub, & Roberts, 2015).

In summary, the role of T cells in dengue infection is complex and could depend on the T cell subtypes, resident tissues and the general host immune state. Specifically, the role of CD1a-autoreactive T cells in dengue infection has not been studied so far.

CD1a-autoreactive T cells are defined as T cells able to response to CD1a-presented endogenous lipid ligands. This T cell population was found to be a common fraction of the human T cell repertoire (de Jong et al., 2010). Lipidomic analysis was used to show that common self-lipids that could be recognised by TCR via CD1a presentation, including SM, free FA, DAG, PC and LPC (Annemieke De Jong et al., 2014). The lipid ligands of CD1a can be either permissive or non-permissive, based on whether the CD1a-ligand complex could bind to TCR and activate T cells or not (Cotton, Shahine,

Rossjohn, & Moody, 2018). It is suggested that by this refined regulation of CD1a-autoreactive T cells, the lipid environment of epidermis is under surveillance (Annemieke De Jong et al., 2014).

Lipids are the major component of dengue virus envelope. Therefore, the lipid metabolism in the infected host cells has critical roles for the replication and release of viral particles. It was reported that the lipid metabolism in the host cells was hijacked by virus in order to benefit the virus replication (Heaton & Randall, 2010; Koh et al., 2020). However, due to lack of virus-encoded enzymes related to lipid metabolism, dengue virus infected host cells are not able to synthesise exogenous lipid species. Therefore, if the change of lipid profile could be detected by the CD1a system, any involved lipid species could be self-lipid and thus potentially activate or inhibit CD1a-autoreactive T cells.

We hypothesized that the function of CD1a-autoreactivated T cells would be affected during dengue infection and might play roles in the immunology or immunopathology of dengue infection based on the following observations: 1) Langerhans cells were one the target cell type of dengue virus in skin (Wu et al., 2000); 2) dengue virus infection altered the lipid profile of the host cells (Heaton & Randall, 2010; Koh et al., 2020), and might alter the ligand repertoire of CD1a; 3) CD1a-autoreactive T cells were a common

fraction of human skin T cell population and blood T cell population. The function of these T cells were regulated by the type of self-lipid ligands presented by CD1a (Annemieke De Jong et al., 2014); 4) CD1a-autoreacitve T cells were reported to have multiple functions which might contribute to the immune response to dengue infection, such as cytotoxicity and production of various cytokines, including IFN-gamma and GM-CSF.

The main aim of this chapter was to identify the influence of dengue infection on the response of CD1a-autoreactive T cells. To achieve this aim, the following challenges needed to be solved: 1) to determine an antigen presenting cell line with CD1a surface expression could be effectively infected by dengue virus *in vitro*; 2) to develop T cell functional assays with co-cultured DENV-infected APCs and CD1a-autoreactive T cells; 3) to refine the design of control groups and to specify the CD1a-related alteration of T cell response.

K562 cells were frequently used as artificial APCs in the studies of CD1-reactive T cells with the benefit of lacking surface expression of MHC protein. In this study, K562 cells had been engineered to express CD1a at cell surface at high density (K562-CD1a), and were kindly donated by Branch Moody (Harvard Medical School). This system largely bypasses MHC alloreactivity and therefore facilitates the study of CD1a-specific T cell

response. In addition, K562 cells are permissive to dengue virus and used in various dengue studies as target cells *in vitro*, although the receptor of dengue virus in K562 cells was not clear (Cruz-Oliveira et al., 2015; I. Kurane, Kontny, Janus, & Ennis, 1990).

As discussed before, the study of T cell function focused on the change of cytokine production and cytotoxicity. Cytokine secretion assays and ELISA were chosen to assess the change of cytokine production in CD1a-autoreactive T cell clones. ELISpot or Flurospot assay have been used to detect a CD1a-autoreactive response in blood polyclonal T cells due to their high sensitivity.

T cells were also reported to be permissive to dengue virus infection which could influence their proliferation *in vitro* and in a humanised mouse model (Mota & Rico-Hesse, 2011; Silveira et al., 2018), although limited evidence has been found that T cells could be infected by dengue virus *in vivo*. The possibility that T cell function could be affected by dengue virus directly needs to be considered and to be excluded by experiments when explaining the influence of DENV-infected APCs on CD1a-reactive T cell response. A low concentration of Glutaraldehyde (GA) is able to fix the cell surface and could be possibly used to inhibit the release of viral particles from infected K562 cells in the culture. In addition, high concentration of anti-flavivirus envelop (E) protein antibody 4G2 could bind to the E protein on the virus surface and stop the viral entry.

Both methods could reduce the direct influence of dengue virus on T cells. However, there were flaws with both methods: GA fixation might affect the T cell activity by altering the molecular structure and function of surface CD1a protein; using 4G2 to block virus infection is not complete and the consequence of ongoing infection needs to be carefully considered.

5.1 CD1a-transduced K562 cells are permissive to dengue infection and can be used as artificial antigen-presenting cells

To investigate the CD1a-related T cell activation, K562 cells transfected with CD1a molecules (K562-CD1a) were used as artificial antigen-presenting cells (APCs). K562 cells have very low levels of HLA-I and HLA-II expression and therefore largely bypass T cell alloreactivity, reduce the background of CD1a-related T cell activation and allow testing the function of T cells from different donors under equivalent conditions. (Wu et al., 2000)

To inoculate K562 cells, the cells were mixed with dengue virus at MOI 1 and incubated at 37℃ for 2 hours (mock infected cells were incubated with media without dengue virus). After virus absorbance, the inoculant was then removed, the cells were washed and cultured in fresh media. Infection rate was determined by intracellular staining of virus envelope (E) protein and accessed by flow cytometry. The DENV E+ gate was set by the mock control.

40-70% of K562-EV cells and around 90% K562-CD1a cells were infected at 2 days post inoculation (dpi) (Figure 5.1, A). CD1a expression level was not affected by dengue virus infection (Figure5.1, B).

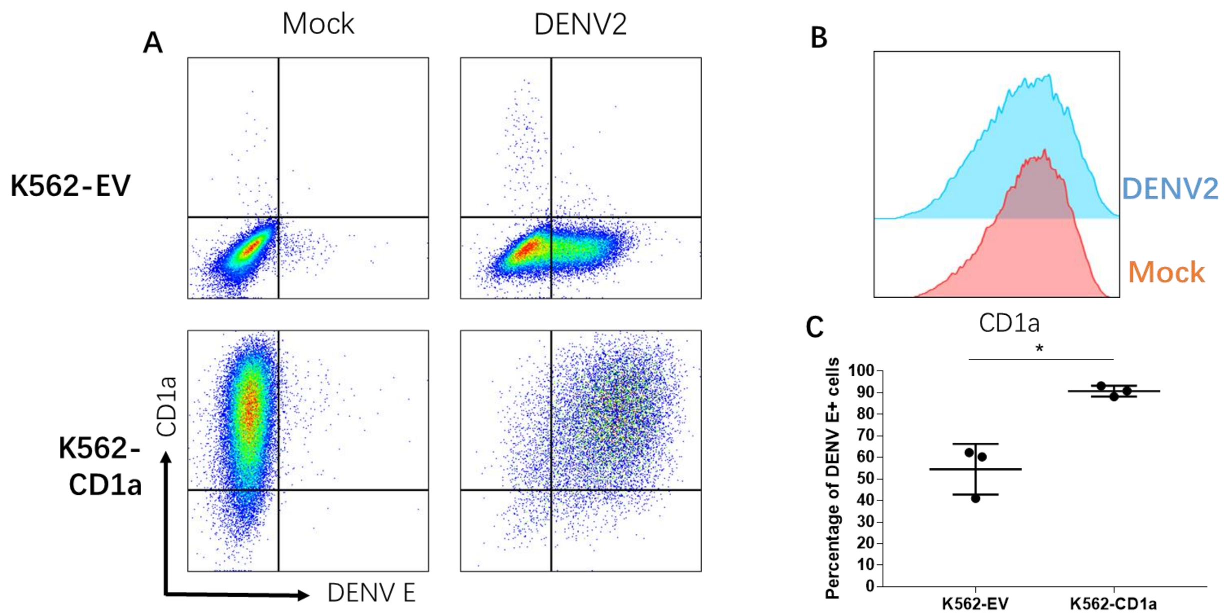

Figure 5.1 K562 cells were able to be infected by dengue virus without alteration of CD1a surface expression. K562-EV (A, upper figures) and K562-CD1a (A, lower figures) cells were inoculated with dengue 2 virus (SL 5-17-04) at MOI 1. Cells were stained with surface BV421 conjugated anti-CD1a mAb (HI149) and intracellular PE conjugated anti-flavivirus E protein mAb (4G2). (B) Histography shows the CD1a surface staining level on dengue infected and mock infected K562-CD1a cells. (C) Infection rate of K562-EV and K562-CD1a cells at 2 days after inoculation. Data represent results from 3 independent experiments. Bars represent standard error. Student T test, * $P<0.05$

Interestingly, although K562-EV and K562-CD1a cells were infected under the same conditions, K562-CD1a cells always showed a higher infection rate than K562-EV cells (Figure 5.1, C). The reason of the different infection rate is not clear, possible explanations include the different proliferation rate or other phenotype alternations during and after

genetic manipulation. However, there were also possibilities that CD1a might be able to promote the process of dengue virus infection in host cells.

5.2 T cells are infected after co-culture with dengue-infected K562 cells

Silveira and colleagues (Silveira et al., 2018) reported that T cells are permissive to dengue virus infection. To assess if T cells was able to be infected by dengue virus release from infected K562 cells, T cells were either co-cultured with dengue infected K562 cells or incubated with supernatant derived from dengue infected K562 culture. These cells were intracellularly stained for DENV E protein after overnight co-incubation. Figure 5.2 shows mixing T cells with infected K562 cells or supernatant from dengue infected K562 culture were both able to lead to detection of dengue antigen in a subset of T cells.

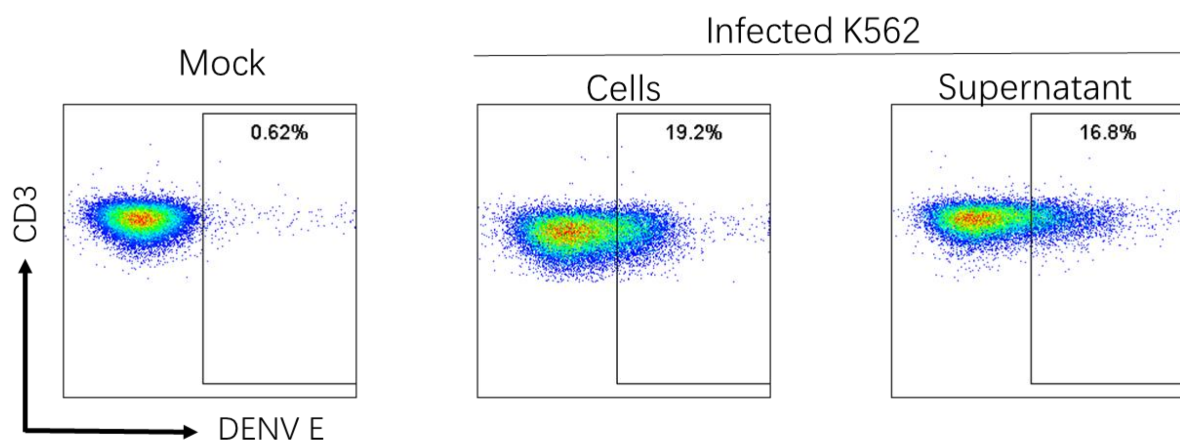

Figure 5.2 Dengue viral antigen were detected in T cells after incubated with infected K562 cells or supernatant from infected K562 culture. T cells were co-

cultured with mock infected K562 (left), or dengue infected K562 cells (middle), or incubated with supernatant from dengue infected K562 culture (right) for 16 hours. T cells were gated as live cells, singlets and CD3+ cells.

However, detecting dengue E protein in the T cells only indicated that some viral particles were present intracellularly in the T cells. It is not enough to claim that T cells are permissive to dengue virus infection, in which virus replication is an essential component (Silveira et al., 2018). Dengue NS3 protein is one of the dengue non-structural proteins and plays essential roles in the virus replication. Therefore, intracellular NS3 staining is used as a marker for the active replication of virus in the host cells. Silveira and colleagues (Silveira et al., 2018) showed that E protein and NS3 protein double-positive T cells were detected in the *in vitro* infection experiments, which indicated that T cells were permissive to the dengue virus infection and replication. However, in our study, the T cells were only cultured with dengue-infected APCs for up to 24 hours, which may be too short for the reliable detection of any virus replication related protein markers. On the other hand, the entrance of dengue virus in T cells without any active replication might be still sufficient to affect the T cell function. Therefore the next key question was how to inhibit the entrance of dengue virus in T cells.

5.3 Glutaraldehyde-fixation of infected K562 cells prevents T cells being infected by dengue virus after co-culture

It is possible that viral particles released from infected target cells may influence the T cell response and confound the interpretation of the functional outcomes via CD1a related mechanism. To avoid this influence, glutaraldehyde was used to fix the surface of K562 cells while retaining the function of CD1a antigen-presentation on the cell surface.

As an active protein crosslinker, glutaraldehyde is toxic to cells and over fixation will affect the function of surface proteins. Therefore, to achieve effective fixation and to retain the function of surface CD1a, several factors in the fixation protocol could be optimised, such as glutaraldehyde (GA) concentration, duration and temperature of fixation. To assess the outcome of different fixation conditions, the GA fixed cells were stained with live/dead dye and anti-CD1a mAb (HI149). The best combination of parameters could be determined by the highest cell viability after 24 hours post fixation (the longest duration of co-culture experiments) and the best staining effect of HI149 antibody, which could possibly reflect the functional state of surface CD1a protein.

Based on the structural studies, the interaction between CD1a and some TCRs relies on the surface structure of CD1a molecule only and is independent on the lipid ligand (Richard W Birkinshaw et al., 2015; Sarah Nicolai et al., 2020). HI149 was reported to be able to block the T cell autoreactivity related to CD1a. Therefore, the HI149 binding

capacity after fixation could possibly reflect the structural consistency between GA fixed or natural CD1a.

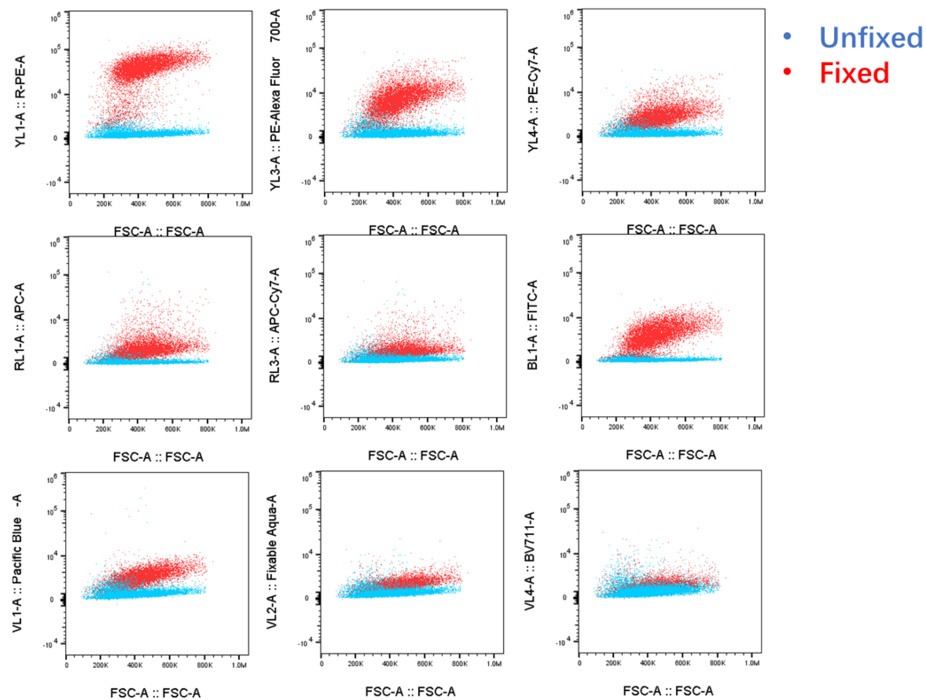

Figure 5.3 Autofluorescence of glutaraldehyde fixed cells. Baseline fluorescence of GA-fixed K562 cells (red) and unfixed K562 cells (blue) were tested under several fluorescent settings for common fluorophores, including PE, AF700, PE-Cy7, APC, APC-Cy7, FITC, Pacific Blue, Aqua and BV711.

Based on the literature (Duarte C Barral et al., 2008; Sarikonda et al., 2008), the following conditions were chosen to optimise the fixation protocol (concentration, fixation duration): 0.01%, 1min; 0.01%, 2min; 0.01%, 5min; 0.05%, 1min; 0.05%, 2min; 0.05%, 5min. For the convenience of operation, the experiments were conducted at room temperature.

In the initial experiment, PE-conjugated HI149 was used for CD1a staining, however, strong autofluorescence background was found in the GA-fixed cells and largely affected the gating of CD1a+ cells. By subsequently searching for publications, it was identified that the strong autofluorescence caused by GA fixation was related to the Schiff base formed between GA and free amino groups of the surface proteins (Collins & Goldsmith, 1981). The authors also reported the spectra of the autofluorescence caused by GA fixation. The spectra displayed a wide excitation wavelength and peaked around 540nm. While the strongest emission was found at wavelength 540-580nm (Collins & Goldsmith, 1981).

To minimise the influence of GA caused autofluorescence, the fluorophore chose for the staining need either have a short excitation wavelength (<450nm) or have a long emission wavelength (>700nm). Several fluorescent channels were tested on the GA-fixed and unfixed K562 cells to assess the baseline of autofluorescence related to GA fixation (Figure 5.3). The result was consistent with the theoretical prediction: fluorescent settings for fluorophores with short excitation or/and long emission wavelength, such as APC-Cy7(640nm/780nm), Aqua(405nm/510nm) and BV711(405nm/710nm) displayed less autofluorescence, among which BV711 had minimum autofluorescence background as a result of the combination of preferred excitation and emission wavelength. Whereas

fluorescent setting for PE (561nm/585nm) gave out the most strong autofluorescence due to the large spectral overlap to the GA formed Schiff base.

Based on the analysis of autofluorescence related to GA fixation, Aqua live/dead staining dye and BV711 conjugated HI149 were used for the staining in the following experiments.

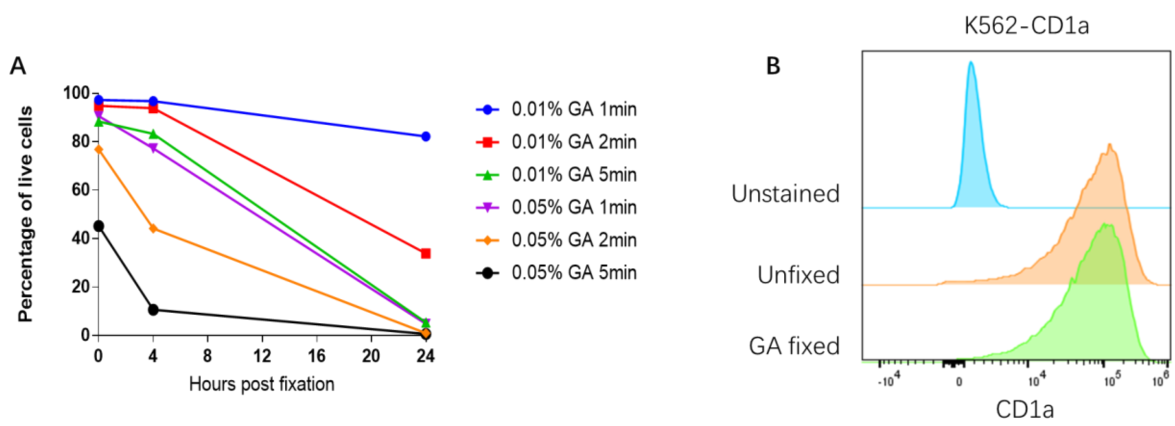

Figure 5.4 Optimisation of GA fixation conditions. K562-CD1a cells were fixed at room temperature with GA under various conditions. After fixation, the cells were washed twice with RPMI media and cultured in complete media for up to 24 hours. Cells were stained with Aqua live/dead dye and fluorophore conjugated HI149 antibody at 0h, 4h, 24h after fixation. (A)Percentage of live cells in the culture at 0h, 4h and 24h after fixation with different conditions. Live cells were gated on live/dead dye negative singlets. (B)Histography of CD1a staining on unstained, unfixed and GA fixed K562-CD1a cells (0.01% GA, fixed for 1min at room temperature).

Due to the cell toxicity of GA, cell viability was reduced with the increased GA concentration and prolonged fixation duration. Fixed with 0.01% GA for 1min gave the best viability with ~99% live cells after 4 hours and >90% live cells after 24 hours (Figure 5.4, A). Fixation with higher concentration of GA (0.05%) largely increased the ratio of cell death, killed ~5% and ~60% immediately after fixation for 1 min and 5 min and left almost no live cells after 24 hours. Therefore, the condition using 0.01% GA and fixing for 1min was chosen for all the following experiments. In addition, under this circumstance, the CD1a staining with HI149 antibody was well retained after fixation (Figure 5.4, B).

Since viral antigen was detected in T cells after co-culture with dengue-infected K562 cells (Figure 5.2), checking the presence of dengue E protein in T cells after co-cultured with GA fixed cells was a meaningful method to evaluate the effect of GA fixation on limiting virus particle release.

To test the effect of GA fixation on blocking dengue virus release, T cells co-cultured overnight with GA-fixed or unfixed dengue-infected K562 cells were stained for intracellular dengue E protein. The result showed that after co-culture with GA fixed dengue-infected K562 cells, the rate of DENV E+ T cells was reduced to the same level to the with mock-infected cells (Figure 5.5), indicating that fixing the K562 cells with GA before co-culture could effectively block the release of viral particles from K562 cells.

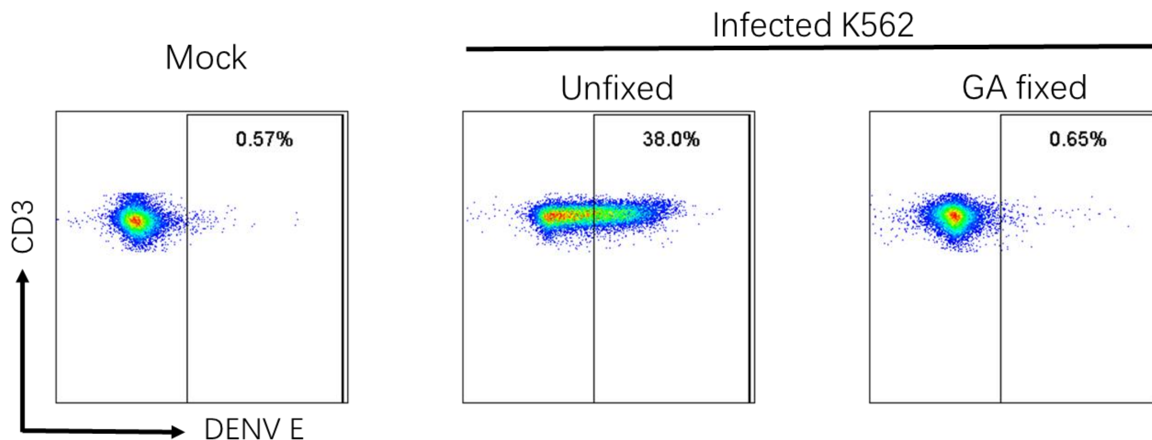

Figure 5.5 GA fixation on infected K562 cells effectively blocked the presence of dengue virus in T cells. T cells were co-cultured overnight with mock infected (left panel), unfixed dengue-infected (middle panel) and GA-fixed infected K562 cells (right panel). The mixture of cells was then stained for surface CD3 and intracellular dengue E protein. DENV E+ gates were draw based on the T cells culture with mock infected K562 cells. T cells were gated as singlets, live cell and CD3+ cells.

5.4 Cytokine production of CD1a-autoreactive T cells is affected by dengue infected APCs

In the experimental settings of using K562-CD1a cells as artificial APCs, T cell autoreactivity against CD1a could be measured as the increased T cell activation in the culture with K562-CD1a cells comparing to the culture with K562-EV cells.

CD1a-autoreactive T cells were reported to be able to secrete various cytokines, including both type I cytokines (IFNγ) and type II cytokines (IL-4, IL-13) and other cytokines (GM-CSF, IL-22, IL-17) (Sarah Nicolai et al., 2020). To investigate the effect of dengue infection on CD1a-reactive T cells, we screened a pool of cytokine-secreting T cell lines/clones developed in our lab, which includes GM-CSF, IFNγ, IL-22 and IL-13 secreted lines/clones generated by Dr Yi-Ling Chen.

T cells were co-cultured with mock-infected/dengue-infected K562-EV or K562-CD1a cells for 24 hours. To induce the production of certain cytokine, IL-2 and combinations of helper cytokines were added into the culture: for IFNγ clones, IL-12 and IL-18 were added; for GM-CSF clones, IL-1β and IL-12; for IL-22 clones, IL-6 and TNFα; for IL-13 clones, IL-4 and IL-33.

After co-culture, supernatant was collected and frozen for ELISA assay. Cells were stained to assess the expression of T cell co-receptors and activation markers.

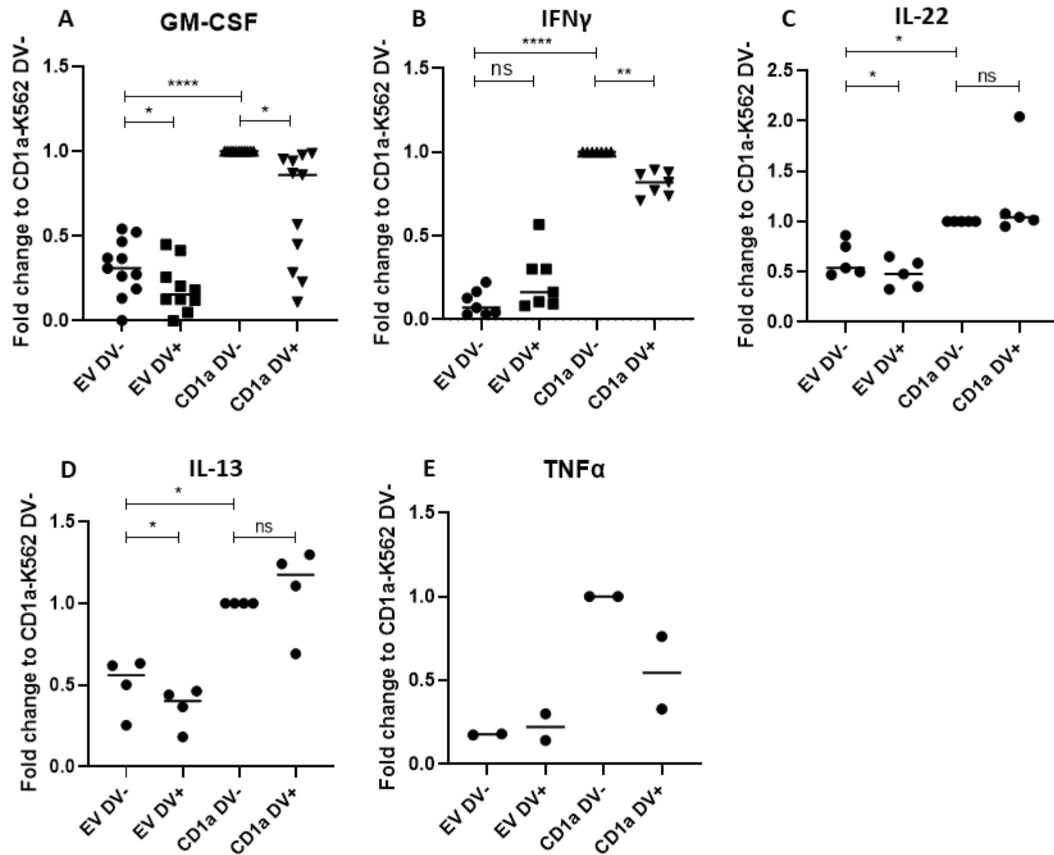

Figure 5.6 Cytokine production of CD1a-autoreactive T cell clones cultured with dengue infected K562-CD1a. CD1a-autoreactive lines/clones were co-cultured with mock-infected or dengue-infected K562-EV or K562-CD1a cells in the presence of certain helper cytokines. GM-CSF (A), IFNγ (B), IL-22(C), IL-13(D) and TNFα(E) level in the supernatant from the culture of according lines/clones was measured by ELISA. The data were the mean of duplicates and were adjusted to the fold change of cytokine production in the culture with mock-infected K562-CD1a cells. One-way ANOVA with Sidak's multiple comparisons test, *P<0.05, **P<0.01, ****P<0.0001.

Due to the stimulation with IL-2 and helper cytokines, a background of cytokine production in the culture with K562-EV cells was detected in most of the line/clones tested. Since all the lines/clones tested are CD1a-autoreactive, comparing to the T cell response with K562-EV cells, increasing level of secreted cytokines was detected with K562-CD1a cells (Figure 5.6). However, the level of GM-CSF and IFNγ were reduced in the culture with dengue-infected K562-CD1a cells comparing to the culture with mock-infected K562-CD1a cells (Figure 5.6, A and B). While this reduction was not found in the IL-22 and IL-13 lines/clones (Figure 5.6, C and D).

Although in the context of dengue virus infection, much studies focused on IL-6, TNFα, IL-10, these cytokines are not typically studied in the context of CD1a-autoreactive T cells. However, we did noticed that two CD1a-autoreactive T cells clones (32γ-3G6, 11HDM-1G8) screened for IFNγ production also secreted TNFα at a much lower level (10-500 pg/ml) comparing to the IFNγ level measured in the same clones (1000-100,000 pg/ml). But the changing pattern of TNFα production with different APCs were similar to IFNγ: increased TNFα secretion was induced by mock-infected K562-CD1a cells, but reduced by dengue virus infection in the K562-CD1a cells (Figure 5.6, E). In addition, it has to be mentioned that the cytokine secretion profile of the T cell lines/clones tested might be polarised by the helper cytokines added in the culture. The IFNγ clones tested might produce higher level of TNFα with different combination of helper cytokines.

CD25 and CD69 were T cell activation makers. However, co-culturing with CD1a expressing APCs was not the only factor that could affect the expression of CD25 and CD69. After being recovered from the frozen stock, the T cell lines/clones were incubated with irradiated PBMCs as feeder cells for 2-3 weeks to promote the cell proliferation before conducting the functional test. The prolonged effect of this stimulation could confound the expression level of CD25 and CD69 in the following functional assays. Therefore, the expression level of CD25 and CD69 in the tested T cell clones/lines might not be able to reflect the activation state of T cells. However, an increase of CD25 and CD69 MFI in the T cells cultured with mock-infected K562-CD1a cells comparing to T cells cultured with K562-EV cells was found in most of the GM-CSF secreted lines/clones tested and was not observed in lines/clones secreting other cytokines. The upregulation of CD25 and CD69 in the GM-CSF secreting lines/clones indicated more T cell activation against CD1a and was consistent with the cytokine production assessed by ELISA. The same pattern was found in the reduction of CD25 and CD69 expression in the culture of dengue-infected K562-CD1a cells against mock-infected K562-CD1a cells. It is difficult to explain this particular feature of GM-CSF lines/clones on CD25 and CD69 regulation. However, this observation indicated that CD1a-autoreactive T cell lines/clones with the same cytokine production capabilities might share some communal responding mechanism against CD1a.

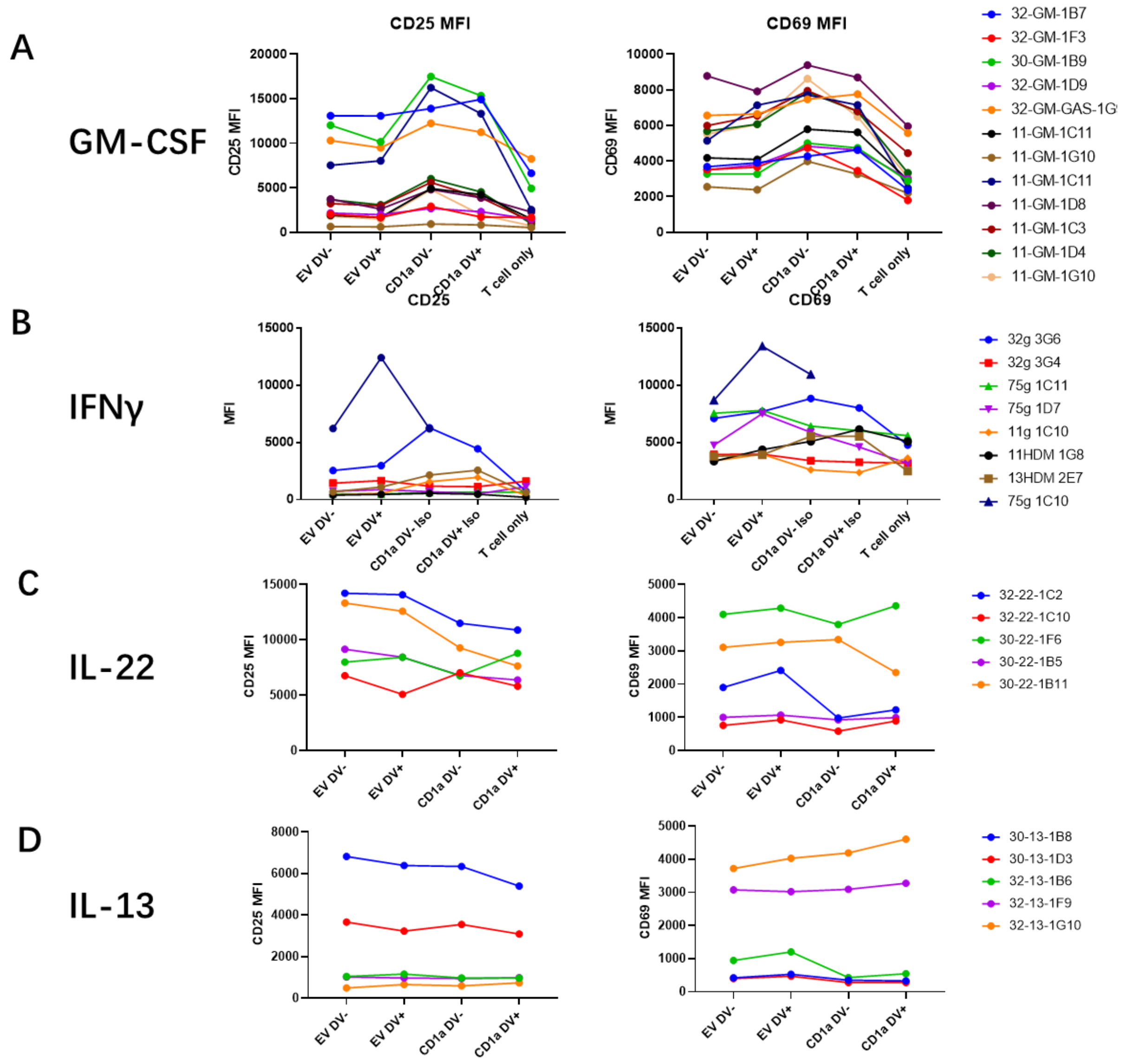

Figure 5.7 Surface activation maker expression of CD1a-autoreactive T cell clones cultured with dengue infected K562-CD1a. CD1a-autoreactive lines/clones were co-cultured with mock-infected or dengue-infected K562-EV or K562-CD1a cells in the presence of certain helper cytokines. Expression level of CD25 and CD69 were assessed by surface antibody staining and measured by flow cytometry. Figures showed the change of MFI of CD25 and CD69 in the CD3+ population, each line indicated the data from a single line/clone. The lines/clones secreted the same cytokines were showed in

the same figure: (A)GM-CSF lines/clones, (B) IFNγ lines/clones, (C) IL-22 lines/clones, (D) IL-13 lines/clones.

The results with CD1a-autoreactive lines/clones suggested GM-CSF and IFNγ-producing T cells were more potently affected by dengue infection. Therefore, to investigate the influence of dengue infection on CD1a-related activation in human blood polyclonal T cells, GM-CSF and IFNγ secretion were chosen as the readouts of T cell function.

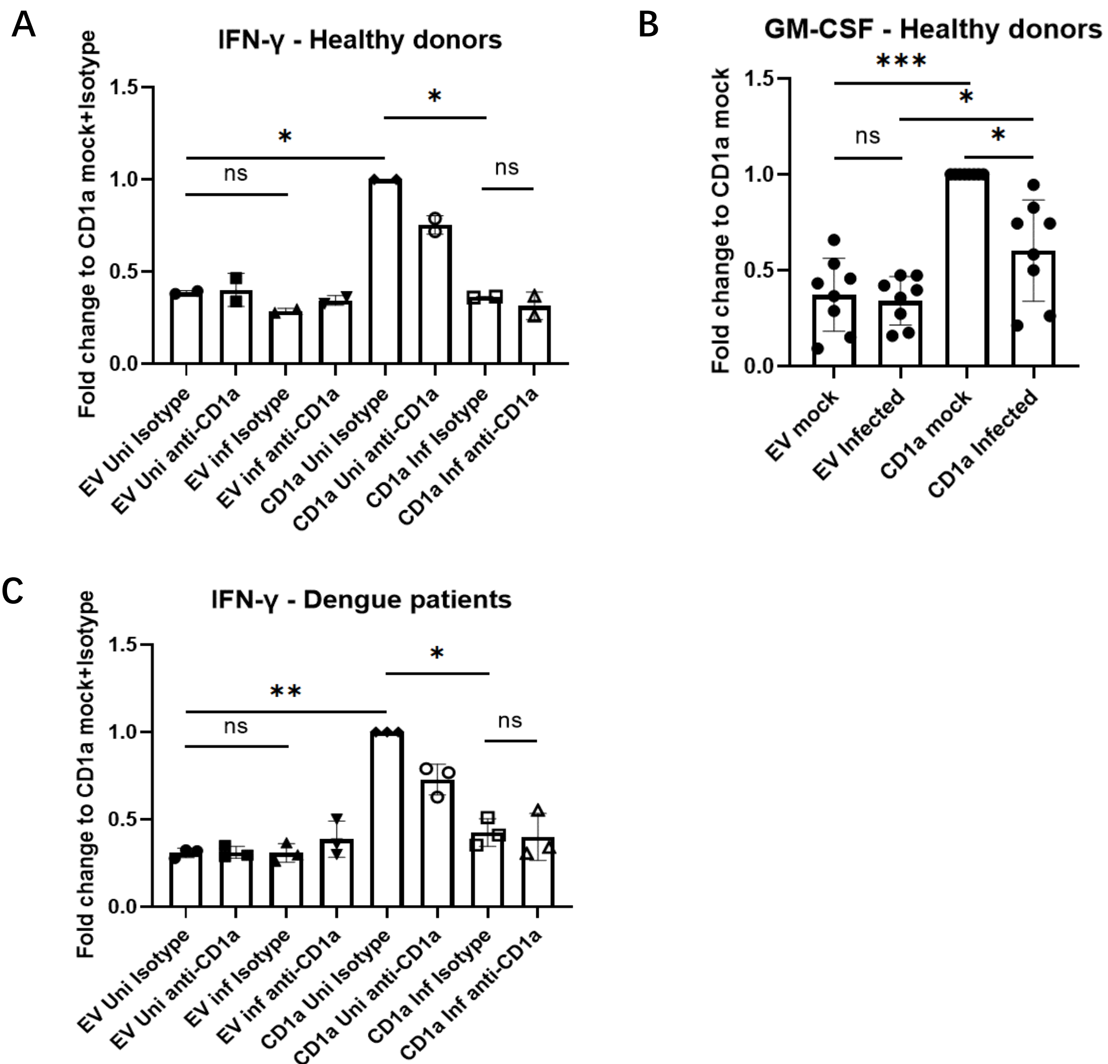

Figure 5.8 Dengue infection reduced the autoreactive IFN γ and GM-CSF production in blood polyclonal T cells. T cells were isolated by CD3 MACS beads from human PBMCs and co-cultured with mock-infected or dengue-infected K562 cells (EV or CD1a-trnsfected) overnight. T cells production of IFNγ (healthy donors (A) or dengue patients (C)) and GM-CSF (B) was measured by ELISpot. Spot numbers in each condition were adjusted to the fold change against mock-infected K562-CD1a condition. One-way ANOVA with Sidak's multiple comparisons test, *$P<0.05$, ***$P<0.001$.

In the result of IFNγ ELISpot assay (Figure 5.8, A, C), T cells from all the donors displayed 1-3 fold increased IFNγ-producing cells in the culture with mock-infected K562-CD1a cells comparing to the negative control (culture with mock-infected K562-EV cells). T cells from dengue-infected patients displayed a larger extent of activation with mock-infected K562-CD1a cells comparing to the T cell response from healthy donors, although it will be important to study more individuals. In all the donors, adding CD1a blocking antibody partially blocked the activation of T cells by CD1a. Surprisingly, the large increase of IFNγ-producing cells was not observed in the condition with dengue infected K562-CD1a cells. While the number of activated T cells had no difference between mock or dengue infected K562-EV conditions. Like IFNγ-producing response, dengue infection in K562-CD1a induced less GM-CSF production in human blood polyclonal T cells, while background response was not affected by virus infection (Figure 5.8, B).

As discussed previously, it is possible that virus released from infected target cells may influence the responding T cells. To assess the effect of the direct interaction between virus and human blood polyclonal T cells in the ELISpot assays, 4G2 antibody was added in the cultures to reduce the entrance of dengue virus into T cells.

Intracellular staining of dengue antigen showed that the infection of T cells was effectively reduced by 4G2 antibody (Figure 5.9, A). Whereas no difference of activation level was observed between the co-culture condition with 4G2 antibody or isotype antibody (Figure 5.9, B), indicating that the reduction of GM-CSF-producing cells in the culture with dengue infected K562-CD1a cells was not caused by the idengue virus infection of T cells.

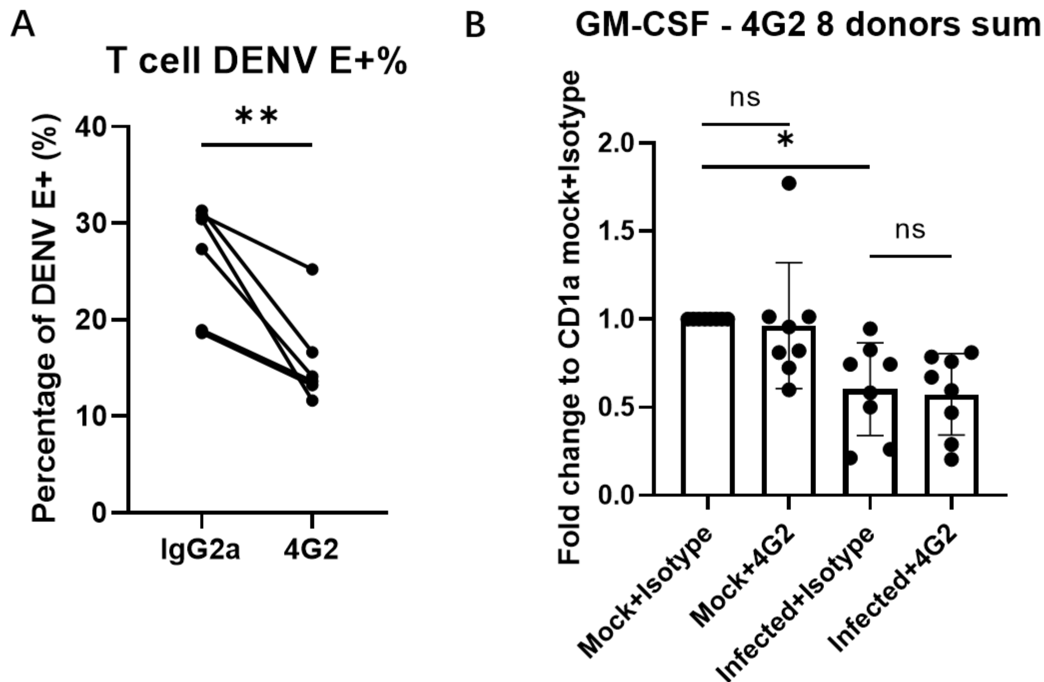

Figure 5.9 Adding 4G2 to reduce the virus presence in T cells had no effect on T cell GM-CSF production in response to CD1a. 4G2 antibody and paired isotype antibody were added to the culture of T cells and K562 cells. (A)Percentage of intracellular DENV E+ cells in the T cell population with the presence of 4G2 or isotype antibody were measured by flow cytometry after overnight co-culture with dengue-infected K562-CD1a cells. (B) Number of GM-CSF-producing cells in different co-culture conditions were measured by ELISpot, the results were adjusted to the fold change comparing to the negative control condition (mock-infected K562-CD1a cells with isotype antibody). (A) Student t test: **P<0.01 (B) One-way ANOVA with Sidak's multiple comparisons test: ns, not significant; *P<0.05

5.5 Cytotoxicity of CD1a-autoreactive CD8+ T cell clones against dengue infected APCs

Some CD1a-autoreactive T cell clones have been reported to display cytotoxicity against CD1a expressing cells (Stenger, 1997). To access if the cytotoxicity of T cell lines/clones was affected by dengue infection, CD8+ CD1a-autoreactive T cell line/clones were co-cultured with K562 cells for 2 days and cytotoxicity of the T cells was measured using CellTox dye (Promega), which enters the cells through leaking plasma membrane caused by granzymes and can be detected by green fluorescence emission after excited with blue laser (488nm). In the cytotoxicity assay, the cells were also stained by PE conjugated Annexin-V to stain apoptotic cells. Dead cells were gated as CellTox and Annexin-V double positive cells.

Since the tested CD8+ T cell lines/clones were also selected by cytokine production (GM-CSF or IFN-γ), specific helper cytokines as well as IL-2 were also added in the co-culture stage to enhance the T cell response (IL-12 and IL-1β for GM-CSF lines/clones; IL-12 and IL-18 for IFNγ lines/clones). K562 cells only with media supplied with relevant combinations of helper cytokines was added in the experiment as negative controls.

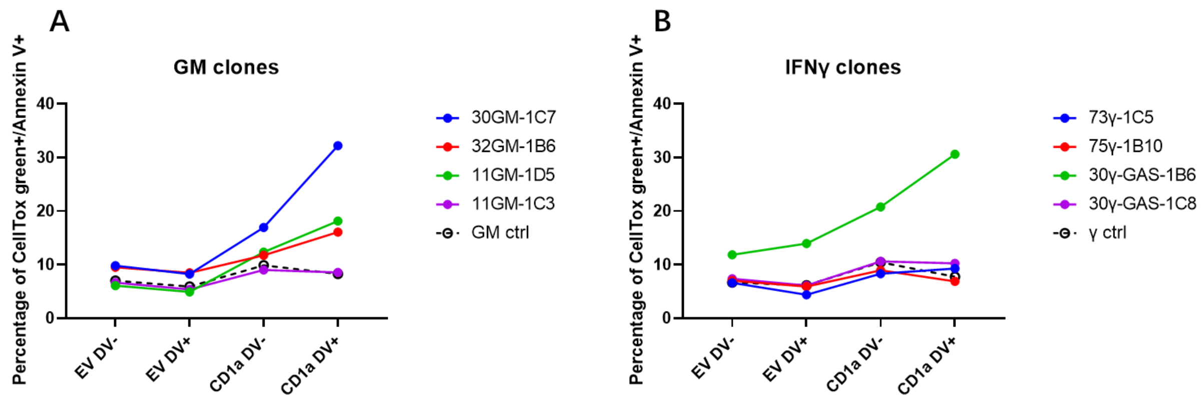

Figure 5.10 Cytotoxicity of CD8+ CD1a-autoreactive T cell lines/clones against dengue infected K562 cells. Cytotoxic CD8+ CD1a-autoreactive T cell lines/clones were co-cultured with K562 cells at 1:1 ratio for 2 days. K562 cells were labeled with CellTrace violet before mixing with T cells. After co-culture, cells were stained with CellTox Green and PE conjugated Annexin-V and assessed by flow cytometry. Dead K562 cells were gated as CellTrace violet+, CellTox Green+ and Annexin-V+ cells. Cytotoxicity were measured by the percentage of dead K562 cells in the total K562 cells (CellTrace violet+ cells). (A) T cell lines/clones selected with GM-CSF production. (B) T cell lines/clones selected with IFNγ production. Dot lines indicated the result of K562 only conditions supplied with IL-2 and helper cytokines to boost the function of certain T cell lines/clones: (A) IL-12 and IL-1β, (B) IL-12 and IL-18.

A preliminary screen was done using several CD8+ GM-CSF or IFNγ producing lines/clones on the specific cytotoxicity against CD1a-expressing cells. Four of each type

of lines/clones with specific killing ability were used in the experiments with dengue infected K562 cells (Figure 5.10).

The data of the control groups (K562 only conditions) showed that cell viability had no difference between mock-infected or dengue-infected, EV- or CD1a- K562 cells (Figure 5.10). Consistent with previous observations, all of the CD8+ T cell lines/clones killed more K562-CD1a cells than K562-EV cells. Although some lines/clones (e.g. 30GM-1C7, 32GM-1B6, 30γ-GAS-1B6) also caused more cell death in K562-EV cells comparing to the control conditions (Figure 5.10). The unspecific cytotoxicity against K562-EV cells might be due to the background activation boosted by helper cytokines.

Interestingly, in contrast to the decline of T cell cytokine production induced by dengue-infected APCs, the cytotoxicity of CD8+ T cell lines/clones was increased when co-cultured with dengue-infected K562-CD1a cells, although variability was noted (Figure 5.10). No difference of cytotoxicity was observed between mock-infected K562-EV cells and dengue-infected K562-EV cells, indicating that dengue infection in K562 cells was not able to increase the nonspecific cell death caused by T cells. However, it is still not clear whether the increased death of dengue-infected K562-CD1a cells was caused by the increased activation of CD1a-reactive T cells or was due to the increased sensitivity of the CD1a related killing in the dengue-infected K562-CD1a cells. These possibilities

could be dissected by future experiments: 1) Staining T cells for surface CD107 expression, which is an activation marker of cytotoxic T cells. The MFI level or the percentage of CD107+ cells could directly represent the level of cytotoxicity of the T cell lines/clones and therefore bypass the indirect measurement of cell death in K562 cells; 2) Measuring the concentration of granzymes in the supernatant of co-culture, as another direct measurement on the T cell cytotoxicity.

Chapter 5 Summary

Overall, these data show that dengue infection in K562 cells expressing CD1a was able to influence the response of CD1a-autoreactive T cells, and the specific functional change was dependent on the subtype of T cells. For IFNγ and GM-CSF producing CD1a-autoreative T cell lines/clones, dengue infection in APCs induced less cytokine release, while the function of T cell lines/clones producing IL-13 and IL-22 was not affected. Using ELISpot and Fluorospot, it was found, in the human blood polyclonal T cells, the number of IFN-γ and GM-CSF producing cells was also decreased when co-cultured with dengue infected K562-CD1a cells. Interestingly, for CD8+ CD1a-autoreactive T cell clones which were able to specifically kill CD1a+ cells, instead of inhibiting the function of T cells, dengue infection in the APCs potentially increased the cytotoxicity of these cells, but the data were hard to conclusively interpret.

To assess the influence of dengue infection in APCs on the function of CD1a-autoreactive T cells, several technical issues were solved and the data of related experiments were described in this chapter: 1) K562 modified to stably express CD1a were used as artificial APCs for activating CD1a-reactive T cells. The permissive nature of dengue virus infection in the K562 cell lines was confirmed. And the surface expression of CD1a on the K562 cells was also confirmed to be not affected by virus infection. 2) Dengue virus was found to be present in the T cells after co-culture with infected K562 cells. Treatments including GA fixation or 4G2 antibody blocking were used to reduce the entrance of dengue virus into T cells and to avoid any possible influence on T cell function.

This is the first time that dengue infection in CD1a-expressed APCs could affect the response of CD1a-autoreactive T cells has been described in the literature. However, the underlying mechanism and the specific roles of the functional change of CD1a-autoreactive T cell populations in the context of dengue infection still need to be further studied.

The role of IFNγ

In viral infections, IFNγ is usually considered to have a protective role. Many studies in the context of dengue virus infection indicated the similar protective function of IFNγ (Fagundes et al., 2011; Gunther et al., 2011; Ho et al., 2005; Daniel H. Libraty, Pichyangkul,

Ajariyakhajorn, Endy, & Ennis, 2001; Prestwood et al., 2012; Sujan Shresta et al., 2004). However, there might be more complexity in the case of dengue immunity. Some studies also provided evidence indicating a negative role for IFNγ (Bozza et al., 2008; Michael S. Diamond et al., 2000; Kontny, Kurane, & Ennis, 1988; I Kurane et al., 1991; Patro et al., 2019).

The protective role of IFNγ is demonstrated in using various models. Studies using either IFNγ deficient (Fagundes et al., 2011) or IFNγ receptor deficient mice model (Prestwood et al., 2012; Sujan Shresta et al., 2004) demonstrated that IFNγ had important roles to limit virus replication and decrease the disease severity after virus challenge in the animal models. Some studies used human monocyte-derived dendritic cells (moDCs); for example, one study showed that IFNγ could increase the IL-12 p70 production from dengue virus infected moDCs and therefore enhance the cell-mediated immunity (Daniel H. Libraty et al., 2001). Another study showed that IFNγ pre-treated moDCs were less vulnerable to the virus infection, and viral production in the cells was shown to be limited (Ho et al., 2005). Importantly, a study using a human challenge model also provided some evidence on the protective role of IFNγ (Gunther et al., 2011). In this study, PBMCs were collected at various stages after virus infection. The cells were stimulated with viral particles *ex vivo* and the culture media were then collected and analysed for cytokine production. Interestingly, among all the cytokine tested, only IFNγ was associated with protection against fever and/or viremia (Gunther et al., 2011).

The evidence of negative role for IFNγ could be summarised into two categories. Some studies reported that in the *in vitro* infection model with human myeloid cell lines, IFNγ might augment antibody-dependent enhancement via increase the expression level of some Fc receptor (Michael S. Diamond et al., 2000; Kontny et al., 1988). Other studies tested the cytokine level in the serum of naturally infected patients and found that IFNγ level significantly correlated with disease severity and could serve as potential predictor for disease severity (Bozza et al., 2008; I Kurane et al., 1991; Patro et al., 2019).

Although evidence of the role of IFNγ seems to be conflict from different studies, it is important to note that the studies indicated the protective or negative roles of IFNγ actually used quite different models, while the studies using the same or similar models provided consistent results. When considering the detailed settings in different studies, the answer to this question seems not be clear. The roles of IFNγ could be either protective or negative depending on the conditions, such as the phase of disease or the infection history (primary infection vs. secondary infection). Especially for the studies finding the increase level of IFNγ was associated with severe disease, it is important to mention that the severe dengue infection is associated with higher viremia (Morsy et al., 2020; Vaughn et al., 2000), therefore the increased level of IFNγ in serum could just be a result of stronger immune response against higher titre of dengue virus in the blood and tissues.

The role of GM-CSF

Similar to IFNγ, higher level of GM-CSF was found in the sera from acute dengue patients, and the cytokine concentration was also associated with disease severity (Bozza et al., 2008; I Kurane et al., 1991; Patro et al., 2019). However, unlike IFNγ, role of GM-CSF in dengue infection is not well studied. Most related literature focused on the plasmid-expressing GM-CSF as adjuvant for experimental DNA vaccine against dengue virus (Chen et al., 2014; Raviprakash et al., 2003; Raviprakash et al., 2001; Zheng et al., 2011). In 3 out of 4 studies (2 studies used mice model and 1 study used Aotus monkeys), GM-CSF played positive roles to promote the protection induced by the vaccine (Raviprakash et al., 2003; Raviprakash et al., 2001; Zheng et al., 2011). However, in a mouse study meant to comprehensively test the function as adjuvant with DNA vaccine against different flavivirus (including dengue virus, hepatitis C virus and Japanese encephalitis virus), the role of GM-CSF seemed to be complex with suppression of immune response in the context of vaccine against dengue and Japanese encephalitis virus and an enhanced protection with HCV vaccine (Chen et al., 2014). Therefore, whether the role of GM-CSF in the dengue infection is negative or protective is still an open question. Although most evidence with the vaccine studies indicated a positive role of GM-CSF in promoting antibody response, however, it should be noted that the context of real virus infection could be fundamentally different to the context of DNA vaccine administration.

CD8+ T cells in dengue immunity

CD8+ T cells are considered to be protective in dengue infection. A study in children with secondary dengue infection demonstrated that comparing to the individual who developed symptoms, the subclinical cases was associated with higher frequency of DENV-specific CD8+ T cells (Hatch et al., 2011). Consistent with this observation, studies on the specific HLA type associated with lower risk of severe dengue disease also revealed that higher magnitude of CD8+ T cells was correlated with the protective HLA alleles (de Alwis et al., 2016; Weiskopf et al., 2013).

Closely related to the function of CD1a in the skin, dengue-specific CD8+ T cells from patients in acute phase were reported to express skin homing makers (CLA) (Laura Rivino et al., 2015). These CD8+ T cell populations were also found in the skin of acutely infected patients (Laura Rivino et al., 2015). Consistent with this result, another study using both skin biopsies and blood samples from patients with dengue shock syndrome revealed that skin CD8+ T cells were activated (Duyen et al., 2017). The above evidence suggests that CD8+ T cells could respond to dengue infection in the skin. However, the mentioned studies were either on peptide-specific CD8+ T cells (Laura Rivino et al., 2015) or on the general CD8+ T cell population. When it comes to CD1a-specfic CD8+ T cells, if these cells could be activated and play effector functions and kill infected CD1a+ APCs in the skin during infection is still an open question.

Possible mechanisms of functional change of CD1a-autoreactive T cells induced by dengue infected APCs

As important component of virus envelope, lipids play critical roles at multiple stages of virus infection, replication and release. Dengue virus was found to hijack the lipid metabolism in the infected host cells to provide energy (ATP) for virus replication (Cloherty et al., 2020; Heaton & Randall, 2010). Also, a study on dengue infected mosquito cells also revealed that the lipid repertoire of infected cells were largely changed, which linked to the membrane remodelling facilitating virus replication (Rushika Perera et al., 2012). In this study, several types of phosphatidylcholine (PC), sphingomyelin (SM), ceramide (CER) and lyso-phosphatidylcholine (LPC) were found to elevated in the infected cells comparing to the mock-infected cells (Rushika Perera et al., 2012). Importantly, PM, SM, CER and LPC were also found to be CD1a-ligands that could be either permissive or disruptive in the interaction between CD1a and specific TCRs. There is a possibility that the changed lipid repertoire in the infected host APCs affect the repertoire of lipid ligands carried by the CD1a to the cell surface and subsequently affect the response of CD1a-autoreactive T cells. While the study of lipid repertoire comes from mosquito cells, this result may still shed some light on the possible mechanism of the functional changes of CD1a-reactive T cells. However, more experiments need to be done in the mammalian cells to support this hypothesis, such as investigate the alteration of lipid repertoire in the dengue infected human dendritic cells or Langerhans cells.

However, to specifically detect the change of CD1a ligand profile after dengue infection of the host cells, it is important to develop some tools to study the lipid species carried by CD1a on the cell surface. A K562 cells expressed genetically modified CD1a is under development in our lab. In the design of this cell line, the extracellular part of CD1a can be removed from the cell surface by enzymes and collected for lipidomic analysis. If this system was tested to show enough sensitivity in the future, it could be a powerful tool to study the difference of CD1a ligands between the infected and uninfected host cells, which could be a most direct evidence to test our hypothesis.

6 Dengue glycoproteins bind langerin

Introduction and Aims

The C-type lectins are a superfamily of proteins that contain C-type lectin-like domains. The name of this family originally came from their Ca2+ dependent binding ability to carbohydrates (Brown, Willment, & Whitehead, 2018). Based on the phylogeny, structural and functional properties, members of C-type lectins have been classified into 17 subgroups (I-XVII) (Monteiro & Lepenies, 2017). In mammals, there are both secreted forms of C-type lectins as well as transmembrane receptors (C-type lectin receptors, CLRs) (Brown et al., 2018).

Many types of immune cells, including lymphocytes and all myeloid cells, express a broad range of C-type lectins. Myeloid CLRs are a group of CLRs that are predominantly expressed by APCs, such monocytes, DCs and macrophages. These CLRs play critical roles in pathogen recognition, endocytosis, and antigen processing in APCs (Monteiro & Lepenies, 2017). According to the classification of C-type lectin, most myeloid CLRs belong to group II, V and VI. In the immune response against some virus infections, CLRs can recognise the glycan on the virus surface and eliminate the virus by degrading the virus through lysosomal or autophagy pathways (Bermejo-Jambrina et al., 2018). However, some viruses can hijack the function of CLRs; they can escape from the degradation pathway and instead, use the CLRs as a handle to invade the cells (Bermejo-Jambrina et al., 2018).

As discussed in the previous chapters, myeloid cells are the primary targets of dengue virus. In dengue virus infection, several type of myeloid CLRs have been identified that promote virus infection, such as dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN) (Tassaneetrithep et al., 2003), liver/lymph node-specific ICAM-3-grabbing integrin (L-SIGN) (W. Dejnirattisai et al., 2011), macrophage mannose receptor (MMR) (Miller et al., 2008). Through binding to the dengue glycosylated envelope protein, the CLRs facilitate the entry of virus into myeloid cells.

Langerin is a CLRs exclusively expressed in Langerhans cells (LCs). Besides expression on the surface of Langerhans cells, Langerin also forms Birkbeck granules, a Langerhans cell specific organelle, with CD1a inside the cells. It is reported that langerin facilitated the CD1a-dependent presentation of antigens from Mycobacterium leprae to T cells (Hunger et al., 2004). In HIV-1 infection, in contrast to the role of DC-SIGN which helps transmission of HIV-1 to T cells, langerin displays a antiviral role via binding to HIV-1 and promoting the degradation of virus in LCs through a TRIM5α-dependent autophagic degradation, thus limiting the transmission of HIV-1 (de Witte et al., 2007; Ribeiro et al., 2016). Langerhans cells are among the primary targets of dengue virus infection in the skin. However, the role of langerin in the dengue infection of Langerhans cells is still not known.

Except for dengue E protein, NS1 protein is another glycoprotein encoded by the genome of dengue virus. Modified with N-linked glycosylation containing high mannose (Yap et al., 2017). Therefore, in theory, dengue NS1 protein can also bind to multiple C-type lectins. Indeed, it has been demonstrated (Thiemmeca et al., 2016) that NS1 binding to mannose binding lectin (MBL) is an escape strategy of dengue virus, by trapping the MBL-dependent virus neutralisation with NS1. Therefore, in addition to the binding of langerin with E protein, it is also interesting to assess the capability of langerin of binding to NS1 protein.

The main aims of this chapter include, 1) to investigate if langerin can bind to dengue glycoprotein, E and NS1 protein; 2) to investigate the function of dengue glycoprotein binding to langerin in the process of virus entry and infection.

6.1 Purified dengue glycoproteins bind langerin *in vitro*

To investigate if recombinant langerin (rlangerin) can bind to recombinant dengue glycoproteins (dengue E protein and NS1 protein) *in vitro*, an ELISA-like binding assay was designed. In this assay, dengue glycoproteins were coated in the ELISA plate, followed by adding recombinant langerin into the plates. The bound langerin was then

detected by adding the HRP conjugated antibodies which recognized the recombinant langerin and the substrate of HRP (TMB).

Due to the availability of reagents, two versions of experimental design were tested (table 6.1). In the second experimental design, rlangerin purified from mammalian cell line (HEK293 cells) with a FLAG tag was used instead of the recombinant protein derived from *E.coli*, which should have better conserved structure and function. And anti-FLAG antibody was used instead of anti-langerin antibody, which should have less steric hindrance of the protein function. Although recombinant langerin derived from different resources and different detection antibodies was used, the result was consistent between the two designs. The difference of the designs was summarized in the table 6.1.

	Design I	**Design II**
Coating	Dengue NS1/ E	Dengue NS1/ E
Primary	Langerin (**from *E.coli***)	Langerin (**from HEK**)
Secondary	**HRP Anti-Langerin (blocking clone)**	**HRP Anti-FLAG**

Table 6.1. Differences in experimental design in the two versions of ELISA-like binding assay.

In the first version of experimental design, HRP-conjugated anti-langerin antibody was used as detection antibody. The HRP conjugation on the antibody was conducted with the antibody HRP conjugation kit from Abcam. To test the efficiency of HRP conjugation and the specificity of antibody after conjugation, different concentration of recombinant langerin were coated in the ELISA plate. After removing unbound antibodies, the level of bound antibody were measured by the absorbance at 450nm after adding TMB substrate of HRP. The more bound antibody molecule left in the well, the higher absorbance at 450nm.

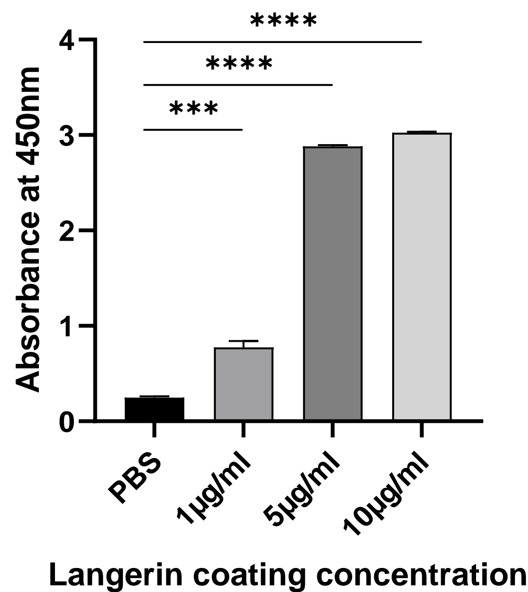

Figure 6.1. Test the efficiency of HRP conjugation and the binding specificity of anti-langerin antibody after HRP conjugation. Binding of HRP-conjugated anti-langerin antibody to the rlangerin coated ELISA plate were detected by the ELISA-like binding assay. The coating concentrations of rlangerin were 1μg/ml, 5μg/ml and 10μg/ml, Black

bar indicated negative control condition, using PBS to coating the ELISA plate wells. Data indicated results from three technical repeats. One-way ANOVA and Dunnett's multiple comparisons test, ***$P<0.001$, ****$P<0.0001$.

The result of this experiment (figure 6.1) demonstrated the absorbance at 450nm was increased with the coating concentration of rlangerin, which indicated that the binding between detection antibody and the coated protein is specific and the HRP conjugation on the antibody is efficient. This experiment also indicated that 5ug/ml could be a useful indicator as the concentration of rlangerin used in the ELISA-like binding assay to bind coated dengue protein, although less rlangerin should be detected binding to dengue protein comparing to the direct rhangerin coating.

To test if dengue NS1 and E protein can bind to rlangerin, different concentrations of dengue glycoprotein were coated in the ELISA plate microwell. After blocking and incubating with 5μg/ml rlangerin and HRP-conjugated anti-langerin antibody, TMB substrate were added and absorbance at 450nm were detected by the ELISA plate reader.

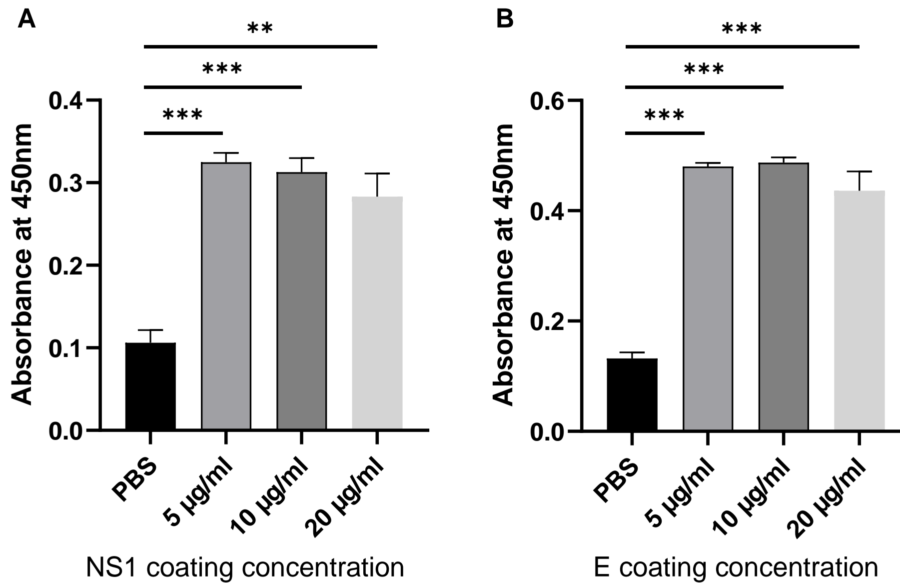

Figure 6.2 E. coli-derived rlangerin bind to dengue NS1 and E protein *in vitro.* Binding of E. coli-derived rlangerin and recombinant dengue NS1 (A) and E protein (B) were detected by ELISA-like binding assay. The wells were coated with increasing concentration of recombinant dengue glycoproteins: 5 μg/ml, 10 μg/ml, 20 μg/ml. Black bar indicated negative control condition, using PBS to coating the ELISA plate wells. Data indicated results from three technical repeats. One-way ANOVA and Dunnett's multiple comparisons test, **P<0.01, ***P<0.001.

Compared to the well coated with PBS, wells coated with dengue glycoprotein demonstrated significant higher absorbance at 450nm, which indicated that rlangerin bound to both dengue NS1 and E protein under the experimental conditions *in vitro*.

However, interestingly, there was not a significant difference of absorbance at 450nm detected between various coating concentration of dengue glycoprotein, which might indicate that for the dengue glycoproteins the plate coating was already saturated at a concentration lower then 5μg/ml (the lowest concentration had been tested). The highest concentration (20μg/ml) even displayed slightly lower read out than the other two concentrations (5ug/ml and 10ug/ml). This observation could be explained by the competition of plate-bound glycoprotein and the free glycoprotein redissolved from the plate surface into the liquid phase. However, the higher absorbance in all the wells coated with glycoprotein comparing to the wells coated with PBS is enough to draw the conclusion that dengue glycoprotein could bind to rlangerin *in vitro*.

To test if the binding between dengue glycoproteins and the rlangerin was specific, mannan, a blocking reagent of langerin, was used to pretreat and block the rlangerin.

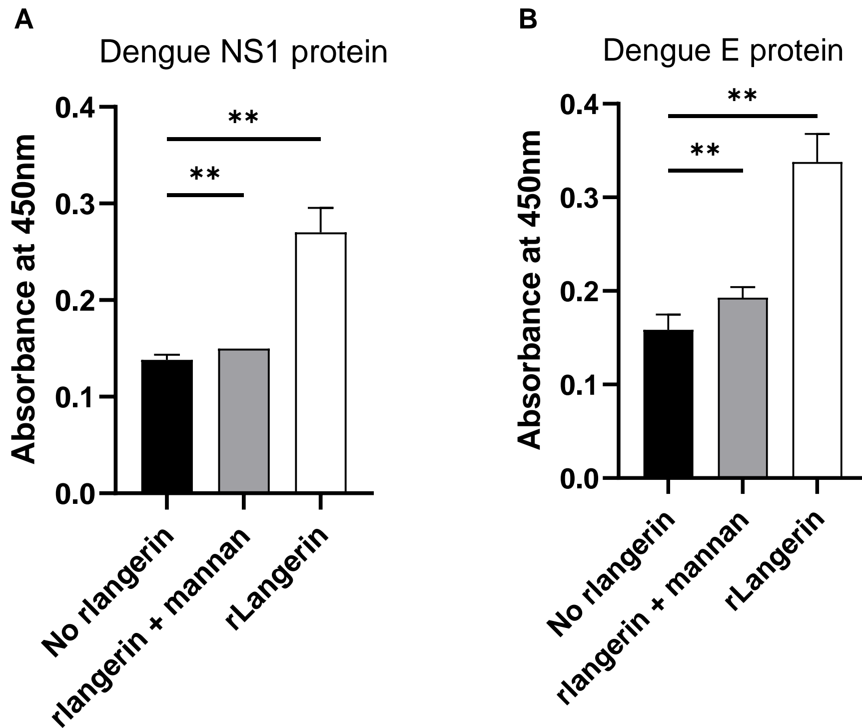

Figure 6.3 Binding of rlangerin with dengue glycoproteins was specific and could be blocked by mannan. The specify of the ELISA binding assay and the mannan blocking was tested with ELISA plate coated with 5ug/ml dengue NS1 protein (A) or 5ug/ml dengue E protein (B). Black bars indicated wells incubated with no rlangerin, grey bars indicated wells incubated with 5ug/ml rlangerin pre-treated by mannan, white bars indicated wells incubated with 5ug/ml rlangerin without any pre-treatment. Data indicated results from three technical repeats. One-way ANOVA and Dunnett's multiple comparisons test, **P<0.01.

The results (figure 6.3) show that pre-treating langerin with mannan reduced the absorbance reading to the level of no langerin controls, which indicated that the binding

detected between dengue glycoprotein and rlangerin was dependent on the oligosaccharide-binding domain . The mannan blocking of binding ability of rlangerin to dengue glycoprotein indicated that these interactions were dependent on the C-type lectin-like domains of the langerin.

In the second version of experimental design, the rlangerin derived from HEK293 cells were used to substitute the recombinant protein derived from *E.coli*. Also, the detection antibody was changed to the HRP-conjugated anti-FLAG antibody. The HRP conjugation was still conducted with Abcam antibody HRP conjugation kit and the efficiency of conjugation was also tested by the ELISA-like assay with plate-bound rlangerin.

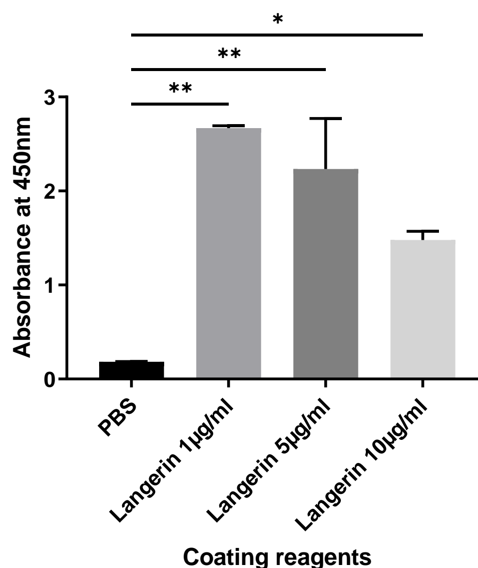

Figure 6.4 Test the efficiency of HRP conjugation and the binding specificity of anti-FLAG antibody after HRP conjugation. Binding of HRP-conjugated anti-FLAG antibody

to the rlangerin coated ELISA plate was detected by the ELISA-like binding assay. The coating concentrations of rlangerin were 1μg/ml, 5μg/ml and 10μg/ml, Black bar indicated negative control condition, using PBS to coating the ELISA plate wells. Data indicated results from three technical repeats. One-way ANOVA and Dunnett's multiple comparisons test, *$P<0.05$, **$P<0.01$.

The wells coated with rlangerin displayed significantly higher signal compared to the negative control coated with PBS only, which indicated the HRP conjugation was successful, and the conjugated antibody conserved its specificity. Interestingly, different to the results from the previous experimental design, in this experiment, the lowest coating concentration of rlangerin (1ug/ml) displayed the highest signal. The decrease of signal in the higher coating concentration could also be explained by the competitive binding to the antibody between the surface-bound rlangerin and the re-dissolved rlangerin. Although the core results remain the same, the difference between the results from two experimental designs might be caused by the different physical properties of the rlangerin from difference sources.

To optimise the coating concentration of the dengue glycoproteins under the new experimental settings, 5μg/ml to 20μg/ml dengue NS1 protein and E protein were coated

in the ELISA plate and the binding affinity were tested by adding rlangerin as described previously.

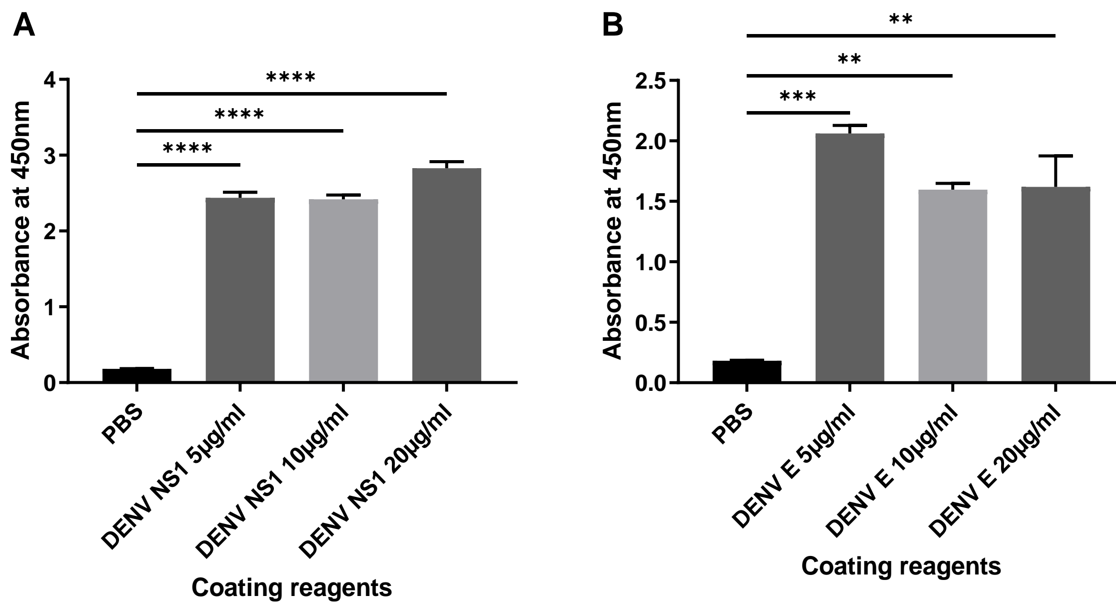

Figure 6.5 HEK293-derived rlangerin bind to dengue NS1 and E protein *in vitro.* Binding of HEK293-derived rlangerin and recombinant dengue NS1 (A) and E protein (B) were detected by ELISA-like binding assay. The wells were coated with increasing concentration of recombinant dengue glycoproteins: 5 μg/ml, 10 μg/ml, 20 μg/ml. Black bar indicated negative control condition, using PBS to coating the ELISA plate wells. Data indicated results from three technical repeats. One-way ANOVA and Dunnett's multiple comparisons test, **P<0.01, ***P<0.001.

Consistent with the result with the previous experimental design (using *E.coli* derived rlangerin and anti-langerin as detection Ab), the wells coated with dengue glycoprotein all displayed a higher readout comparing to the negative control wells which coated with PBS only. Also, like the previous dosing experiments, there is no significant difference in the signal between the different coating concentrations of glycoproteins.

Interestingly, the signal of the dengue glycoprotein coated wells under the new experimental settings was much higher comparing to the first experimental design, while the readout of the PBS only controls were not changed. This might indicate that the mammalian-derived rlangerin had better binding affinities to dengue glycoproteins. This finding could also be explained by a better binding affinity of the detection Ab, which anti-FLAG Ab was used instead of the anti-langerin Ab. However, the contribution of these two factors could not be distinguished and tested in this experimental design.

Although in the previous experiments, the result that the dengue glycoprotein coated wells gave out higher absorbance comparing to the PBS coated well already indicated that the reagents added into the wells later (rlangerin and detection Ab) were capable to bind to the coated proteins. However, although less likely, there was a possibility that the detection Ab could bind to the coated protein directly. To rule out this possibility, new negative controls with no rLangerin applied to the wells were added into the

experimental design. Specifically, after coating and washing, PBS instead of rLangerin was added into the control wells and were incubated under the same conditions. To verify the background of rlangerin bound to the plate directly, PBS coated negative controls were also included in these experiments.

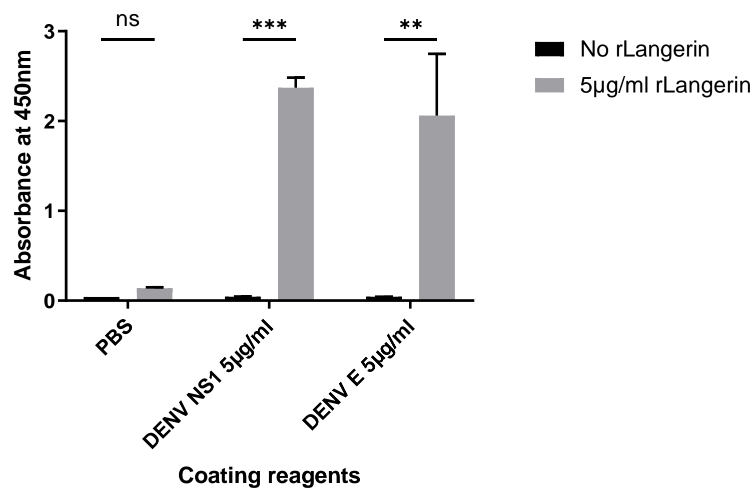

Figure 6.6 No unspecific binding between detection reagents and dengue glycoproteins was detected. The specify of the ELISA binding assay was tested with ELISA plate coated with PBS, or 5ug/ml dengue NS1 protein, or 5ug/ml dengue E protein. Black bars indicated wells incubated with no rlangerin, grey bars indicated wells incubated with 5ug/ml rlangerin. Data indicated results from three technical repeats. Two-way ANOVA and Sidak's multiple comparisons test, ns, non-significant, **P<0.01, ***P<0.001

From the result (figure 6.6), the PBS-coated wells incubated with no rLangerin displayed the lowest signal. The absorbance of the PBS-coated wells incubated with rLangerin were a bit higher than the no rlangerin controls and this additional signal could be explained as the residue binding of rLangerin to the plate after washes. However, since the readout of this control group was still quite low, this unspecific binding could not explain the binding between dengue glycoproteins and rLangerin. In the wells coated with dengue glycoproteins, the no rLangerin conditions display similar readouts as the PBS coated controls. While the wells incubated with rLagnerin displayed much higher readouts, which indicated that the detection reagents showed no unspecific binding to the coated dengue glycoprotein and therefore the signal of protein binding could all be explained as the binding between dengue glycoproteins and rLangerin.

C-type lectins usually interact with glycoprotein by binding to the oligosaccharides. The *in vitro* binding experiments demonstrated that pre-treating rLangerin with mannan could efficiently block the interaction between rLangerin and dengue glycoprotein (figure 6.3). To further investigate the role of glycosylation in the interaction of langerin and dengue glycoproteins, PNGase F (New England Biolabs) was used to remove the *N*-linked oligosaccharides on the dengue glycoproteins. PNGase F is the most effective enzyme to remove almost all *N*-linked oligosaccharides from glycoproteins.

Firstly, the efficiency of PNGase F on both dengue NS1 protein and E protein was tested. After mixing and incubating to allow the enzyme function on the target proteins, the samples was separated by protein gel electrophoresis and stained by Coomassie blue for total protein staining. Due to the large molecular weight of the oligosaccharides, the glycosylated protein forms a lower band in the protein gel comparing to the native protein.

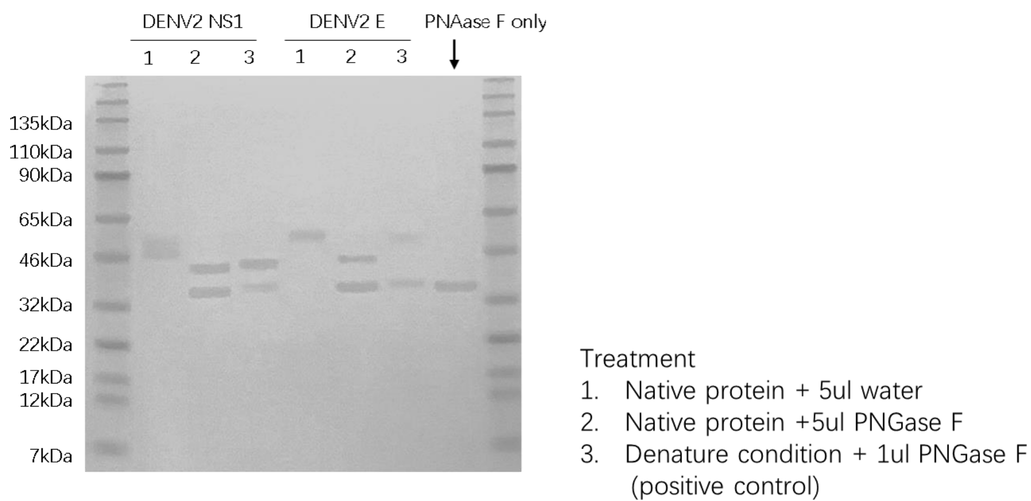

Figure 6.7 PNGase F efficiently removed oligosaccharides from DENV2 NS1 and E protein under native condition. Coomassie blue stained protein gel of native dengue glycoproteins, native dengue glycoproteins treated with PNGase F, denatured dengue glycoporteins treated with PNGase F and PNGase F only. The molecule weight of protein standard ladder was labelled at the left side.

The protein gel with Coomassie blue staining demonstrated that PNGase F efficiently removed the oligosaccharides on DENV2 NS1 and E protein under both native and denatured conditions. According to the manufacturer (NEB), the efficacy of the enzyme was usually better when it used with denatured protein. Therefore, the denatured conditions were added as positive controls. However, in my experiment design, native protein was preferred to avoid any complexity in the following *in vitro* binding assay.

The protein gel showed that both naive recombinant dengue NS1 and E protein had a molecular weight around 50kDa. In the samples treated with PNGase F, both dengue protein band migrated to a position around 46kDa and bands of PNFase F protein appeared at the lower position around 35kDa. The band swich of dengue protein indicated the removal of heavy chain of oligosaccharide. For the dengue NS1 protein, the native condition and denatured condition displayed the similar bands on the protein gel, indicating that the deglycosylation was successful under both conditions. However, interestingly, for dengue E protein, the upper band (E protein) in the denatured condition did not shift to a lower position comparing to the non-treated sample, while the deglycosylation of E protein in the native condition was very efficient.

After proving that PNGase F could efficiently remove the *N*-linked oligosaccharides from both dengue glycoproteins under the native condition, the next step was to prepare deglycosylated NS1 and E protein for the binding assay.

To avoid the influence of PNGase F in the system, the His-tagged dengue proteins were purified from the mixture. The same volume of water instead of PNGase F was added in the control samples.

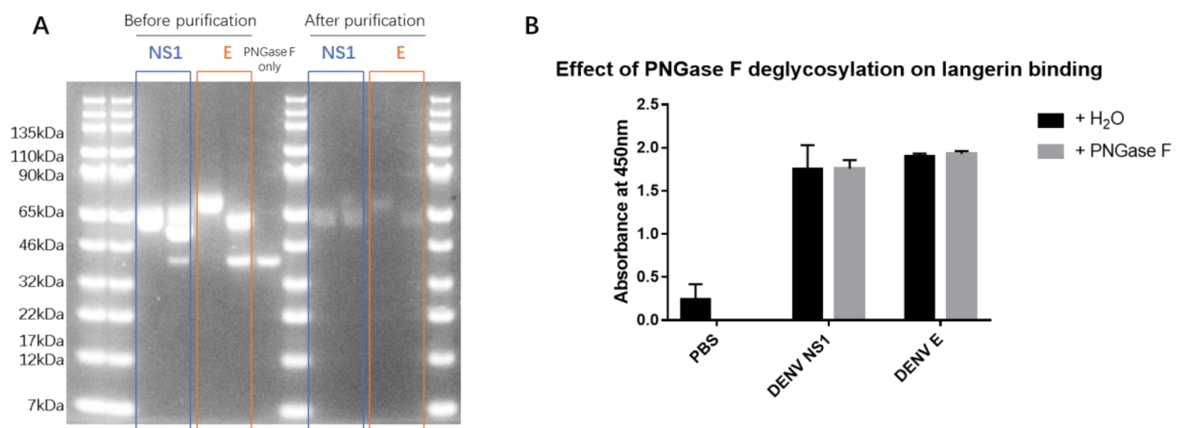

Figure 6.8 PNGase F deglycosylation did not affect the binding of dengue glycoproteins and rlangerin in the *in vitro* binding assay. (A) Coomassie blue stained protein gel with control recombinant dengue glycoproteins (added with water) and glycoproteins treated with PNGase F. The left channels were loaded with protein mixture before His-tag purification. The right channels were loaded with samples after purification. The molecule weight of protein standard ladder was labelled at the left side. (B) Binding of native or deglycosylated dengue glycoproteins with rlangerin *in vitro*. Black

bars indicated native glycoproteins (mixed with water), grey bars indicated deglycosylated glycoproteins (treated with PNGase F).

The protein gel showed that after incubation with PNGase F, deglycosylated protein band could be found in both treated NS1 and E protein samples, although the deglycosylation of NS1 protein is not 100% efficient in this experiment (Figure A, left channels). The right channels showed the sample components after His-tag purification: all the bands around 35kDa were absent, indicating that PNGase F was removed from the mixture after purification; in the channels with E protein, the band from the PNGase F treated samples was lower than the band treated with water, demonstrating that the purified E protein was successfully deglycosylated; however, in the NS1 samples, the two bands in both the control sample (+H_2O) and the deglycosylated sample (+PNGase F) were at the same position, while the lower band in the left panel indicating the deglycosylated NS1 protein was not present. This result indicated that the deglycosylated NS1 protein was lost during the purification and the purified NS1 protein used in the binding assay was not deglycosylated.

In the *in vitro* binding assay, although the *N*-linked oligosaccharides was efficiently removed from the dengue E protein, the binding of rLangerin to the deglycosylated E protein has no significantly change comparing to the native protein, indicating that the

interaction between E protein and rLangerin protein was not through *N*-linked glycosylation. This interaction might take place via *O*-linked glycosylation or through other domain on the E protein while still depends on the oligosaccharide-binding domain of the langerin since mannan could block the interaction.

6.2 Function of langerin in dengue virus infection

As discussed previously, in the context of HIV-1 infection, langerin acts as natural barrier of the virus infection in Langerhans cells. After binding to viral surface protein, gp120, langerin facilitates the virus internalisation and autophagic degradation, and therefore, also limits virus transmission (de Witte et al., 2007; Ribeiro et al., 2016). To investigate the role of langerin in dengue virus infection, an *in vitro* infection test was conducted with monocyte-derived Langerhans-like cells (moLCs) with the presence or absence of langerin blocking antibody (clone 10E2).

To derive moLCs, monocytes isolated from PBMCs were cultured with media supplied with IL-4, GM-CSF and TGF-β for 4 days. The moLCs were then inoculated with dengue virus (MOI = 1). At 2 days after inoculation, the cells were collected and stained for surface marker expression (CD1a, DC-SIGN and langerin) and intracellular dengue antigen (dengue E protein).

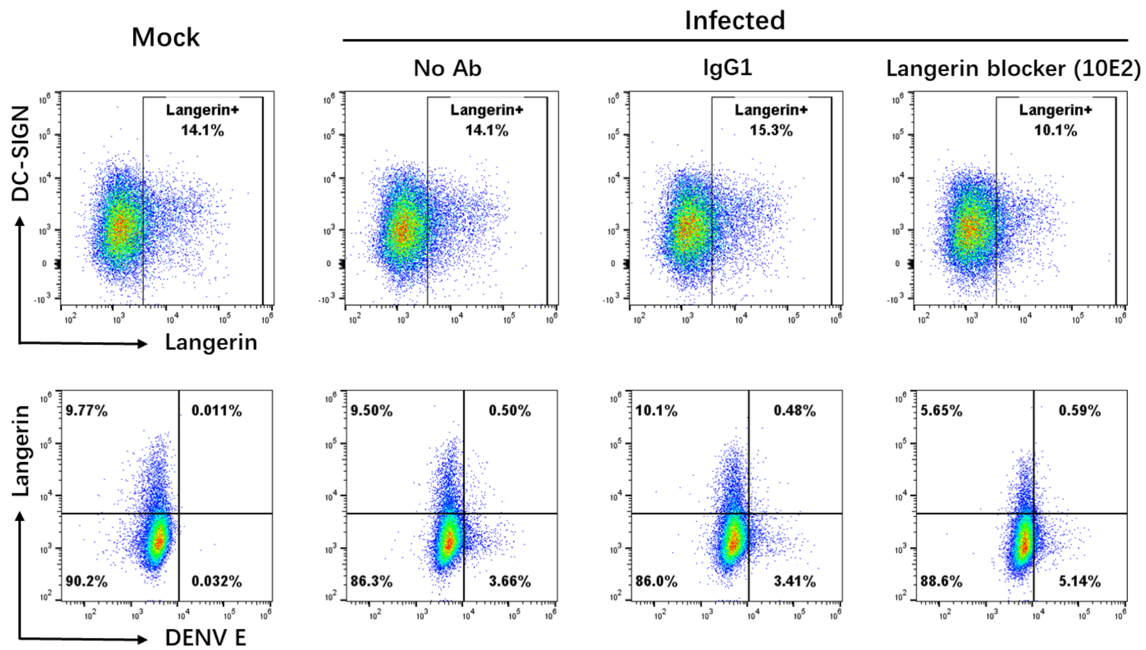

Figure 6.9 Langerin blockade increases the dengue virus infection in moLCs. Monocytes were separated from PBMCs by CD14 MACS beads. Cells were cultured with RPMI media supplied with FCS and cytokine cocktail (IL-4, GM-CSF and TGF-β) for 4 days. Langerin blocking antibody (clone 10E2) or isotype control antibody were added into the culture at 10μg/ml 1 hour before virus inoculation. Cells were stained and analysed by flow cytometry at Day 2 after inoculation. The upper figures show the DC-SIGN vs. langerin plot, the lower figure show the langerin vs. DENV E plot. The langerin positive gate was drawn based on FMO control, the DENV E positive gate was drawn based on mock infection control.

After 4 days culture with IL-4, GM-CSF and TGF-β, the langerin expression is induced in a subset of moLCs (figure 6.9). The percentage of langerin+ cells was typically lower in

the cells treated with langerin blocking antibody compared to other conditions, which is likely to be caused by the competition binding of blocking antibody against langerin staining antibody. Interestingly, although the DENV E+ percentage was not changed significantly from langerin blocking condition to isotype control condition, the MFI of DENV E staining was increased in the cells treated with blocking antibody. (figure 6.9) However, this experiment result is not enough to drive any conclusion regarding the role of langerin in dengue virus infection in moLCs. To achieve a clear conclusion, cell lines with artificially expressed langerin could be a better model to answer this question.

Chapter 6 Summary

C type lectins serve as important attachment factors for dengue virus binding the target cell surface. However, the role of langerin in dengue virus infection has not been studied. In this chapter, binding of rlangerin with purified dengue glycoproteins (NS1 and E protein) was confirmed by *in vitro* ELISA-like binding assay. While two different combination of reagents was tested, the conclusions were consistent. By using mannan to treat rlangerin, the binding of rlangerin and dengue glycoproteins were proved to depend on the oligosaccharide-binding domain of langerin. However, using PNGase F to remove the glycosylation of dengue E protein did not affect its binding to rlangerin in the *in vitro* assay, indicating that the binding between E protein and rlangerin was not dependent on the *N*-linked oligosaccharides on dengue E protein and suggesting that other forms of glycosylation are important.

Dengue virus proteins acquire differentiated glycosylation from different cell types. Dejnirattisai and colleagues (W. Dejnirattisai et al., 2011) have demonstrated that dengue virus produced in insect cells, tumor cells lines and primary human DCs bind to different C type lectins. DC-derived dengue virus lost the ability of binding DC-SIGN while still infecting L-SIGN-expressing cells. In the initial DENV infection stage, the primary invading virus are all produced by mosquito cells. While at the later stage of infection, when new virus particles were released from human cells, these viruses bear envelope protein with differential glycosylation patterns. In humans, DENV can infect several types of skin cells, including keratinocytes, Langerhans cells and dermal DCs and macrophages (Duangkhae et al., 2018; Rathore & St John, 2018; Surasombatpattana et al., 2011). Resident at the skin epidermis, Langerhans cells may contact both primary invading viruses produced by mosquito cells and viruses produced by other type of human cells. It is of interest to ask if the virus produced by different host and cell type have differentiated tropism against Langerhans cells, and the role of langerin.

In the *in vitro* assay discussed in this chapter, the dengue NS1 and E proteins were both purified from HEK293 cells. In the moLC infection experiments, the virus used for inoculation was produced by mosquito C6/36 cells, while during the 2 days post-inoculation culture, moLCs may also be infected by virus released from initially infected moLCs. Therefore, when considering the DENV infection of Langerhans cells in the

physiological conditions, the observations from *in vitro* experiments need to be interpreted with caution in regarding of the sourcing cell type of virus.

Overall, the data presented in this chapter show that langerin is previously unrecognised cell surface receptor for dengue virus proteins and that the interaction is dependent on glycosylation. Both dengue envelope and NS1 protein could bind langerin suggesting that langerin can mediate interactions with whole virus, but also with dengue secreted NS1 protein. It is of note that langerin blockade increased dengue viral infection of moLC, suggesting that langerin binding can lead to shuttling of dengue virus to intracellular sites of inactivation. A further possibility is that NS1-mediated binding to langerin could act as a decoy to reduce such inactivation. These explanations will be tested in future experimental work. However, given that langerin is known to contribute to CD1a-restricted T cell responses, these data further implicate the langerin-CD1a pathway in relevance to dengue pathogenesis.

7 Discussion

As discussed in detail in the introduction, dengue is caused by at least 4 different serotypes of dengue virus (DENV 1-4) and is the most prevalent mosquito-borne disease causing a heavy global health burden. The clinical presentation of dengue infection is a spectrum from asymptomatic or mild infections to potentially fatal dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). However, there are currently no anti-viral therapeutics approved for dengue infection and usage of the only approved vaccine is limited by vaccine-related ADE effect in dengue-naive individuals. Because most dengue infections are mild and self-limiting, understanding of the pathology of severe dengue is critical to the development of treatments. The host immune system has been found to play dual roles in both defence and immunopathogenesis of severe disease.

Like other viral infections, innate immunity plays critical roles in the early stage of infection control and initiates the adaptive immune response. However, as a successful pathogen causing human disease, dengue virus has evolved a series of mechanisms to escape the host innate immune response, which emphasises the function of adaptive cellular and humoral immunity. While there is increasing evidence to support the ADE hypothesis and explain the increased risk of severe disease in secondary dengue infection, the role of T cells in disease control and pathogenesis is still under debate. In addition most published dengue T cell studies address conventional peptide-specific T cell activation through antigen presentation by MHC molecules.

CD1a is an MHC I-like molecule highly and specifically expressed on the surface of Langerhans cells in skin epidermis. As one of the members of the CD1 family, CD1a presents foreign and endogenous lipid antigens to responsive T cells (de Jong & Ogg, 2021). CD1a-autoreactive T cells have been shown to be a common component of the blood T cell repertoire in healthy individuals, comprising up to 10% of circulating T cells (de Lalla et al., 2011). While CD1a-expressing Langerhans cells have been found to be one of the first targets of dengue virus infection in the skin after a mosquito bite, the role of CD1a and CD1a-reactive T cells in dengue infection has never been investigated. Lipids form an important component of dengue virus envelope, and host lipid synthesis and metabolism can be hijacked by dengue virus to facilitate virion replication. As such we hypothesised that CD1a+ infected APCs present altered lipid antigens during dengue infection tuning the response of CD1a-reactive T cell populations.

This thesis aimed to address questions regarding CD1a function in the human immune response to dengue virus infection. In these studies, we have shown that a component of dengue patient serum downregulates CD1a expression of moDCs, and that CD1a-autoreactive T cells produce less IFN-γ and GM-CSF when co-cultured with DV-infected CD1a+ APCs. This reduction of cytokine production from CD1a-autoreactive T cells was confirmed with CD1a-autoreactive T cell lines/clones and polyclonal blood T cells from both healthy donors and dengue patients. Interestingly, the cytotoxic function of CD1a-

reactive CD8+ T cell lines/clones potentially increased when co-cultured with DV-infected CD1a+ APCs, killing more cells compared to the uninfected CD1a+ control and DV-infected CD1a- control. However, it was noted that there was nonspecific cytotoxicity and variability, and so the CD8 assays should be interpreted with caution. The differential regulation of T cell effector functions, anti-viral cytokine production or cytotoxicity, may reflect the dual function of CD1a in host immunity against dengue virus.

We sought to address the regulation of CD1a-autoreactive T cell activation in the context of dengue infection and how infection affects CD1a antigen presentation. Studies of endogenous ligands of CD1a (Richard W Birkinshaw et al., 2015; Cotton et al., 2021) revealed that lipid ligands bound to CD1a could be divided into two classes based on their interaction with specific CD1a-autoreactive TCRs including BK6. Permissive ligands, such as phosphatidylcholine (PC) and phosphatidylinositol (PI), which insert in to the ligand groove of CD1a and allow the direct interaction of TCR and CD1a. And non-permissive ligands, such as long chain sphingomyelin (e.g. SM 42:2), which sterically hinder the TCR approach, with the lipid phosphocholine group riding above the display platform of CD1a.

Lipids are vital virus components, for example making up the lipid bilayer of the viral envelope. Sphingolipids were reported to be crucial to the composition of the West Nile Virus (WNV) envelope, and a significant increase of sphingolipids was noted in WNV-infected cells (Martin-Acebes et al., 2014). Analysis of the lipid content of WNV envelope

revealed an enrichment of SM and reduced level of PC compared to total cellular membranes of model host cells (Martin-Acebes et al., 2014). In addition, infected host cell lipid metabolism was found to be hijacked by flavivirus to facilitate virus replication and reassembly (Cloherty et al., 2020). On comparative lipidomic analysis of lipid extracted from mock-infected and DV-infected mosquito cells, SM was found to be dramatically increased after DV infection (Rushika Perera et al., 2012). Based on these findings, it is reasonable to hypothesise that dengue virus infection alters the lipid profile of APCs and increases the contribution of CD1a non-permissive ligands, therefore impacting the CD1a-reactive T cell response. To further address this hypothesis, CD1a-reactivity of lipids extracted from mock-infected and DV-infected APCs (such as K562 cells or moDCs) could be assessed using CD1a-autoreactive T cells line/clones and polyclonal blood T cells. With this experimental design, confounding CD1a-independent T cell activatory factors, such as dengue virus or soluble or membrane-bound signalling molecules can be controlled for with mock-infected and DV-infected conditions in addition to EV CD1a-negative and anti-CD1a controls.

It is of note that the alteration of lipid composition in the DV-infected APCs may not necessarily affect the repertoire of CD1a ligands. To investigate this question directly, we must analyse the lipid ligands bound to cell surface CD1a. Our group has developed a K562 cell line that expresses an engineered CD1a protein, with an ectodomain that can be cleaved from cell surface with HRV 3C-protease, purified and analysed using lipidomic techniques. If an increase in non-permissive CD1a ligands bound to CD1a derived from

DV-infected cells were detected, it could explain the altered CD1a-autoreactivity of T cells.

However, the hypothesis of altered CD1a ligand profile does not fully explain why T cell effector functions, cytokine production and cytotoxicity, showed differential regulation after co-culture with DV-infected APCs. As discussed in Chapter 5, increased cell death in DV-infected K562-CD1a cells may be a factor increasing the sensitivity of K562 cells to CTL-induced cell death. However, the DV-infection of T cells may also contribute to this observation and could not be excluded by the experiments described in this thesis. The influence of DV-infection of T cells was controlled for in the cytokine production assays by GA-fixation or 4G2 antibody blocking. However similar control conditions were not employed in the cytotoxicity assays using CD8+ T cell lines/clones due to limitations in cell availability. CD8+ T cells were found to be more susceptible to DV infection than CD4+ T cells (Silveira et al., 2018). In addition, T cell activation markers, including CD69, CD38 and HLA-DR, were shown to be increased on the DV-infected T cells (Silveira et al., 2018). Therefore, it is possible that DV-infection of CD8+ T cells may synergise with CD1a-dependent T cell activation and enhance the killing capacity of these T cell lines/clones. If this were found to be the case, it would be important to investigate whether DV-infection could increase the killing capacity of peptide-specific CD8+ T cells. To answer this question, dengue-specific CD8+ CTL lines/clones could be co-cultured with APCs in the absence or presence of peptide antigen and dengue virus.

In addition, it is noteworthy that some CD4+ T cell subsets have cytotoxic activity (Takeuchi & Saito, 2017). The protective role of these CD4+ CTLs has been highlighted in dengue infection (Y. Tian, Sette, & Weiskopf, 2016). CD4+ T cells were demonstrated to mediate DV-specific killing *in vitro* and *in vivo* using a DV-infection mouse model (Gagnon, Ennis, & Rothman, 1999; A. L. Rothman, Kurane, & Ennis, 1996; L. E. Yauch et al., 2010). The frequency of CD107a+ CD4+ T cells is higher in DF patients compared to patients with more severe disease (Duangchinda et al., 2010). Although CD1a-reactive cytotoxic T cell lines were reported early in the study of CD1a antigen presentation (Porcelli et al., 1989), most studies so far have focused on cytokine production as the major read-out of CD1a-dependent T cell activation. In this thesis, we demonstrate that CD8+ CD1a autoreactive T cells can kill APCs in a CD1a-dependent manner. However, whether some CD4+ CD1a-reactive T cells could also have cytotoxic function is still an open question. Indeed CD4+ T cells were reported to produce Granzyme A after DV-infection, while CD8+ T cells did not (Silveira et al., 2018). It would therefore be of interest to test the effect of DV-infection on the cytotoxicity of CD4+ T cells, including the CD4+ CD1a-autoreactive T cells.

It is of interest to discuss the CD1a-induced cytotoxic T lymphocytes (CTLs) activation with more details. Although the CD1a-reactive CTLs were described in the early time of CD1 research history, however, most studies on CD1a-reactive T cells by far focused on

the cytokine releasing from T helper cells. One of the potential reasons could be the limited cell types expressing CD1a, i.e. Langerhans cells and some subtype of dermal DCs. Professional APCs are known to have natural mechanism to resist CTL/NK cell induced cytotoxicity(Bladergroen et al., 2001). Therefore, if CD1a-reactive CTLs can efficiently kill pathogen-infected or transformed (in the case of Langerhans cell histiocytosis) primary Langerhans cell is a key question regarding the roles of these CD1a-specific CTLs. On the other hand, instead of killing CD1a+ APCs, there is a possibility that the CD1a-reactive CTLs may kill the bystander CD1a- cells, similar to what was observed with MHC-reactive CTLs(Burrows, Fernan, Argaet, & Suhrbier, 1993). To date, there is no report on the bystander killing of CD1a-reactive CTLs yet. Therefore, it is of importance to test if the same phenomenon can be induced by CD1a-reactive CTLs.

It was reported that the IL-10 produced by metastatic melanoma cells can induce downregulation of CD1 expression on DCs in metastatic tumour lesions(Gerlini et al., 2004). The authors suggested that this is an escaping strategy used by tumour cells. If the bystander killing is true for CD1a-reactive CTLs, this could be an additional immune surveillance mechanism suppressed by the tumour cells besides of the activation of CD1a-reacitve helper T cells. The same mechanism might be shared by other pathological conditions in skin, including dengue infections.

In addition, there could be several other critical questions to be answered on CD1a-reactive CTLs:

- Are there CD1a-reacitve memory CTLs existing in skin and blood?
- Do CD1a-reactive CTLs prefer different CD1a ligands comparing to CD1a-reactive helper T cells?
- The CD1a-reative CTL clones described in this thesis recognised self-lipid ligands. How is the activity of these CD1a-autoreactive CTLs regulated *in vivo*?
- No protein-protein interaction between CD1a and CD8 or CD4 molecule has been reported to date. What are the similarities and differences between the CD1a-induced and MHC I-induced CTL activation at molecular level?

The study on the CD1a-reactive CTLs will be an important complement to the current studies on CD1a-reactive helper T cells. In addition, it will also help to develop a more comprehensive understanding of CTL-induced cytotoxicity in various pathological conditions.

Langerin was reported to facilitate CD1a ligand loading in LCs (Hunger et al., 2004), and was found to co-localise with CD1a in the LC-specific organelles, Birkbeck granules (Sugita et al., 1999). In this thesis, protein-protein interaction was found between purified human recombinant langerin and purified dengue envelope (E) protein. Furthermore, neutralising antibody blockade of langerin led to decreased dengue virus infection in

moLCs. Therefore, it would be of interest to investigate if dengue virus can be trapped in Birkbeck granules by binding langerin, and whether such co-localisation of dengue virus and CD1a could increase the CD1a presentation of the dengue virus derived lipid antigens. K562 cells used as model APCs in this thesis do not express langerin and lack Birkbeck granules, therefore the potential function of langerin in dengue envelope-derived lipid antigen presentation by CD1a was unaddressed and did not impact upon the differential T cell function observed. To test the contribution of langerin to CD1a antigen presentation in the context of dengue infection, a Langerhans cell model would need to be used in the APC/ T cell co-cultured experiments. However, the limited langerin expression of the moLCs might hinder such experimental design. Instead, genetically engineered K562 cells with CD1a and langerin dual-expression could be a useful model. However, the formation of Birbeck granules in such an engineered K562 cell line would need to be confirmed.

Polymorphisms of *CD1A* have been observed in many different populations (D. Cerny et al., 2016; Rahman et al., 2018; Seshadri et al., 2013; Seshadri et al., 2014; Taheri et al., 2019; Wang et al., 2019). The association of *CD1A* single nucleotide polymorphisms (SNPs) with susceptibility to several diseases was studied (Rahman et al., 2018; Seshadri et al., 2014; Taheri et al., 2019; Wang et al., 2019). Polymorphisms rs366316 and rs411089 were found to be significantly associated with susceptibility to tuberculosis (Seshadri et al., 2014; Taheri et al., 2019). The other three SNPs (rs16840041, rs2269714, rs2269715)

were found to be associated with blood neurofilament light (NFL) level, which is a marker of neuroaxonal injury in neurodegenerative diseases (Wang et al., 2019). The association between Guillain-Barré syndrome and *CD1A* SNPs was also studied in a Bangladeshi population, but no significant association was found (Rahman et al., 2018). To date, little is understood of the functional consequence of different *CD1A* SNPs. However, 2 SNPs were reported to result in CD1a deficiency in Langerhans cells. A SNP in the 5' untranslated region (rs366316) leads to low CD1a expression at both mRNA level and protein level (Seshadri et al., 2013). Another SNP in the exon area (rs761269454) was found in a Vietnamese family that lead to virtually complete deficiency of CD1a on the surface of Langerhans cells (D. Cerny et al., 2016).

In conclusion, we have investigated the potential roles for CD1a protein in the context of dengue infection and how dengue infection may in turn influence the expression level of CD1a in APCs. We demonstrated that IL-10 in serum from acute phase dengue patients induced downregulation of CD1a expression by moDCs *in vitro*. In addition, dengue virus infection was also found to decrease surface CD1a levels in moDCs. Interestingly, CD1a was significantly downregulated within DV moDC culture by DV-uninfected bystander cells, not DV-infected moDCs. Although CD1a expression level was not significantly reduced in DV-infected APCs we found that the production of anti-viral cytokines IFN-γ and GM-CSF from CD1a-autoreactive T cells was decreased when co-cultured with DV-infected K562-CD1a cells, in which the CD1a expression was not affected by dengue virus

infection, suggesting that CD1a lipid antigen presentation may play a role in modulating Th cell dengue responses. In contrast to cytokine production, the cytotoxic potential of some CD8+ CD1a-reactive T cells was increased leading to more lysis of DV-infected than bystander APCs in co-culture experiments, although these data were variable and require validation.

Finally, we also found that purified dengue NS1 and E protein can bind to recombinant human langerin *in vitro*. By blocking langerin with antibody in moLCs, the dengue virus infection ratio was slightly decreased. However the data was not enough to drive a clear conclusion that if langerin plays protective role in dengue virus infection as what has been demonstrated in HIV-1 infection (de Witte et al., 2007; Ribeiro et al., 2016).

The data described in this thesis lead to more open questions and several directions are worthy to be further explored. The background and details of the most questions and directions have already been discussed in the other part of this thesis. Presented here is a reorganised summary to better illustrate the full scope and rationale.

- CD1a-reactive T cells in dengue infection
    - Changes of CD1a expression level on Langerhans cells and dermal DCs in dengue patients.

- Changes T cell repertoire regarding CD1a reactivity in dengue naïve individuals and individuals with infection history.
- Do CD1a-autoreacitve memory CTLs exist in normal blood and skin samples and if these CTLs activate and expand during dengue infection?
- Can CD1a-autoreactive CTLs kill dengue infected CD1a- cells (e.g. keratinocytes) via bystander killing in skin?
- Changes of CD1a ligands profile in dengue-infected APCs.
- For the naturally occurred CD1a-dysfunction individuals in South Asia and Southeast Asia population, how is their immune response against dengue virus different from the individuals with functional CD1a alleles? What are the T cell repertoires in these individuals regarding CD1a autoreactivity?

➢ Function of langerin in dengue infection

- Can langerin protect Langerhans cells via trapping dengue virus and guiding the virus into degradation pathway?
- If langerin do have protective function, since Langerhans cells were still found to be infected, how does the virus escape from this trapping mechanisms? Can NS1 protein play as decoys to occupy and saturate langerin in this pathway?
- Can the differentiated glycosylation of dengue E proteins and NS1 proteins produced by mosquito cells ("primary" dengue products in mosquito saliva) and

human cells （“secondary” dengue products from infected human cells) lead to different binding capability to langerin?

- How can langerin affect the CD1a-related T cell activity change in dengue infection?

To develop a safe and efficacious vaccine and to identify therapeutic targets for treatment of dengue we need to understand the molecular and cellular mechanisms that control the host immune response to dengue virus in individuals who experience mild and severe disease. The findings presented here expand our understanding of the function of T cells during dengue infection, highlighting the role of unconventional CD1a-restricted T cells and potential of targeting this pathway.

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