Molecular mechanisms underlying the pathogenesis of dengue fever

Dengue virus co-opts innate type 2 pathways to escape early control of viral replication



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A thesis submitted for the degree of Doctor of Philosophy in Medical Sciences - 2022

Acknowledgements

The work in this project has been completed under the supervision of Professor Graham Ogg and Dr Clare Hardman. Being a full-time doctor in clinical medicine from Sri Lanka, I never imagined myself having the opportunity to read for a DPhil in molecular medicine at University of Oxford. I am eternally grateful to Professor Graham Ogg for giving me this life time opportunity and for the constant guidance provided. I am privileged to work under such an outstanding scientist. His humble, respectful and friendly demeanour taught me numerous life lessons which I would carry through my life. Thank you for being a role model and sensing what is the best for the student and gently guiding us towards the goal. I perceived science as fascinating and learnt to enjoy small successes with passion because of Professor Ogg. In addition, I am lucky to have the opportunity to work on one of the most problematic tropical diseases in Sri Lanka, dengue infection.

Dr Clare Hardman has been a delightful colleague and a graceful scientist who taught me various techniques and helped to take the first steps during my infancy in science. It was a hard task teaching a medical doctor on the subtle delicate nature of scientific experiments, not to forget the patience and resilience that must be developed over time. I am humbly thankful for her teaching and guidance.

In addition, working in the WIMM, University of Oxford has afforded me with great experiences alongside great scientists. I would like to thank Linacre College for the support given and the University of Oxford for providing a unique learning environment among diverse passionate scientists coming from all around the world. I would like to thank all past and present Ogg lab members who have been amazing colleagues who inspired and showed me what good science is. I would extend my special thanks to Dr Janina Nahler, Dr Jiahe Yang, Dr Randeep Singh, Dr Jeongmin Woo, Dr Yi-Ling Chen, Dr David Cain who have been instrumental in my project success. Additionally, I would

specially thank Dr Craig Waugh of the flow cytometry facility for performing ILC2 sorting. I would like to thank Alastair Waugh, Kathryn, Zoe and Malcolm from lab management and health and safety for facilitating the wet lab and CAT3 lab work.

Importantly, I would like to thank Professor Neelika Malavige who is an icon of science in Sri Lanka who encouraged me to pursue this venture and her words of encouragement still resonate in my mind. It is amazing how life gives you surprises and opportunities. It is through Dr Nilanka Perera who finished her DPhil at University of Oxford that I received the inspiration to start this journey.

I would have not been here without my beautiful family. Firstly, I would like to thank the most important person to grace my life, my mom, Kusum Fonseka. She is my soul, my energy and the force of encouragement for all things I have achieved. Her guidance and unconditional love through the difficult times in our lives gave me strength to conquer any challenge. I thank my father, late Mr George Fonseka who is the reason I am an all-rounder who loves sport and entertainment. This helped me to enjoy science while enjoying life. Following his sportsmanship, I could qualify to obtain Oxford University sports honours. Although my mom and I unexpectedly lost him, I know that his blessings were always with us.

It is an unforgettable memory where COVID-19 pandemic disrupted all of our scientific activities and halted progress of my DPhil. Every dark cloud has a silver lining! Myself and my wife, Dr Dayakshi Abeyaratne, had our son, Devin (Georgie), during the lockdown and we spent the most memorable period of our lives together in Oxford. During this combined ‘PhD-like’ project, I received invaluable support from my supervisors and friends. Thank you so much.

Finally, I am extremely grateful to, Dr Dayakshi Abeyaratne, my charming and loving wife, who gave me all the strength and support while in Oxford. Her simplistic way of looking at complex matters and unconditional love helped me to push boundaries. Her constant listening and advice on my sometimes failed ILC2 experiments, although she did not understand what I was talking about, was the reason that I could carry out my work on a brighter note.

Effects of COVID-19 pandemic on scientific work

Unexpectedly, the COVID-19 pandemic affected the progress of my DPhil. In the initial period of the pandemic, I had to reduce academic activities related to the institute social distancing limitations and then closure of institution for non-COVID related projects for number of months. I used this lockdown period in reading and writing the sections of the thesis. Due to the necessity of wet lab and CAT3 access for my project I had to stop all experiments during this period. After re-opening of the labs I had to work in a restricted environment with limited number of sessions per week. Due to the fact that ILC2 require a 6-8 weeks expansion period and the lengthy process of viral stock generation, the nature of the restricted access had a particular impact on my research. Finally, I am happy that I could work hard to compensate as much as possible for the time lost.

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Abstract

Dengue is a mosquito borne tropical infection caused by the dengue virus with a dramatic global spread over the last few decades. Dengue infection can present as dengue fever (DF) or the severe forms such as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). There is a recognised clinical link between asthma and other allergies associating with severe dengue. Furthermore, mast cells are known to be activated in dengue infection and high levels of type 2 cytokines are known to be associated with DHF. Group 2 Innate Lymphoid Cells (ILC2) are type 2 cytokine secreting cells that respond to different stimuli including lipid mediators such as prostaglandin D_2 (PGD_2) and leukotrienes. Here, the hypothesis was tested that ILC2 contribute to dengue pathogenesis. First, increased peripheral blood ILC2 frequency was observed in dengue-infected individuals compared to healthy controls (HC). From *ex vivo* RNA sequencing data of ILC2 sorted from peripheral blood mononuclear cells (PBMC) of patients with DF and DHF, it was found that ILC2 were activated and infected, supporting viral dissemination. *In vitro*, ILC2 were permissive to infection particularly when activated and led to productive viral replication. From *ex vivo* RNA sequencing data it was evident that in DF the type I interferon related genes were highly expressed by ILC2 and in DHF this response was impaired.

Blockade of CRTH2, a PGD_2 receptor, reduced dengue viral infection in ILC2 by inhibiting action of both exogenous and endogenously produced PGD_2; and PGD2 reduced the ability of ILC2 to respond to type I IFN. Moreover, LTE_4, another lipid mediator in the group of leukotrienes produced by mast cells increased infectivity of ILC2, and this could be inhibited by leukotriene receptor blockade. It was found that activated ILC2 supernatants promoted differentiation of monocytes to monocyte-derived dendritic cells (MoDCs) and could upregulate expression of DC-SIGN and MMR, which

are key receptors facilitating attachment of dengue virus. Additionally, activated ILC2 supernatants increased dengue virus infection in differentiating monocytes in a GM-CSF dependent mechanism. Lastly, urinary PGD2 metabolites were elevated in severe dengue when reaching the 'critical phase' of the disease.

In summary, ILC2 are highly activated during acute dengue infection and can be infected by dengue virus *ex vivo* and *in vitro*. Dengue virus co-opts the innate type 2 pathways to escape protective type I interferon responses. We propose CRTH2 inhibition could be a novel mechanism to reduce viral replication in dengue infection.

Abbreviations

AD	Atopic dermatitis
ADE	Antibody dependent enhancement
ALT	Alanine transaminase
AST	Aspartate transaminase
CCL	Chemokine (C-C motif) ligand
cGAS	Cyclic GMP/AMP synthase
CLEC5A	C-Type Lectin Domain Containing 5A
COX	Cyclooxygenase
CPE	Cytopathic effects
CRTH2	Prostaglandin D2 receptor 2
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DEG	Differentially expressed genes
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DP1	PGD2 receptor 1
DSS	Dengue shock syndrome

E	Envelope
ELISA	Enzyme-linked immunosorbent assay
EP2	Prostaglandin E2 receptor 2
ER	Endoplasmic reticulum
FDR	False detection rate
FFA	Foci forming assay
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA-3	GATA Binding Protein 3
GDP	Gross development product
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GO	Gene ontology
H-PGDS	Hematopoietic prostaglandin D synthase
HRP	Horseradish Peroxidase
HS	Heparan sulfate
ICAM-3	Intracellular adhesion molecule 3
IFITM3	Interferon Induced Transmembrane Protein 3
IFN	Interferon
IL	Interleukin
IL4Rα	IL-4 receptor alpha
ILC2	Group 2 innate lymphoid cells

iNKT	Invariant natural killer T cells
ISG15	Interferon-stimulated gene 15
LRT	Likelihood ratio test
LTE4	Leukotriene E4
MAIT	Mucosal associated invariant T cells
MC	Mast cells
MCP-1	Monocyte chemoattractant protein-1
MMR	Macrophage mannose receptor
MoDC	Monocyte derived dendritic cells
MOI	Multiplicity of infection
NCR	Natural cytotoxicity receptors
NK	Natural killer
NS1	Non-structural protein 1
OAS	2'-5'-Oligoadenylate Synthetase
PAF	Platelet Activating Factor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2

prM	Pre-membrane protein
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RSAD2	Radical S-Adenosyl Methionine Domain Containing 2
RSV	Respiratory syncytial virus
SEARO	South-East Asia Regional Office
STAT1	Signal transducer and activator of transcription 1
STAT2	Signal transducer and activator of transcription 2
T-bet	T-box transcription factor TBX21
Tc2	Type 1 CD8(+) T cells
TEER	Trans-epithelial/transendothelial resistance
TGF	Transforming growth factor
Th2	Type 1 CD4(+) T cells
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSLP	Thymic stromal lymphopoietin
TXA2	Thromboxane A2
TXB2	Thromboxane B2
UTR	Untranslated region
VEGFR	Vascular Endothelial Growth Factor receptor

WHO	World health organization

1 Chapter 1: Introduction

1.1 Introduction to Dengue Fever

1.1.1 Dengue virus and structure

Dengue fever is an arboviral disease caused by the dengue virus. The dengue virus (DENV) belongs in the genus Flavivirus and under the family Flaviviridae. This has four distinct, but closely related, serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). Over the last few decades dengue has evolved from a sporadic disease to a major public health problem with substantial impact due to the global spread and disease severity.

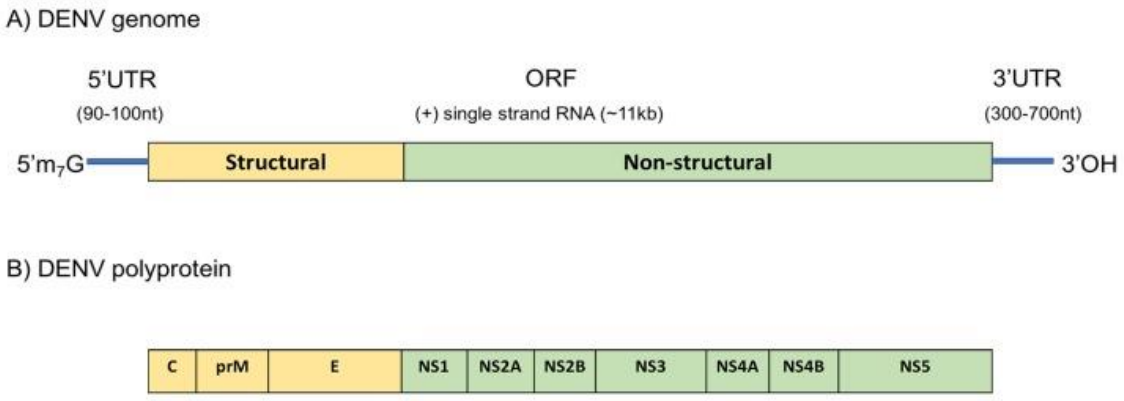


Figure 1: Dengue virus structure. A) Dengue virus genome and B) DENV polyprotein containing structural and non-structural proteins. *Obtained from* (1)

Dengue virus is an RNA virus consisting of a positive-sense RNA genome of approximately 11 kilobases. The DENV genome encodes three structural proteins (capsid, pre-membrane and envelope) and seven nonstructural proteins (NS1, NS2A/B, NS3, NS4A/B and NS5)(2) (Figure 1). The genomic RNA also carries 2 noncoding regions, known as the 5' untranslated region (UTR) and 3' UTR, that are highly structured and play an essential role in viral replication(3). Dengue virus has 3 structural proteins: the capsid (C) protein, responsible for viral RNA recognition and assembly; the pre-membrane protein (prM), implicated in blocking immature virus fusion; and the

envelope glycoprotein (E), responsible for viral interaction with the cellular receptor and for viral entry into the target cell. Mature DENV capsid is a highly basic protein of 12 kDa that forms homodimers in solution with affinity for both nucleic acids and lipid membranes. Structural studies showed that the monomer has four α-helices (α1 to α4). This protein is located in the cytosol and is the very first protein translated by the viral genome followed by prM(4). Capsid is one of the least conserved proteins among the flaviviruses, therefore capsid based vaccines would be unlikely to provide balanced protection against all serotypes. The prM protein, also termed precursor membrane protein, comprises an N-terminal domain followed by an M-domain, stem region and two transmembrane helices. The prM protein is believed to be a chaperone for the envelope protein to assure its conformational changes during different steps of secretory pathway and to produce infectious viral particles. Stem region of prM is reported to have a significant role in viral maturation. This is due to the fact that prM has shown higher affinity toward E protein, as well as with liposomes under acidic condition, which induces conformational changes in surface protein observed during maturation of the dengue virus(5). The prM protein alone does not act as a strong immunogen, therefore, it is always used in combination with E protein for development of vaccines.

The E glycoprotein has important functional roles in virus attachment to cells and fusion with membranes, and is the major target for neutralizing antibody. DENV infectivity depends on E protein binding to target cell heparan sulfate(6). It contains the main epitopes recognised by neutralizing antibodies (virus-specific and cross-reactive epitopes). This protein has three structural and functional domains: domain II contains the internal fusion peptide (responsible for the fusion of flaviviruses to their target cells) and the domain III contains cellular receptor-binding motifs. Domains I and III contain predominantly subcomplex and type-specific epitopes, whereas domain II contains the

major flavivirus group and subgroup cross-reactive epitopes(7). The DENV E protein gene is considered a molecular marker for viral pathogenicity. The E protein gene sequences of DENV pathogenic strain and nonpathogenic strain are different. Antibodies that deactivate one type of DENV may be ineffective against other DENV strain owing to the structural variations in E proteins(6, 8, 9).

There are 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) involved in the regulation of protein synthesis, viral replication, pathogenesis, and the control of host antiviral responses(10). DENV non-structural protein 1 (NS1) is 43-48kDa, multifunctional, dengue glycoprotein synthesised by infected cells as a monomer (48 kDa). It is then glycosylated in the endoplasmic reticulum (ER), and released into the extracellular milieu as a hexamer (~310 kDa). Secreted DENV NS1 circulates in the blood during acute illness and serum NS1 level has been shown to correlate with dengue disease severity(11). Therefore, NS1 can be efficiently used as a diagnostic marker for early diagnosis of dengue as it is secreted into blood plasma before the production of antibodies against the virus and it is the only NS dengue viral protein that is secreted. Upon secretion, NS1 accumulates in extracellular fluids in hexameric conformation stabilised by a lipid core. In a murine model, secretory NS1 is directly involved in inducing features of DHF/DSS through destabilizing tight junctions by stimulating glycocalyx degradation, which leads to tissue specific endothelial hyperpermeability and vascular leakage(12). NS1 is also reported to activate macrophages, platelets, and mononuclear peripheral cells via binding to Toll-like receptor 4(13).

Each NS protein has specific roles to assist viral replication and act together to exacerbate viral replication and to inhibit host mediated viral defense mechanisms. NS2A protein (NS2A-D125) of DENV2 is considered essential for host protease recognition and mutations in this sequence could hamper viral replication. NS2A acts synergistically with

NS4B in immune suppression by inhibiting phosphorylation of TANK binding kinase (TBK), hence, raising viral titres in host cells. NS2B can combat host immune responses by inhibiting type I interferon synthesis through targeting cyclic GMP/AMP synthase (cGAS)(14). NS3 is one of the most conserved proteins among DENV viruses as 77% amino acids of NS3 are similar in all four serotypes, which makes it a potential target for vaccine development. Mutations in this region reduce the polyprotein processing and viral replication efficiency due to attenuation of both protease and helicase activities of NS3(15). Protease and helicase regions of NS3 are separated via a linker region, which comprises 11 amino acids. Mutational analyses have revealed that the linker region has evolved to its optimal length, which imparts enough flexibility to NS3 so that it can optimally perform both its helicase and protease activities. Targeting helicase activity of NS3 could significantly attenuate viral replication and can be exploited for the development of antiviral drugs. Use of molecular beacons along with suramin can also be used as a tool for identifying DENV helicase inhibitors (16, 17). It is believed that NS4A is involved in inducing modifications in host membranes in a way that facilitates the formation of a replication complex, which is essential for viral replication. Similar to NS4A, NS4B also has a prominent role in viral replication. NS4B resides in the ER of DENV infected cell unless recruited by NS4A for participation in the replication complex. Additionally, NS4A, NS4B, and NS1 are reported to inhibit autophagy in several cellular models, thereby preventing self-degradation of virus infected cells and promoting viral infection(18). NS5 protein is the largest and the most conserved of the flavivirus proteins, and functions as a RNA-dependent RNA polymerase(19). NS5 protein harbors the key catalytic enzymes of the replication complex involved in viral genome replication and capping. The N-terminal region of NS5 contain a methyl-transferase domain, which is capable of catalyzing all activities necessary for 5′-RNA

capping. Capping contributes to viral evasion from host cell sensors. The C-terminal region of NS5 contains a conserved domain for RNA dependent RNA polymerase for *de novo* synthesis of viral RNA(20). NS5 is directly involved in the reduction of STAT2 levels, which is a key regulator for interferon (IFN) signaling. IFN signaling provides a first line of defense against viral infection. But, viruses have evolved to escape this signaling pathway. NS5 protein contributes to evasion of IFN signaling pathway through binding to STAT2. In addition to NS5, NS2A, NS4A and NS4B are reported to hamper IFN signaling by preventing the phosphorylation of STAT1(5, 21).

The dengue genomic RNA also carries 2 noncoding regions, known as the 5' untranslated region (UTR) and 3' UTR, that are highly structured and play an essential role in viral replication(3). At the 5' end, the genome possesses a type I cap that allows ribosome recognition and the genome does not have a polyadenylated region at the 3' end(5).

1.1.2 DENV serotypes

All four serotypes of dengue virus (DENV) share similar but distinct antigenic properties. Polyclonal serum antibodies raised to one DENV serotype can bind to all four DENV serotypes, but may be minimally neutralizing. However, such antibodies do efficiently neutralize and provide long- term protection against the same serotype. DENV serotypes have ~50–60% sequence homology(22). In addition to the serotype differences, genotypes have been associated with characteristically mild or severe disease(22). Each serotype has four to six distinct genotypes. For DENV3 five genotypes have been described, named I-V(23). Close monitoring of the emergence of new genotypes would be important for prediction of future outbreaks and to study the molecular epidemiology of the dengue virus.

1.1.3 Dengue viral vector characteristics

Dengue virus is transmitted to humans through the day biting mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. Both species are sensitive to changes in environmental conditions such as temperature, precipitation and humidity and prefer tropical climates. Climate change is thought to impact vector dynamics, and it is predicted that dengue fever could pose risk to a wider population and geographical locations in future. Aedes-borne diseases spread by *Aedes aegypti* and *Aedes albopictus* have both emerged and spread worldwide throughout tropical, sub-tropical, and temperate zones in urban areas. *Ae. aegypti* is restricted to warm, urban environments where it breeds in human-made containers in and around houses. Further, it bites during the daytime exclusively on humans, and is a highly competent vector for dengue, chikungunya, zika, yellow fever, and other viruses. In contrast, while *Ae. albopictus* can adopt this urban ecology, it is far more ecologically flexible and can breed in suburban, rural, residential, and agricultural habitats, which could be both natural or human-made. It bites humans and a wide range of mammals and birds and is a moderately competent vector for dengue, chikungunya, and Zika(24).

It has been predicted that we could expect poleward shifts in Aedes-borne virus distributions. However, the differing thermal patterns of the two vectors may produce different patterns of shifts under climate change. More severe climate change scenarios could result in larger population exposure to transmission by *Ae. aegypti*, but not by *Ae. albopictus* in the most extreme cases. Climate-driven risk of transmission from both mosquitoes could increase substantially for most of Europe. In contrast, significant reductions in climate suitability are expected for *Ae. albopictus*, most noticeably in southeast Asia and west Africa. Within the next century, nearly a billion people are threatened with new exposure to virus transmission by both *Aedes spp*. in the worst-case

scenario. Additionally, a global shift of dengue towards seasonal risk across regions has been projected (24). A study from 2002 estimated that the population at risk (PAR) from dengue infection would rise from 1.5 billion in 1990 to 5–6 billion by 2085 as a result of climate change(25). A more recent study added GDP per capita as a predictor in dengue distribution models, and found that climate change would increase the global dengue PAR by 0.28 billion by 2050 if GDP held constant compared to today(26).

1.1.4 Epidemiology of dengue

Dengue viruses (DENV) are mosquito-borne flaviviruses that have plagued humans for centuries. A global pandemic of dengue begun in Southeast Asia after World War II. The first reported epidemics of dengue fever occurred in 1779-1780 in Asia, Africa, and North America with a simultaneous occurrence of outbreaks across three continents. Usually, there were long intervals (10-40 years) between major epidemics, mainly because the virus and their mosquito vector could only be transported between population centers by sailing vessels(27). In Southeast Asia, by 1975 it became a leading cause of hospitalization and death among children in many countries. In the 1980s, DHF began a second expansion into Asia when Sri Lanka, India, and the Maldive Islands had their first major DHF epidemics. In 1995, dengue was the most important mosquito borne viral disease affecting humans; its global distribution is comparable to that of malaria, and an estimated 2.5 billion people are currently living in areas at risk for epidemic transmission. Each year, tens of millions of cases of dengue fever occur and depending on the year, up to hundreds of thousands of cases of DHF. Before 1995, the case-fatality rate of DHF in most countries was about 5%: most fatal cases being among children(27). DENV infections have been endemic in Sri Lanka since the mid-1960s. DF was serologically confirmed in the island in 1962. The presence of DF in all of the major towns situated below 1200 m elevation was confirmed in 1966 and in 1976–1978(28).

Epidemic dengue was first recognized in Colombo, the capital of Sri Lanka, in 1965–1966. Outpatient, clinic-based surveillance at Colombo's Lady Ridgeway Children's Hospital in Colombo during 1980–1984 found dengue accounted for 16% of acute febrile illness, among which 66% were secondary (recurrent) dengue cases. A 1980–1985 school-based study found baseline DENV seroprevalence was 50% in Colombo and 6-month dengue incidence was 15.6%, of which 37% were secondary cases. In the early 1980s, severe dengue was rare in Sri Lanka. However, since 1989, many cases of DHF have been reported from the heavily urbanized western coastal belt of Sri Lanka(29). Later, urbanization and human population growth in the tropical regions of the world have produced favorable conditions for DENV transmission. Now, there is a dramatic increase in DENV infections with an expanding geographic distribution of both DENV and the mosquito vectors. In southern Sri Lanka, 6.3% of all patients presenting with acute febrile infection had acute dengue, with 50% having primary and the rest having secondary infection. Fifty-four percent of patients had either acute or past dengue giving an overall seroprevalence of 50.9%(30). In a hospital-based study from Colombo, DF was seen in approximately 30% and DHF in nearly 70%. Of the 34.3% with primary dengue infections, 51.4% developed DF and 48.6% developed DHF. Overall, 38.9% had bleeding manifestations. Secondary dengue infections were significantly associated with development of severe disease and mortality was 3.7%(31). The WHO has named dengue as one of the top 10 threats to global health in 2019 due to the burden of infection in low- and middle-income countries, particularly as there has been a 15-fold increase in dengue over the last 2 decades. The annual global cost of dengue was estimated to be a staggering US$8.9 billion in 2013(32). Costs of dengue control activities and hospitalization were estimated to be US$3.45 million in year 2012 in Sri Lanka(33), which highlights the economic burden of this disease in resource-poor countries. The case fatality rates (CFRs)

due to dengue have declined in Sri Lanka from approximately 1% in 2009 to <0.3% in 2018(31, 32). This was achieved through early presentation and diagnosis and timely hospital admission for detection of plasma leakage and meticulous fluid management in hospitals to prevent deaths due to shock or fluid overload.

1.1.5 Dengue Fever – clinical classification and management

Manifestation of dengue can range from asymptomatic infection, dengue fever (DF) to the more severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The severe forms of dengue infection are characterised by vascular plasma leakage through the 'leaky' capillaries leading to accumulation of fluid in pleural and abdominal cavities. This leads to depletion of the intravascular space resulting in hemodynamic shock and hemorrhage causing significant morbidity and mortality(34).

DENV infection is transmitted via a mosquito bite, after which there is an incubation period of 4–6 days before fever develops. The clinical presentations of dengue viral infections range from asymptomatic to severe illness that may lead to death if not properly managed. The symptomatic cases are categorized as undifferentiated febrile illness (UF), dengue fever (DF), dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS) and unusual dengue (UD) or expanded dengue syndrome (EDS)(35). Many patients experience a prodrome of chills, erythematous mottling of the skin, and facial flushing (a sensitive and specific indicator of dengue fever). The prodrome may last for 2-3 days. Classic dengue fever begins with sudden onset of fever with headache, retro-orbital pain, arthralgia, myalgia, vomiting, abdominal pain with mild hemorrhagic manifestations (eg, petechiae, bleeding gums, epistaxis, menorrhagia, hematuria). The fever lasts 2-7 days and patients develop rash and hemorrhagic manifestations including petechiae and bleeding at venipuncture sites. Dengue hemorrhagic fever (DHF) or dengue shock syndrome are the severe forms of dengue disease(36). The distinct feature of DHF is the

increase in vascular permeability (plasma leakage) that differentiates DHF from DF. Plasma leakage denotes selective leakage of fluid into the pleural and peritoneal cavities that results in pleural effusion and ascites. In DSS, presentation is the same as in DHF but plasma leakage is more severe such that the patient develops hemodynamic shock(36). Features of DHF are presented in modified WHO/SEARO 2011 criteria which include plasma leakage: elevation of Hct ≥ 20%, detection of ascites, pleural effusion by physical examination, chest film (right lateral decubitus position) or ultrasound and bleeding manifestations. The most common bleeding manifestations are petechiae and other symptoms (epistaxis, gum bleeding, hematemesis, melena, hypermenorrhea, hemoglobinuria)(36). Clinical symptoms of bleeding, low platelet count (<50,000mm3), melena, rash, delayed admission (>5days after onset) elevated hematocrit, lymphadenopathy, hepatomegaly, splenomegaly, convulsions and mortality were significantly associated with severe dengue(37). In a meta-analysis, five symptoms demonstrated to associate with increased risk for severe dengue, included bleeding, vomiting/nausea, abdominal pain, rashes, and hepatomegaly(38). Onset of DHF is characterised by the phase termed as the 'critical phase', which lasts for 24–48 hours and is associated with plasma leakage; whereas patients with DF directly proceed to the recovery phase without developing plasma leakage. Severe dengue is associated with a transient increase in vascular permeability due to endothelial dysfunction in the critical phase. Increase in vascular permeability is associated with vascular leakage, resulting in accumulation of fluid in pleural and peritoneal cavities, and with reduction in blood pressure and pulse pressure, resulting in poor organ perfusion(39).

Currently, there is no effective specific drug for treatment of dengue and the efficacy of available vaccines are still doubtful (40, 41). Therefore, management of this viral infection solely depends on administering fluid and blood transfusions in a meticulous

manner. Meticulous fluid balance is achieved through monitoring indicators of shock and hemoconcentration. Measuring haematocrit/pack cell volume in blood and hemodynamic parameters such as blood pressure, pulse pressure, capillary refilling time and urine output are useful parameters to decide on fluid management.

1.1.6 Antibody dependent enhancement

Due to the presence of 4 distinct serotypes a person can acquire dengue infection up to 4 times during the life time. These infections are termed as primary, secondary, tertiary and quaternary. Primary dengue infections may cause a rash and fever, but many infections are asymptomatic. In certain hyperendemic settings, the overall seroprevalence of asymptomatic dengue infection was measured as 25% in children aged 1–12 years, but the prevalence could vary from 15 to 35% depending on endemicity and density of the population (42). Recovery from infection typically provides lifelong immunity against that particular serotype. However, four cases of homotypic infection (re-infection with the same serotype) have been reported in a case study suggesting that this can occur occasionally (43). Cross-immunity to another serotypes after recovery from dengue infection is only partial, and temporary. Therefore, the immune responses induced against one serotype of DENV does not adequately protect against infection by other serotypes, allowing secondary infection with heterotypic DENV. In addition, dengue co-infection from several serotypes has been described and estimated to be around 15% of total infections (44). Also, phylogenetically distinct DENV1 viruses were detected in the same person (45). Epidemiologic studies suggest that acute cases of dengue illness, including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), occur mostly among secondarily infected patients and cause severe symptoms(46). Subsequent infections (secondary infection) by other serotypes increases the risk of developing severe dengue(47-50). The exact cause of this is unknown, but it is accepted that the

phenomenon of antibody-dependent enhancement (ADE) may cause increased pathogenicity. ADE occurs when antibodies from a previous heterotypic infection do not neutralize a secondary infection with a different serotype but still bind to viral proteins. This creates a virus–antibody complex phagocytosed by cells that are not usually infected via Fcγ receptors, especially by monocytes via FcγII receptor. This results in increased viremia and pathology. Therefore, in a dengue endemic tropical setting, it is evident that a significant portion of the population of asymptomatic dengue cases are at risk of developing dengue haemorrhagic fever and dengue shock syndrome with secondary dengue infection, in addition to the recognizable population who will have symptomatic dengue fever. In contrast, less is known about tertiary and quaternary infections of dengue. It has been shown that the number of dengue admissions caused by a third or fourth DENV infection was extremely low (0.08-0.8%). Once admitted, the risk for DHF relative to dengue fever was not different for those experiencing third or fourth DENV infections over those with a second DENV infection(51).

It is imperative to study the factors responsible for development of severe dengue disease. Secondary dengue infection, obesity and pregnancy are thought to contribute to increased morbidity and mortality(52, 53). Additionally, severe dengue fever has been reported to be associated with atopy, asthma and a type-2 immute state(54, 55). However, epidemiological studies have shown that most DENV infections are either asymptomatic or lead to uncomplicated dengue fever (DF), even among patients secondarily infected with a heterotypic DENV serotype(56). Symptomatic cases appear in only 1% to 5% of infections; however, such cases often result in DHF(49). Severe disease is seen in only 1% of DENV cases; however, the rate of mortality in severe cases can be up to 20%(19). Thus, ADE might not always be solely responsible for severe disease in dengue patients. An epidemiological study of a large number of infants born to DENV-seropositive

mothers showed that, although ADE could be detected, it did not correlate with the incidence of DF and DHF. The infants who developed DHF did not have significantly higher frequencies or levels of DENV3 ADE activity compared to symptomatic infants without DHF(57). ADE suggests that the binding of virus-antibody immune complexes to Fc receptors on the surface of phagocytic cells facilitates virus entry and replication. Fcγ receptor is critical in binding to the DENV immune complex, enhancing virus uptake by DCs and macrophages. These Fcγ receptors connect the innate and the adaptive immune responses by transmitting activating signals to natural killer lymphocytes and myeloid cells upon recognition of IgG. The recognition of viral particles by cross-reactive IgG antibodies leads to increased absorption of partially neutralised viruses. This absorption is mediated by Fcγ receptors present on dendritic cells (DCs), monocytes and macrophages, resulting in increased viral infectivity and ultimately disease severity. Additionally, in Brazil where the population has a high levels of antibodies against dengue virus (indicating previous infection), even during widespread epidemics of dengue fever, the proportion of cases that progress to DHF is small, ranging from <0.05% to 4% of all cases(58). This may indicate that other factors may be involved contributing to disease severity. Additionally, secondary immune enhancement has been a major impediment to the development of dengue vaccines. Although, there is evidence that sequential infections by different dengue viral serotypes plays an important role in the development of severe disease (49, 50, 59), still it is not clear why some cases of dengue progress to DHF. Therefore, it is imperative to uncover the mechanisms that lead to severe disease in dengue infection.

Usually, the peak viraemia correlates with high fever and coincides with the initiation of IgM and IgG production, which promotes clearance of virus infection. When viraemia is resolving or has subsided, some patients may experience haemorrhage and/or develop

shock. This phenomenon is thought to be immune-mediated(60, 61). Clinically, vascular leakage is observed when fever is resolving. During secondary heterologous DENV infection, the period of viraemia is shorter, likely owing to cross-reactive immunity, due to pre-existing antibodies. Cross-reactive epitope-specific T cells promote an earlier and potentially stronger T cell response than that seen during primary infection(62). Owing to the presence of pre-existing immune memory that may adversely affect immune responses to secondary heterologous DENV infection, the risk of severe dengue is higher in patients who experience secondary heterologous DENV infection(63). Notably, immunological memory to a heterologous serotype alters the infection course such that viraemia likely peaks at higher levels, increasing the risk of developing DHF, but viraemia has also been shown to subside more quickly. The maximum daily rate of viremia clearance was significantly higher in patients with secondary infections than primary(64-66).

Vascular leakage which is a phenomenon prominently seen in severe dengue viral infection is thought to occur as a result of complex interactions between the virus, host genetics and the immune system. The resultant cytokine storm is known to play a fundamental role in disease contributing to the clinical manifestations, which is a consequence of endothelial dysfunction or hyper-permeability resulting in hemoconcentration, hypotension and shock(40, 67).

1.1.7 Predisposing factors to development of severe disease in dengue infection

In addition to secondary dengue infection, many other clinical predictors have been defined in dengue. Metabolic diseases such as diabetes, hypertension, and obesity are known to be independent risk factors that are associated with DHF, and diabetes has also shown to be an independent risk factor for shock and severe organ involvement in acute dengue(68, 69). Obesity was noted to be significantly associated with hospitalization >3

days, presence of increasing haematocrit, and decreasing platelets. Both peak and admission levels of leukocyte counts, AST, ALT and creatinine were significantly higher in the obese group(69). Several hypotheses have been thought to contribute to severe disease in dengue infection in the setting of obesity. Downregulation of AMP kinases leading to increased availability of lipids for viral replication, long-term adipokine exposure leading to endothelial and platelet dysfunction and dysregulation of innate and acquired immune responses are possible factors(70). In addition, older age is also found to be a risk factor for severe dengue(68). Elderly patients are more likely to have diabetes and hypertension and have a significantly higher risk of developing severe disease and hospitalisation. Usually, they can present with atypical manifestations to the hospital(71). In addition, the probability of having secondary dengue infection is twice as high comparing the <60 years group(72). Therefore, as the presence of diabetes and such metabolic diseases increase dengue disease severity in symptomatic patients, this could also increase the likelihood of occurrence of symptomatic infection, rather than asymptomatic infection.

Interestingly, early studies suggested that type 2 cytokines are associated with severe disease and type 1 immunity has been associated with the milder forms of dengue disease(73, 74). Cytokine profiling from an outbreak in Taiwan demonstrated that levels of interleukin (IL)-6, IL-4, IL-13, IL-1β, interferon gamma (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were significantly higher in DHF patients compared to DF patients(68). There have been efforts to identify predictive biomarkers of severe dengue, although the results have not yielded reliable markers that could predict severe disease. It is known that IL-7, IL-8, IL-10, TGF-β, and VEGFR2 display peak differences between DF and severe dengue infections during or before the critical phase (on day 4-5)(75). Additionally, through a systematic search of whole-

genome expression datasets that examined whole blood or peripheral blood mononuclear cells (PBMCs) from patients with acute dengue infection, a 20-gene (3 over-expressed, 17 under-expressed) panel was recognised in various aspects of antiviral innate immune responses and found to be predictive of severe dengue. These include genes related to adhesion and/or opsonization (*SPON2, PTPRM, CX3CR1*), oxidative killing (*GBP2*), NK cell function (*CX3CR1, NCR3*) and signaling (*GRAP2, CACNA2D2, GBP2*). *CHD3*, a chromatin-remodeling factor, may exert a role in DENV infection through interaction with the NS3 and NS5 DENV proteins. These genes distinguished DHF/DSS from dengue fever upon presentation and prior to the onset of severe complications (76). However, bulk transcriptomic data are difficult to interpret because blood consists of multiple cell types that may react differently to infection, masking cell intrinsic differential gene expression. Multiple IFN response genes, particularly *MX2* in naive B cells and *CD163* in CD14+ CD16+ monocytes, were up-regulated in a cell-specific manner before progression to severe dengue(77).

Interestingly, presence of allergy and asthma is considered to be a risk factor for severe disease(54, 78). This may indicate that a type 2 inflammatory state could predispose individuals to develop severe disease (55). Dengue patients with pre-existing asthma have twice the risk of symptomatic dengue infection than patients without asthma. Also, asthma could be an independent risk factor for development of severe organ involvement in dengue(54). It was observed that dengue virus presence in peripheral blood leukocytes was greater among a group with asthma compared to a group without asthma and this could promote higher viraemia leading to severe organ involvement(79). It is recognised that asthmatic patients could have impaired innate type I and type III interferon responses. Some studies have shown a defective or impaired type II interferon (IFN-γ) response or lower Th1/Th2 ratios in atopic asthmatic patients in comparison with control

subjects(80). Also, chronic elevation of Th2 cytokines seen in patients with asthma has been associated with a marked deficiency in IFN- λ1 production(81). Moreover, asthmatic epithelial cells have impaired TLR3-mediated interferon-β (IFN-β) and IFN-λ production, which may increase propensity to have severe dengue(54, 82). With the increasing prevalence of allergic, atopic diseases and asthma over the recent decades, there could be a tendency to develop severe dengue when these predisposing factors are present. This emphasises the need for research studies capable of explaining these mechanisms. As the T Helper 2 (Th2) cell-type cytokines play a dominant role in the allergic inflammatory cascade in asthma(83), we hypothesize that this might potentially activate the allergic inflammatory cascade in dengue and contribute to organ damage.

1.1.8 Dengue viral life cycle and infection mechanisms

During natural infection, cells of the mononuclear phagocyte lineage monocytes, macrophages and dendritic cells including the skin-resident Langerhans cells are primary targets for DENV infection(84, 85). There is a wide range of DENV infection permissive cells indicating that either the virus must bind to a ubiquitous cell-surface molecule, or it exploits multiple receptors or mechanisms to mediate infection. Several candidate receptors and/or attachment factors have been identified, which suggests that DENV is capable of utilizing multiple molecules to enter the cell. In mosquito cells, DENV has been shown to interact with heat-shock protein 70 (Hsp70), R80, R67, and a 45-kDa protein. Heparan sulfate, Hsp90, CD14, GRP78/BiP and a 37/67-kDa high-affinity laminin receptor have been identified as receptors for dengue virus in mammalian cells. C-type lectin receptors are involved in the interaction of DENV particles with human myeloid cells. These include DC-specific intracellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN, CD209), mannose receptor (MR) and C-type lectin domain family 5 member A (CLEC5, MDL-1)(86-88). Additionally, it has been shown

that MR/ DC-SIGN first attracts the virus with high affinity, and the virus concurrently interacts with CLEC5A in close proximity to form a multivalent complex and facilitate CLEC5A mediated signal transduction(89).

Membrane fusion is the central molecular event during the entry of enveloped viruses into cells. The critical agents of this process are the viral surface proteins, primed to facilitate bilayer fusion by the conditions of viral interaction with the target cell. DENV as well as other flaviviruses use clathrin-mediated endocytosis for cell entry(86, 90). It has been shown that the DENV contacts the cell surface and migrates in a diffusive manner toward a pre-existing clathrin-coated pit(91). This suggests that DENV particles move along the cell surface by rolling over different receptors, or migrate as virus–receptor complexes. Upon internalization, the particles are delivered to Rab5-positive early endosomes, which mature into Rab7-positive late endosomes, where membrane fusion primarily occurs(91). Depending on the serotype and the target cell type, DENV is able to utilize an alternative internalization pathway independent of clathrin. It is postulated that the acidic pH in endosomes triggers dissociation of E protein homodimers, which then leads to the outward projection of domain II segment exposing the hydrophobic fusion peptide to the target membrane(86, 92). Subsequently, the hydrophobic residues in the fusion loop would insert into the outer leaflet of the target membrane, triggering the assembly of E protein trimers(8, 93). Next, domain III is assumed to shift and fold back toward the fusion peptide into a hairpin-like conformation. This folding-back mechanism would force the target membrane and the viral membrane to bend towards each other and eventually to fuse, releasing the nucleocapsid into the cell cytosol.

After virus entry in to the cytoplasm and uncoating of the nucleocapsid, the virus genome is then released in to the cytoplasm and is translated as a single polyprotein. The DENV

RNA genome is similar to cellular mRNA, and translates a polyprotein precursor in a cap-dependent manner. The polyprotein is processed by cellular and virus-derived proteases into three structural proteins (C, prM, and E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Figure 1). Upon protein translation and folding of the individual proteins, the NS proteins initiate replication of the viral genome. The newly synthesized RNA is subsequently packaged by the C protein to form a nucleocapsid(86). The prM and E proteins form heterodimers that are oriented into the lumen of the ER. Then, the prM/E heterodimers associate into trimers and these oligomeric interactions are believed to induce a curved surface lattice, which guides virion budding(86).

Viral polyprotein translation is performed at the rough ER. After RNA replication, the genomic RNA associates with the C protein and buds into the ER lumen, acquiring a lipid membrane that contains heterodimers of embedded E and prM proteins constituting immature particles. Viral RNA is replicated by the viral RNA-dependent RNA polymerase NS5 in the replication complex. Structural proteins are assembled with the DENV RNA genome in the ER and then transmitted to the Golgi apparatus. These virions are then transported along the cellular secretory pathway and in the trans-Golgi network undergo a maturation step mediated by furin-dependent cleavage of prM(94, 95). Finally, the mature form of the virus is released from the cell by exocytosis(10) and awaits the next round of infection.

1.1.9 Role of type I/III IFN and mechanisms of dengue escape

The innate immune response constitutes the first line of defense against pathogenic microorganisms, and is important in the early control of viral infections. DENV components, such as single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) replication intermediates act as pathogen-associated molecular patterns (PAMPs).

PAMPs are recognized by a series of innate immunity receptors known as pattern recognition receptors (PRRs), and induce an early response in innate immune cells. The interactions between PAMPs and their corresponding PRRs activate various signaling pathways that finally lead to the production of pro-inflammatory cytokines and type I interferon (IFN-I)(96). However, DENV has also acquired various strategies that provide it with the capacity to inhibit IFN-I responses and thereby to escape this main antiviral innate immune response of the host(97). The IFN-α levels were found to be significantly higher in DF than DHF patients irrespective of the infecting serotype (DENV1 or 2), and were found to decline rapidly at day 3 after fever onset. For DENV2 infections, higher IFN-α levels were found during primary than secondary infections. These results suggest that an early strong interferon response correlates with a better clinical condition(98). Dengue virus has been shown to express a weaker interferon response compared to other viruses(3, 99) , consistent with evading protective type I interferon responses.

PRRs, essential for host defense surveillance, recognise viral RNA and then initiate IFN induction. There are several distinct classes of PRRs including RIG-I-like receptors (RLRs), Toll-like receptors (TLRs), Nod-like receptors (NLRs), AIM2-like receptors, C-type lectin receptors (CLRs), and intracellular DNA sensors such as cGAS(100, 101). Intracellular PRRs, RIG-1 and MDA5 typically carry out important functions to induce IFNs from infected cells. Degraded DENV RNA, sfRNA dampens ubiquitination-mediated RIG-I activation, so that it hinders IFN production(97). Additionally, DENV NS4A binds to MAVS and thus prevents the interaction of RIG-I and MAVS, and reduce IFN induced protection(102). Also, STING is involved in both DNA and RNA pathogen-sensing pathways and can interact with RIG-I and MAVS to enhance the antiviral response. DENV protease NS2B3 was found to cleave human STING(103) and reduce interaction with pathogen sensing pathways. There are 1-10 TLRs described in humans.

TLR7 senses ssRNA whereas TLR3 recognizes dsRNA derived from DENV replication. In addition to the RIG-I–MAVS pathway, TLR3 and TLR7 are important for recognizing DENV RNA in the endosome. Both TLRs are involved in producing a type I IFN response during DENV infection, TLR3 is more effective than TLR7 in IFN induction and DENV inhibition. Therefore, administration of combined TLR3 and TLR7 agonists could decrease DENV replication. In contrast, DENV has been shown to block TLR-mediated antiviral signaling by targeting downstream immune mediators, IκB kinase epsilon (IKKε) and TANK-binding kinase-1 (TBK1)(104, 105). Increased expression level of TLR3 and TLR9 in mDCs, and of TLR2 in pDCs was found during the acute phase of DENV infection. Dengue virus has been shown to reduce interferon secretion from DCs when stimulated with TLR9 agonists(105). Several viral proteins of DENV are involved in suppressing IFN-induced signaling. In the presence of IFN, the DENV NS2A, NS4A, and NS4B proteins were found to enhance the replication of an IFN-sensitive recombinant reporter gene in Newcastle disease virus, whereas only DENV NS4A and NS4B significantly reduced the expression of a reporter gene driven by IFN sensitive response element(106). The NS4B was found to inhibit IFN induced STAT1 phosphorylation and nuclear translocation, which impairs the transcriptional activity of ISGF3 to turn on antiviral genes(106). Moreover, DENV NS5 can bind and inhibit the transcription factor STAT2 activated by IFN treatment(21). Also, NS5 protein of the dengue virus has a methyltransferase activity that methylates viral RNA that helps to disguise RNA from degradation(107).

1.1.10 Lipid mediators and mast cells

The available knowledge indicates that the outcome of DENV infection depends on several factors produced during the initial stages of the viral infection such as viral load, presence of non-neutralizing antibodies, immune cells recruitment and production of

immune mediators. Lipids have been shown to be of great importance in the viral infection process. Evaluation of the serum lipidome of patients infected with DENV-4 identified lipids related to the precursors of Platelet Activating Factor (PAF), phosphatidylcholine derivatives and four triglycerides as characteristic for the DENV group compared to healthy subjects(108). Additionally, it was found that activity of secreted phospholipase A_2 (PLA_2), an inflammatory enzyme that generates and regulates PAF, is increased in acute dengue(109). Primary or heterologous secondary exposure to DENV-2, was shown to release significant amounts of PAF, thromboxane B_2, and PGD_2(110). All these indicate that the arachidonic acid pathway could play role in dengue viral infection.

In anaphylaxis, activated mast cells release vasoactive mediators, such as histamine and PAF that are known to contribute to the initial anaphylactic response by increasing vascular hyperpermeability leading to capillary vasodilation and endothelial barrier disruption. Subsequently, vascular hyperpermeability and plasma leakage into local tissues could lead to systemic reactions, including increased hematocrit levels and hypotension(111). The clinical manifestations of dengue vascular leakage are similar to the clinical manifestations of anaphylaxis, although the degree of fluid leakage and the sites of detection of fluids are significantly different. Mast cells are found to be activated in dengue and it was observed that mast cell infection and chemokine release are increased through antibody dependent enhancement(112). It has been previously reported that human mast cells are permissive to antibody-enhanced dengue virus infection, in an FcγRII-dependent manner(113). Infection of mast cell lines KU812 and HMC-1 results in infectious virus production and the induction of significant levels of cytokines, such as IL-1β and IL-6, as well as chemokines CCL3, CCL4, and CCL5, which can recruit additional immune effector cells(112, 114). DENV-2 infection significantly

induced COX-2 expression and prostaglandin E_2 (PGE_2) production in human hepatoma Huh-7 cells. The exogenous expression of COX-2 or PGE_2 treatment dose-dependently enhanced DENV-2 replication(115). In contrast, COX-2 gene silencing and catalytic inhibition sufficiently suppressed DENV-2 replication. This effect could be due to the suppression of downstream signaling mediators in the arachidonic acid pathway. Also, it was shown that NF-κB and MAPK/JNK are critical factors for DENV-2-induced COX-2 expression and viral replication(115).

The vaccinia virus envelope contains sphingomyelin, which is converted to sphingosin-1-phosphate (S1P) and signals through the S1P receptor 2 (SIPR2) G-coupled receptor to cause mast cell degranulation(116). However, how mast cells degranulate in response to other viral infections remains largely unknown. Degranulation occurs in response to A/WSN/1933 infection in RIG-I-deficient mice demonstrating that degranulation could be a RIG-I-independent response. As degranulation occurs within 30 min following inoculation with influenza A virus (IAV), other PRR and/or rapid early signaling events necessary for the virus attachment and/or entry processes are likely important in regulating mast cell degranulation. With dengue virus, degranulation of mast cells occurs prior to RIG-I signaling. Moreover, UV-inactivated dengue virus and IAV retain the ability to activate mast cells, suggesting this occurs early in the viral replication cycle(117, 118). While FcγIII-deficient mast cells are able to degranulate in response to dengue virus, mast cells pre-treated with anti-dengue IgG demonstrate enhanced degranulation in response to all four serotypes of dengue virus compared to dengue virus alone, suggesting that antibody binding enhances degranulation in response to dengue virus(119, 120). Additionally, dengue virus infected epithelial cells secrete IL-33, which is detected by mast cells, resulting in the secretion of IL-6 and TNF-α without degranulation(121). Although mast cell degranulation appears to be pivotal for the

immunopathology associated with dengue virus infections, how degranulation is initiated is still not properly understood.

Mast cells have two distinct phases of activation: immediate degranulation, resulting in the release of pre-synthesized mediators, and delayed secretion of secondary *de novo* synthesized mediators. The delayed secretion of secondary *de novo* effector molecules produced by mast cells can be further segregated into two classes: (1) prostaglandins and eicosanoids released within minutes of activation, and (2) cytokines, chemokines, and growth factors that are released within hours of stimulation. Together, these mast cell outputs can increase epithelial and endothelial cell permeability and activation state, and synergise with chemotactic molecules, resulting in increased inflammatory cell recruitment to infected tissues(118). Additionally, MC products such as chymase (122) and RANTES, MIP-1α, and MIP-1β are found to be higher in dengue infection(112). Furthermore, a recent study detected that urinary histamine and leukotrienes were elevated in severe dengue(123).

1.1.11 Prostaglandin D_2

Prostaglandin D2 (PGD_2) and leukotrienes are two of the major lipid mediators secreted by activated mast cells. Prostaglandin D_2 (PGD_2) is produced by the activity of COX and hematopoietic PGD synthase (H-PGDS). Prostaglandin is a class of lipid mediator synthesised by cells in response to local tissue damage and a previous study demonstrated that mast cells strongly express H-PGDS and produce PGD_2 on a large scale(111, 124). Upon allergen-induced cross-linking of FcεR-bound IgE, mast cells produce high local concentrations of PGD_2 in the nanomolar range. In addition to mast cells, PGD_2 is produced by macrophages, Th2 lymphocytes(125) and Tc2 lymphocytes(126). The effects of PGD_2 are mediated in concert by PGD_2 receptor 1 (DP1) and CRTH2 / DP2 (chemoattractant receptor–homologous molecule) receptor. CRTH2/DP2 is the dominant

receptor mediating the effect of PGD_2 in type 2 cells, as DP1 agonists or antagonists do not exhibit any obvious effect in these cells *in vitro* (127). DP2 is present exclusively on immune cells, including eosinophils, basophils, Th2 and Tc2 cells, and group 2 innate lymphoid cells (ILC2)(128). In contrast, DP1 is broadly expressed in vasculature, central nervous system, retina, and the lungs. Activation of the DP1 receptor, coupled to a $G_{\alpha s}$ protein, induces the production of the second messenger cAMP, which stimulates protein kinase A (PKA) and also induces an influx of Ca2+. The activation of CRTH2 receptors coupled to a $G_{\alpha i}$ protein inhibits cAMP production(128). CRTH2 is a seven-transmembrane G protein coupled receptor structurally related to members of the N-formyl peptide receptor (FPR) subfamily. Furthermore, CRTH2 can mediate intracellular Ca2+ mobilization in response to a factor(s) released from activated mast cells, suggesting that CRTH2 may be closely involved in mast cell mediated allergic inflammation(129, 130).

Both DP1 and CRTH2 receptors have been postulated to contribute to the development of allergic inflammation. The exact role played by each receptor in the process of inflammation remains unclear but the general view suggests that signals through CRTH2 receptor are pro-inflammatory, whereas signals through DP1 receptor may either be pro-inflammatory or anti-inflammatory, depending on the setting and the target cells. However, with respect to activation of Th2 lymphocytes, eosinophils and ILC2, the effects of PGD_2 are mediated by CRTH2(129, 131). It is important to note that PGD_2 rapidly down-regulates cell surface CRTH2 expression on ILC2, T cells and eosinophils. It is proposed that indomethacin, a non-steroidal anti-inflammatory medication, acts as an agonist of the CRTH2 receptor giving rise to unwanted side effects or therapeutic effects of the medication(132). Other nonsteroidal anti-inflammatory medication such as aspirin, sulindac, diclofenac and acemetacin did not demonstrate such effect even at

micromolar concentrations(132). As a result, the predominance of DP signals in these cells may determine inflammatory effects in tissues (133).

PGD_2, like other prostanoids is rapidly metabolised *in vivo* and has an estimated half-life of 30 minutes in the blood. As such, PGD_2 actions are maintained through metabolites, 13,14-dihydro-15-keto-(DK-)PGD_2, PGJ_2, Δ12-PGJ_2 and 15-deoxy-Δ12,14-PGJ_2, binding to CRTH2(128). In addition, dk-PGD_2 was reported to be selective for CRTH2 over DP1 receptor. Furthermore, Δ12-PGJ_2, a plasma metabolite of PGD_2 that can be detected in human urine, can induce mobilization of eosinophils from the bone marrow and prime eosinophils for chemotaxis(134, 135). These data suggest, despite extensive non-enzymatic metabolism of PGD_2 in plasma, its biological activity is maintained through the formation of bioactive metabolites. Moreover, one of the major metabolites of the thromboxane pathway, 11-dehydro-TXB_2, and even the common precursor of all prostanoids, PGH_2, are also potent CRTH2 agonists. Similarly, $PGF_{2\alpha}$ has been found to activate eosinophils through DP2(136).

PGD_2 elicits many pro-inflammatory responses in leukocytes including chemotaxis of eosinophils, basophils, ILC2 and Th2 cells(128), and cytokine production by Th2 and ILC2 cells, and pro-inflammatory protein expression by eosinophils and Th2 cells(131). Additionally, these responses can be amplified through cysteinyl leukotrienes(137). Furthermore, recent studies demonstrated that activation of CRTH2 suppresses Th2 cell apoptosis, a process which is likely to impede the resolution of allergic inflammation(127, 138). Additionally, allergic responses mediated by IgE, mast cells, Th2 cells and eosinophils are dramatically reduced in mice where CRTH2 is genetically ablated or inhibited through small molecule CRTH2 antagonists. Therefore, antagonism of CRTH2 is currently being considered as a potentially useful approach for the treatment of allergic diseases, including asthma, rhinitis and atopic dermatitis, although data

suggest that there are challenges (139, 140). Prior incubation of ILC2 with Fevipiprant (0.01 nmol/L to 1 mmol/L), a CRTH2 inhibitor, significantly reduced IL-4, IL-5, and IL-13 production in a dose-dependent manner at the protein level(131).

Notably, the mode of action of PGD_2 on the regulation of IL-10 production seems distinct from that of IL-12 and IL-1β production. PGD_2 through its interaction with DP inhibits the migration of DCs into regional LNs. In addition, the DP1 signals were able to suppress IL-12 generation in DCs. Treatment of DCs with a DP1 agonist led to suppression of stimulating IFN-γ production from immune LN cells. DP1 signals also inhibited IL-1β production in DCs. This is because PGD_2 regulates IL-10 production through both CRTH2 and DP1, but only the PGD_2 mediated DP1 pathway affects the production of IL-12 and IL-1β. Epidermal DCs, in particular Langerhans cells that produce PGD_2, may act on neighboring DCs and/or dermal DCs, which could interfere with their migration to lymph nodes via DP(133). This could be one of the natural regulatory mechanisms to protect individuals from excessive sensitization to environmental allergens.

PGE_2 is another metabolite in the prostaglandin pathway that predominantly bind to EP4 and EP2 receptors. EP4 receptor has shown rapid desensitization and internalization after PGE_2 stimulation, which is not observed with EP2 receptors. Human plasmacytoid dendritic cells are shown to produce less interferon-α following EP2/EP4 stimulation by PGE_2 and shown to lower their Th1 co-stimulatory capacity(141). Dendritic cells that mature in the presence of PGE_2 preferably drive Th2 polarization, and produce Th2 attracting chemokines. In contrast, PGE_2 suppresses antigen-specific responses, such as proliferation and cytokine secretion of human Th1 and Th2 cells through EP2/EP4 signals(142), suggesting that PGE_2 might limit allergic responses upon allergen re-exposure. In the context of ILC2, PGE_2 has shown to inhibit ILC2 activity and reduces type 2 cytokine production(143).

1.1.12 Proposed mechanisms of vascular leakage

Patients with dengue infection may progress through three clinical phases known as the febrile phase, the critical phase and the recovery phase. However, out of the patients who develop DF, a minority of individuals proceed to the critical phase, which lasts for 24–48 hours and is associated with plasma leakage(36). The majority of DF patients directly proceed to the recovery phase without developing plasma leakage. Severe dengue is associated with a transient increase in vascular permeability due to endothelial dysfunction during the critical phase(40).

Endothelial hyper-permeability is a prominent explanation for vascular leakage in dengue. This phenomenon is observed in other diseases such as anaphylaxis and sepsis(144). It has been found that histamine or PAF induced production of nitric oxide by endothelium is a critical factor in development of anaphylaxis(144). Mast cell-derived histamine and PAF mediate disruption of adherence junctions at the endothelial barrier, whilst PGD_2 enhances formation of endothelial barriers(111). Interestingly, these mediators are found to be high in dengue(145, 146), which prompted consideration that type-2 immunity mediated mechanisms could contribute to the outcome of dengue fever.

It has been thought that cytokines or mediators could be a cause of vascular leakage in dengue, especially due to the transient nature of vascular leakage. Although, type 2 cytokines are shown to be associated with DHF, a direct relationship between type 2 cytokines and vascular leakage has not been found(73). While immunosuppressive cytokines such as interleukin-10 (IL-10) decrease during the critical phase, cytokines that has been associated with vascular leak such as tumour necrosis factor-α (TNF-α), tend to increase. Although TNF-α and many other cytokines are also increased in severe forms of influenza virus infection, the levels of some of these cytokines are lower than those detected in patients with DHF(147). In a murine model (BALB/c mice) of dengue,

treatment with anti-TNF-α serum, obtained by immunizing guinea pigs with recombinant mouse TNF-α, significantly increased the survival of animals compared to dengue infected mice that received control serum. However, viraemia was not different between the groups(148). Also, in a type I and II IFN receptor KO (IFN-α/β/γR KO mice) murine model, treatment with a neutralizing anti-TNF-α antibody increased survival and reduced liver damage without affecting virus production. Additionally, anti-IL-6 neutralizing antibodies partially prolonged their survival. The anti-TNF-α antibody suppressed IL-6, MCP-1 and IFN-γ levels, suggesting that the severe response to infection was triggered by TNF-α. Importantly, treatment with Angiopoietin-1, which is a mediator known to stabilize blood vessels, prolonged the survival of dengue infected mice. Taken together, the results suggest that an increased level of TNF-α together with concomitant upregulation of Tie2/Angiopoietin signaling have critical roles in severe dengue infection(149). Therefore, anti-TNF agents have been suggested as a potential treatment modality for dengue infection. But, the approach has not been tested in humans potentially related to the wider infection risk, and also due to the affordability for use in tropical countries, for a prevalent viral infection.

Although DENV has been shown to be able to infect endothelial cells *in vitro* (150), studies failed to identify widespread endothelial cell infection *in vivo* after fatal dengue. These findings indicate that vascular pathologies could be primarily immune mediated. Many cytokine mediators, mast cell products, inflammatory lipid mediators are implicated for vascular leak in dengue(40). Mast cell mediators, tryptases and chymases are found to be associated with severe dengue(151, 152). Mechanistically, in *in vitro* and *in vivo* models tryptase induced increased vascular permeability and leakage by reducing the expression of the cellular adhesion molecule CD31 on endothelial cells and inducing

breakdown of endothelial cell tight junctions. Furthermore, potent tryptase inhibitor, nafamostat mesylate, has shown to block DENV-induced vascular leakage *in vivo*(153).

Additionally, evidence suggests that leukotrienes increase vascular leakage. Leukotrienes contract endothelial cells in the post-capillary venules in a dose-dependent manner, and upregulate β2-integrin expression in eosinophils and stimulate vascular endothelial cells to produce PAF(154). In dengue models, leukotriene blockade is associated with resolution of vascular leakage(40, 122). St John *et al*, previously demonstrated that mast cells (MC) degranulated in dengue infection and tested a range of mast cell-based interventions for treatment of increased endothelial permeability in dengue fever. The authors used MC-stabilizing compounds that are clinically available such as cromolyn, ketotifen, anti-TNF blocking antibody and montelukast, which is a leukotriene receptor antagonist. At an early time point of administration of 1 hour after systemic infection, both MC-stabilizing compounds (cromolyn and ketotifen) and montelukast significantly reduced vascular leakage compared to untreated DENV. Anti-TNF, at an early time point did not show a significant difference in vascular leakage(122). Interestingly, blocking TNF has previously shown to be effective in limiting vascular leakage at a late time points of infection in immunocompromised mice lacking type I and II Interferon receptors (IFN-α,β,γ-R−/− or AG129)(155). Furthermore, supernatants of DENV-infected MCs from WT and TNF-KO, induced a reduction in the trans-epithelial/transendothelial resistance (TEER) relative to baseline in mouse endothelial cells (EOMA cells). In experimental models reduction of TEER mimics increased endothelial permeability. Furthermore, supernatants from DENV-infected MCs from a 5-lipoxygenase (5-LO-KO) knock out model, which is the enzyme required for leukotriene synthesis, a significant reduction of TEER was not observed(122). Taken together, these studies provide evidence that

signifies the importance of mast cell mediators in the pathogenesis of dengue associated disease.

1.2 Innate Lymphoid cells

1.2.1 Innate lymphoid cell subsets

Innate lymphoid cells (ILC) represent a group of effector lymphoid cells that lack a rearranged antigen-specific receptor (T cell or B cell receptor) and develop from the common lymphoid progenitor in fetal liver and bone marrow(156). They are largely tissue-resident cells and react promptly to stress signals. ILC can be identified in lymphoid and non-lymphoid tissues, and are enriched at epithelial barrier surfaces such as the intestine, lung, and skin(157), with ILC2 being the most preponderant(158). However, ILC2 are present at low frequencies in peripheral blood representing up to 0.1% of circulating lymphocytes(157). Their ability to quickly respond to tissue stress and inflammation underpins their critical role in regulating tissue homeostasis.

The discovery of ILC has been recent, but the pathway of discovery has initiated from mid-1980s. In 1986, a landmark publication by Tim Mossman and Robert Coffman described two subsets of CD4+ T cells, known as T helper 1 (Th1) cells and Th2 cells, that differed in their cytokine production profiles. Th1 cells produced interferon-γ (IFNγ), whereas Th2 cells produced IL-4 and IL-5(159). In 2005, a third subset of CD4+ T cells that produce IL-17 was described (Th17 cells)(160). This subdivision based on cytokine production profiles turned out not to be unique to CD4+ T cells expressing the αβ T cell receptor (TCR), as it was also observed for natural killer T (NKT) cells and T cells expressing the γδ TCR. Furthermore, in 2006, a novel population of IL-4–, IL-5–, IL-13–producing non–B/non–T that were important in helminth expulsion was identified(161). In 2009 and 2010, several papers were published characterising populations of non-T cell, non-B cell lymphoid cells defined by their cytokine responsiveness and production.

The similarities between these 'innate' lymphocytes and the Th cell subsets were described in 2013 and a unifying nomenclature was proposed to this family/type of cells, innate lymphoid cells (ILC)(157, 162). Thereafter, the ILC group of cells were divided in to five subtypes including 3 groups of ILC, NK cells and lymphoid tissue inducer cells(157).

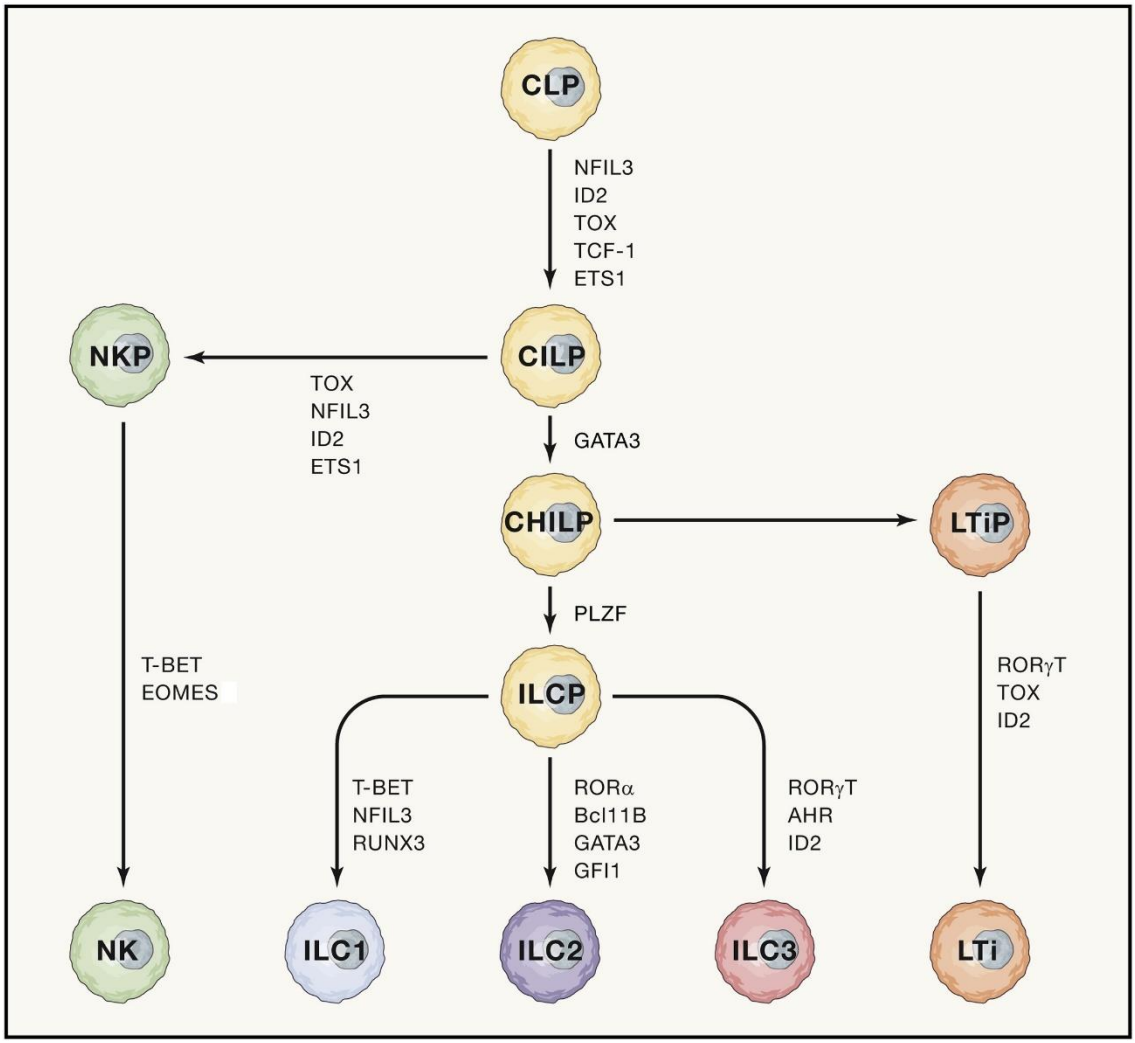


Figure 2: Development of innate lymphoid cells. *Obtained from* (157)

ILC have been classified into groups based on functionality, cytokine production and transcription factor dependence, in which helper ILC resemble the T helper cell subsets, namely ILC1, ILC2 and ILC3 (Figure 2). ILC1 comprise IFN-γ secreting ILC that likely use transcription factor T-bet for lineage commitment, but not eomesodermin (EOMES).

However, the phenotype and functions of ILC1 depend on the tissue microenvironment, and ILC with a mixed NK cell and ILC1 phenotype has been described(163). ILC2 comprise type 2 cytokine producing ILC that require transcription factor GATA3 for their development and function. Group 2 ILC (ILC2), produce type-2 cytokines, mainly IL-4, IL-5, IL-9, and IL-13 but also can produce IL-6, IL-10, GM-CSF, IL-8 and amphiregulin(164, 165). ILC3 are dependent on transcription factor RORγt (*RORC*) for lineage specification. ILC3 comprise subsets that can be distinguished on the basis of expression of the natural cytotoxicity receptors NKp46 (NCR1) and NKp44 (NCR2)(166). The main cytokine produced by ILC3 is IL-22 and a proportion of ILC3 also produce IL-17A(156).

1.2.2 ILC2 development

ILC are an emerging family of immune cells that originate from fetal liver and adult bone marrow (BM) progenitors (167). Prenatally, ILC progenitors occupy peripheral sites from mid to late stages of fetal development and are detectable in peripheral tissues by embryonic day 15.5 in the mouse (168). The effector programming of ILC initiates in the fetal liver in the embryo and later moves to the bone marrow. In the bone marrow, ILC develop from the common lymphoid progenitor (CLP). B cells and early thymic precursors also derived from the CLP. A subset of CLP differentiates into common helper–like ILC precursors or common helper innate lymphoid progenitors (ChlLPs). This precursor is shared among ILC, NK cells and lymphoid tissue inducer–like cells(169). In the next stage of development, upregulation of the transcription factor PLZF occurs and forms an ILC progenitor (ILCP) giving rise to effector ILC. Thereafter, to differentiate in to three main subtypes of ILC they require the transcription factors; T-bet, GATA3 and RORγt, along with complex networks of other signaling molecules and

other transcription factors(167) (Figure 2). Further details of differentiation decisions of ILC counterparts are unclear and is still being elucidated.

1.2.3 ILC2 distribution and migration patterns

ILC are widely distributed throughout barrier and non-barrier tissues including the skin, intestines, lungs, uterus, liver, spleen, and secondary lymphoid tissues; and tissue localization is strongly associated with the phenotype/subset type. Recent studies have revealed that some ILC, specifically ILC1 and ILC3, express lymphoid tissue-homing receptors to migrate into SLT, and can switch expression of homing receptors to migrate to non-lymphoid tissues in a manner similar to T cells(170, 171). ILC2, on the other hand, appear to migrate directly from the hematopoietic site to target tissues, in a manner similar to myeloid cells and some innate T cells(171). It has been shown that ILC2 exhibit amoeboid-like movement and accumulate in the extravascular tissue surrounding large blood vessels rather than alveolar capillaries during IL-33-induced lung inflammation. Also, data suggest that the rapid expansion of ILC2 numbers in tissues of the mice challenged with IL-33 is dependent on recruitment of ILC2 precursors from the bone marrow rather than in situ proliferation of a small population of resident precursors at steady status(172).

1.2.4 Activation and proliferation of ILC2

Group 2 innate lymphoid cells (ILC2) play important roles in the initiation and amplification of type 2 immune responses(173). Under inflammatory conditions, ILC2 are stimulated by the alarmin cytokines IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), which are products of activated epithelial cells and macrophages. Alarmins activate ILC2 potently to produce type 2 cytokines. IL-33 directly acts on ILC2 through ST2 receptor(164). The alarmin IL-33 is known to augment the generation of ILC2. In addition to direct signaling via ILC2 cell surface T1/ST2 receptor, IL-33 induces the rapid

release of TGF-β into the airways to prime ILC2 and enhance their migration. Epithelial-derived TGF-β appears to be essential for the accumulation of ILC2 in the lung and acts downstream of IL-33 to initiate type 2 poised immune responses(174). ILC2 react rapidly and potently to multiple cell-derived factors such as cytokines and eicosanoids, which are produced by other cells in response to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)(175). Type-2 inducer cytokines and lipid mediators such as IL-33, IL-25, TSLP, PGD_2, LTE_4 activate ILC2 to produce type-2 cytokines. Lipid mediators such as PGD_2 and cysteinyl leukotrienes, promote rapid ILC2 activation(176, 177) to induce IL-5 and IL-13 production, as well as migration of ILC2 (Figure 3) (127). PGD_2 activates ILC2 through CRTH2, which is a G protein–coupled receptor for PGD_2 and is a defining cell surface marker of human ILC2, in addition to be expressed on eosinophils, basophils, Th2 and Tc2 cells. Emerging evidence suggests that the activation of CRTH2 leads to pro-inflammatory responses in leukocytes(178). Interestingly, ILC2 have been shown to secrete and respond to PGD_2 in a paracrine/autocrine manner leading to cytokine-induced ILC2 activation(131, 164, 179). Therefore, there is a strong likelihood that ILC2 can contribute to a type 2 cytokine environment in skin.

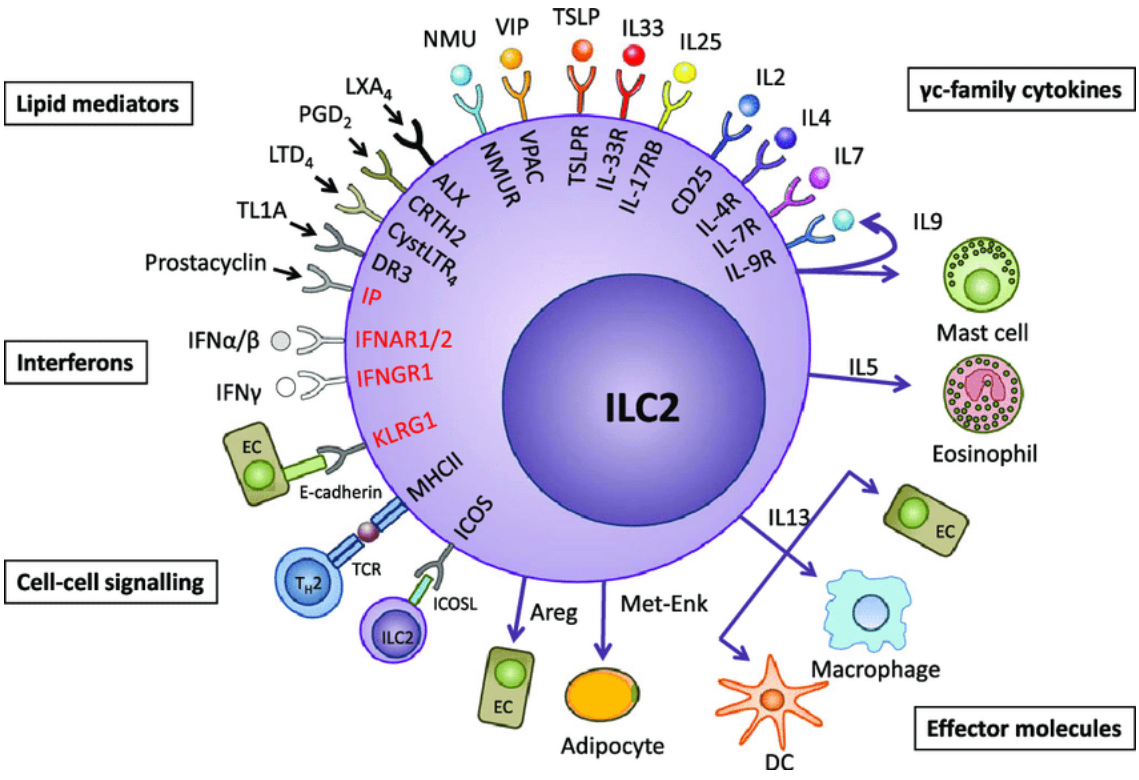


Figure 3: Control of ILC activity, activator and inhibitory signals. *Obtained from* (180)

1.2.5 Inhibition of activity of ILC2

Inhibitory mediators of ILC2 function have been described recently. LipoxinA4 and prostaglandin I_2 was shown to markedly inhibit ILC2 cytokine production(181, 182), thereby reducing the allergic inflammation. Additionally, it was demonstrated that Treg cells directly suppress ILC2 function *in vitro* and *in vivo* either by cytokines through TGF-β and IL-10 or through inducible co-stimulator (ICOS) ligand interactions(183). Work by other investigators has suggested that several cytokines, including Th1 associated cytokines can also suppress ILC2 responses. Type I interferons, including IFN-β, as well as IL-12, IFN-γ, and IL-27 have recently been shown to inhibit mouse and human ILC2 responses (Figure 3). Additionally, IFN-β is shown to increase ILC2 apoptosis and inhibit cytokine production in ILC2(184, 185). Importantly, IL-12 reduces ILC2 GATA-3 expression and transforms ILC2 into IFN-γ producing ILC with increased

T-bet expression(186). This likely demonstrates the capacity of plasticity in ILC2. Interestingly, the pro-inflammatory cytokine IL-1β appears to both activate ILC2 cytokine production and prime ILC2 to produce IFN-γ in response to IL-12(187). Also, another lipid mediator important for the inhibition of ILC2 is PGE_2. PGE_2 has been shown to inhibit GATA-3 expression, as well as the production of the type 2 cytokines, IL-5 and IL-13, from human tonsillar and blood ILC2 and downregulated the expression of IL-2 receptor α (CD25). In line with this observation, PGE_2 decreased ILC2 proliferation. These effects were mediated by the combined action of E-type prostanoid receptor (EP) 2 and EP4 receptors, which are expressed on ILC2(143). Inhibition of the COX-1/2 enzymes with flurbiprofen almost completely prevented IL-5 and IL-13 secretion from cytokine-stimulated ILC2(188). Additionally, a study showed that dexamethasone-treated mice that were administered *Alternaria* species had increased ILC2 apoptosis and markedly reduced lung ILC2 numbers, cytokine production, and eosinophilic lung inflammation indicating a suppressive function of dexamethasone on ILC2(189).

1.2.6 Role of group 2 innate lymphoid cells in health and disease

Currently, the function of ILC2 has been primarily investigated in the context of allergic diseases. It has been demonstrated that ILC2 are involved in allergic airway inflammation in asthma. ILC2 in peripheral blood and bronchoalveolar lavage fluid (BALF) were increased in asthmatic patients compared to control subjects and negatively correlated with airway function(190). In addition, many inhalant antigens could trigger epithelial IL-33 and IL-25 production inducing eosinophilic asthma-like airway inflammation through activation of lung ILC2. House dust mite allergen (HDM) induces IL-33 release from epithelial cells provoking an asthma-like airway inflammation through ILC2 activation(191). Also, ILC2 are increased in peripheral blood in patients with allergic rhinitis after allergen challenge and during grass pollen season(192). Studies in murine

allergic asthma models support a Th2-promoting role of ILC2(193, 194). This signifies that there is a link between allergic inflammation and ILC2.

It has been shown that ILC2 play a major role in helminth expulsion. Type 2 immunity, from innate and adaptive immune responses, is essential for anti-helminth responses. ILC2 are a critical innate source of IL-13 that is important for efficient helminth expulsion in the absence of adaptive immunity(195). Both ILC2 and Th2 cells work together to eliminate helminth larvae in the lungs upon re-infection and prevent their maturation and trafficking to the stomach and intestines(196). Additionally, IL-9 derived from ILC2 plays a vital role in epithelial repair and facilitates helminth expulsion, acting via autocrine and paracrine mechanisms(197).

ILC2 are predominantly tissue resident innate cells that are enriched in the skin and are among the initial immune cells that could respond to inflammation. ILC2 are found in higher frequency in individuals with atopy(164). ILC2 are resident in healthy human skin and it has been shown that ILC2 are increased in abundance and are activated within AD lesional skin (164, 198, 199). ILC2 in AD lesions showed increased expression of IL-25 receptor (R), IL-33R and TSLPR levels and ILC2 potently produce IL-4, IL-5 and IL-13, which are the key effectors of AD immunopathology (198, 200). In addition, IL-33 induced activation of ILC2 promotes wound healing through improved epithelial repair(201).

1.2.7 ILC and viral infections

Innate immune cells, such as macrophages and dendritic cells (DCs), which reside in peripheral tissues, recognize pathogens through the expression of toll-like receptors (TLRs) and produce a wide-variety of effector molecules and play a vital role in early pathogen recognition at the site of infection(202). Additionally, it has been found that

tissue resident lymphocytes are relatively abundant in peripheral organs. Tissue-resident lymphocytes, such as ILC and “unconventional” T cells have been shown to display critical roles during pathogen challenge(157).

Data from a murine model suggested that pulmonary ILC2 are more potent than CD4+ T cells in their induction of type 2 cytokines. It is estimated that ILC2 produce 10 times more cytokine than T cells on a per cell basis, especially with IL-25 and IL-33 stimulation(203, 204). Respiratory syncytial virus (RSV) infection induced a 3-fold increase in the number of IL-13-producing ILC2 in a TSLP and IL-33 dependent mechanism(204). Additionally, RSV-infected TSLPR KO mice had significantly decreased levels of whole-lung IL-13 compared with RSV-infected WT mice, suggesting that TSLP drives ILC2 activation(204). In RSV infection, ILC2 act as a significant driver of the type 2 response stimulated by epithelial-derived IL-33, leading to mucus production and airway dysfunction. Subsequently, IL-13 and IL-4 produced by neonatal mice could polarize dendritic cells towards a Th2 biased response(205). ILC2 have been found to be activated in viral infections such as RSV and Influenza virus. In Influenza infection, ILC2 accumulate in the lungs of mice and plays a role in epithelial regeneration and tissue remodeling through production of amphiregulin(206). In acute exacerbation of chronic obstructive pulmonary diseases, ILC2 and Th2 frequency has found to be increased and ILC2 are thought to function in antigen presentation with higher expression of MHC-II(207).

Moreover, ILC1 are known to have a role in early viral control through production of IFN-γ. For example, rapid production of IL-12 by tissue-resident conventional dendritic cells promoted ILC1 production of IFN-γ in a STAT4-dependent manner to limit early viral burden(208). ILC1 reside abundantly in basal layers of the mucosa compared to few NK cells, and are suggested to distally control viral infection through IFN-γ production

rather than via direct cell-cell contact(209). Additionally, ablation of *Zfp683*-dependent liver ILC1 has shown to increase viral loads of mouse cytomegalovirus, while having intact intact innate and adpative immunity(208).

1.2.8 Role of innate cells in dengue Fever

DENV is transmitted to people via a mosquito bite. These is increasing understanding of the cell types involved in initial surveillance for DENV. DENV is a lymphotropic virus, known to progress from the initial stage of cutaneous infection to infection of the draining lymph nodes and then leading to viraemia, which is vital to propagate the virus to another host through mosquito bite. At the site of the mosquito bite in the skin, various immune sentinels such as dendritic cells (DCs), Langerhans cells (LCs), macrophages and mast cells (MCs) are present. These sentinels are distributed throughout the skin at densities of ~70–100 MCs, ~60 CD11c+ DCs and ~80 $CD163^{+}FXIIIA^{+}$ macrophages per mm^2 of healthy human dermis(210, 211). As opposed to the rare occurrence of ILC in the normal healthy skin, it was found that atopic dermatitis (AD) lesions are prominently populated by AHR^{+}ILC3 and $GATA3^{+}$ILC2. The adaptive Th-cell counterparts consisted mostly of Th2, and to a lesser extent Th22 cells. Particularly striking regarding the potential role of ILC in AD was the observation that T cells were in close proximity to ILC2, the majority belonged to the Th2 subset(212). This may indicate that ILC2 communicate with and may modulate Th2 responses in AD.

Following infection, the virus initially replicates in skin cells, such as keratinocytes and Langerhans cells. This would trigger a variety of host innate immune responses. Innate immune cells are the first to respond using PRR recognising PAMPs (19). Innate cells such as DCs, endothelial cells, fibroblasts, keratinocytes, macrophage, mast cells, and monocytes are permissive for dengue infection(213). As discussed above, the PRRs mainly associated with DENV recognition are cytoplasmic RIG-I and melanoma

differentiation-associated protein 5 (MDA5), along with endosomal TLR3 and TLR7(19). Activation of these receptors by DENV recognition induces type I interferon responses and these perform an essential function of sensing viral replication in the cytoplasm.

Exposure of immature human myeloid DCs to DENV induces activation and maturation of both infected DCs and uninfected bystanders, leading to the production of TNF and IFN-α(214, 215). Monocytes are one of the main targets of dengue infection. CD14 and CD16 expression by DENV-infected blood monocytes was found to be higher compared to controls, and mediated B cell to plasmablast differentiation and production of IgM and IgG(216). Production of type I IFN inhibits DENV infection of monocytes(19). In contrast, monocyte-derived dermal macrophages bind and internalize DENV into early phagosomes without permitting virus replication and lead to sequestration of the virus internally. Such DENV-exposed dermal macrophages apparently do not produce IFN-α(217). Furthermore, it has been shown that primary keratinocytes are permissive to dengue and secrete IFN-α and IFN-γ(218). This signifies that DENV replication in these innate cells contributes to the establishment of antiviral innate immunity that might occur in the shortly after the bite of mosquito.

Also, as above, it has been shown that DENV induces activation and strong mast cell (MC) degranulation *in vitro*, in the skin following dengue infection(219). Additionally, MCs can contribute significantly to the early recruitment, activation, and proliferation of γδ T cells and enhance the clearance of virus *in vivo*(220). As above, PGD_2 and its metabolites are mast cell derived mediators that are released after activation of mast cells especially after provocation with allergens(221).

Further, among the other unconventional T cells, MAIT cells have been shown to be activated in dengue infection with increased co-expression of CD38, HLA-DR and IL-7 receptor. MAIT cell frequencies were increased in dengue(222). There was no difference in the production of IFN-γ in the unstimulated MAIT cells between acute and convalascent dengue samples, but there is a significantly lower IFN-γ production from MAIT cells in the acute phase after *in vitro* bacterial stimulation (222, 223). However, the role of unconventional lymphocytes such as innate lymphocytes in skin is still unclear.

1.3 Type-1 and type-2 cytokine biased responses in Dengue Fever

As discussed above, the balance of type-1 and type-2 immunity in dengue has been proposed as a contributing factor to severe disease(73, 74). It was observed that type 1 immune response is prominent in DF and type 2 immune response appeared to be prominent with the severe form of dengue, DHF. This may indicate that a Th1-type cytokine profile would be protective and Th2-type cytokine profile could be associated with the pathogenesis of DHF (73). A study conducted to assess DENV peptide-specific T cell response in a large cohort of DF and DHF patients found that IFN-γ production was significantly higher in patients with DF as opposed to DHF, especially during early infection. Those who had lower DENV-NS3 specific IFN-γ production by T cells since the onset of illness (before development of fluid leakage), were significantly more likely to subsequently develop DHF(224). This further signifies that lower IFN responses could be associated with a risk of developing DHF. Considering that the majority of patients with both DF and DHF have a very low magnitude of DENV peptide specific Th1 T cell responses(224), there could be a state where another cell type, such as ILC2, could contribute to induce a type 2 immune response at an early stage, for example during initial events in tissue. Initially, it was widely accepted that the exclusive source of type 2

signature cytokines was Th2 cells, a subpopulation of CD4+ helper T cells of the adaptive immune system. However, studies have now identified that ILC2 require the transcription factor GATA-3 for their development and produce large quantities of type 2 signature cytokines(162). Additionally, DENV specific T cells are found to be activated and have Tc1 effector functions. Peripheral blood DENV specific T cells expressed skin homing markers and found to be enriched in skin in patients with dengue infection demonstrating the immune surveillance role and effector functions in the skin(225).

1.4 Main project Aims

It is hypothesised that this meticulous balance of type 1 and type 2 immune responses at an early stage of infection could be critical in the development of severe disease in dengue. Furthermore, given their skin tissue residence at the point of early infection, it is hypothesised that ILC2 contribute to disease pathogenesis. The main project aims are:

- To investigate potential activation and infection of ILC2 during dengue virus infection
- To determine whether ILC2 modulate dengue virus replication and define underlying mechanisms

2 Chapter 2 - Materials and methods

2.1 Innate lymphoid cells

2.1.1 RNA sequencing analysis of ILC2 among healthy individuals (HC), dengue fever (DF) and dengue Hemorrhagic fever (DHF) patient groups

Blood samples were taken from four adult individuals of Sri Lankan origin on day 2-5 of acute dengue infection (DF and DHF). Control bloods samples from healthy individuals were obtained. Blood was taken during a period where dengue 2 serotype was predominant. PBMCs were separated by density gradient centrifugation (using Lymphoprep^{TM} density gradient medium) and frozen in -80°C. Ethical approval for this study was obtained from National Health Service (NHS), National Research Ethics Service (NRES) research ethics committee 14/SC/0106 and Ethics Review Committee, University of Sri Jayewardenepura (741/13). Samples from dengue patients were kindly provided by Professor Neelika Malavige, Dengue research unit, Sri Lanka, and I am grateful for her for sending samples and for providing support throughout. I am also extending gratitude for Dr Clare Hardman for conducting and facilitating RNA sequencing of these samples. Additionally, I am thankful to Welcome Trust Centre for Human Genetics (WTCHG) for carrying out sequencing of the samples.

Frozen peripheral blood mononuclear cells (PBMC) samples from these groups (HC, DF and DHF patients) were thawed and stained for markers to define ILC2 (Live, Lineage - , CD3 -, CD45+, CRTH2 +, IL-7Ra+). After staining, were washed with PBS (endotoxin, RNAse free) and were resuspended in PBS. Then samples were sorted at 100 cells/PCR tube directly into lysis buffer (0.2% Triton X-100 solution containing RNase inhibitor) using BD FACS Aria III flow cytometer in single cell mode. Cell sorting was performed in the flow cytometry facility of Weatherall institute of molecular medicine, University of Oxford. I would like to thank Craig Waugh of the flow cytometry facility for assisting

with sorting samples. Then, samples were snap frozen and was stored at -80˚C. Lysed samples were processed to form cDNA libraries using Nextera XT and SmartSeq2 protocols. RNA-Sequencing was performed in triplicate on an Illumina Hi-Seq 4000 generating 75bp reads. Following quality control with the fastQC package (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and trimming poor-quality reads and adapter sequences, reads were aligned to the human genome assembly (NCBI build38 (hg38) UCSC transcripts) using STAR (version 2.7.3a) with two pass default mapping mode (226). Duplicate sequences as an additional quality control step was performed using Picard tools (227, 228) and aligned reads were counted with Samtools(229). Reads originated from dengue virus were analysed with same procedure using combined genome of human and 4 serotypes of dengue virus (GenBank accession KM204119 for dengue Virus 1, KM204118 for dengue Virus 2, KU050695 for dengue Virus 3 and KR011349 for dengue Virus 4, respectively) as a reference for mapping. Raw read counts were normalised into reads per kilobase per million mapped reads (RPKM values) using the featureCounts function(230) from the Subread package(231) with default parameters(232) and RPKM values were used to compare gene expression levels of individual genes between disease groups. Sample quality metric and raw read counts were imported in R for further processing. The DESeq2 (233) Bioconductor package was used to estimated library size factors, normalize count and perform differential expression analysis. 10,000 most variable genes were used to perform principal component analysis where variance-stabilized transformation expression was used as input.

Differentially expressed genes across 3 groups HC, DF and DHF was analysed using likelihood ratio test (LRT), by comparing ‘full’ model with the disease status as a factor, to the ‘reduced’ model which was the intercept. Benjamini–Hochberg multiple testing correction was used to compute FDR, and genes were considered significantly

differentially expressed at <5% FDR. To carry out differential expression analysis comparing DF vs HC and DHF vs HC Wald test was used. Genes were considered significantly differentially expressed if absolute fold change value was more than 1.5 and FDR value was under 0.05.

GO enrichment analysis

Gene ontology (GO) terms associated with biological processes that are significantly over-represented for DEGs were identified using the cluster Profiler R package (234). GO gene sets were tested for overrepresentation in each set of DEGs by computation of enrichment *P* values with default parameter of 'enricher' function, which implements hypergeometric test. Hypergeometric P values were adjusted for multiple testing using Benjamini-Hochberg correction. The 30-40 GO terms with highest gene ratio, gene number within DEG list to gene number within background reference, were visualized as dotplots using clusterProfiler, enrichPlot and ggplot2 (235). I am thankful for the contributions made by Dr Nicki Gray for facilitating RNA sequencing data analysis along with Dr Clare Hardman and Dr Joengmin Woo, the bioinformatician in our group who helped perform the subsequent bioinformatic analyses.

2.1.2 Frequency of ILC2 among healthy individuals (HC), dengue fever (DF) and dengue haemorrhagic fever (DHF) patient groups

Patients with dengue infection of Sri Lankan ethnicity were enrolled within 2–5 d of symptom onset with positive NS1 antigen and/or anti-DENV IgM antibody. SEARO classification (WHO 2011) was used to classify disease severity. PBMC from Sri Lankan Healthy controls (n=15), DF (n=29) and DHF (n=16) patients stained ILC2 - Lineage-, CD3-, CD45+, IL7R+, CRTH2+ and c-Kit+.

2.1.3 ILC2 isolation by sorting and cell culture

PBMCs from healthy adult donors were isolated using density gradient centrifugation (Lymphoprep^{TM} density gradient medium). Subsequently, T cells and monocytes were depleted from the PBMCs using magnetic-activated cell sorting (CD3 & CD14 MicroBeads, Miltenyi Biotec) and the enriched sample was used for flow cytometric cell sorting. ILC2 were defined as: lineage (CD3, CD16, CD56, CD4, CD8, CD14, CD19, CD11c, CD11b, CD123, and FceRI)–, CD45+, IL7Ra+ and CRTH2+. The ILC2 were sorted into 96-well plates at 100 cells per well.

Next, the cells were resuspended in a mixed leukocyte reaction (MLR) of gamma irradiated PBMCs obtained from three healthy volunteers of diverse ethnic backgrounds with IL-2 (200IU/ml) and PHA (2.5ug/ml). MLR was prepared from PBMCs of the healthy donors which were irradiated with 3000rad and 100ul of the MLR was added to a 100ul of cell suspension containing sorted ILC2. Sorted cells were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat inactivated human serum and 2 mM L-glutamine, 10 ml/liter penicillin-streptomycin (Sigma-Aldrich), HEPES, Non-essential amino acids, sodium pyruvate and 2-Mercaptoethanol (ILC2 Media).

The sorted cells were rested for 5-7 days in MLR suspension and thereafter, half of the medium was replaced with equal volumes of ILC2 media containing 200 IU/ml IL-2. Over the next 4 to 6 weeks the cells expanded and after 4 to 6 weeks, the growing cells were tested by flow cytometry, to ensure a pure population of lineage−CRTH2+IL-7Ra+ ILC2 was obtained. Cells with purity more than 98% were used to conduct experiments.

2.1.4 Cell Isolation from human skin samples

Human skin tissue samples were obtained from healthy donors undergoing plastic/bariatric surgery (under NHS ethics REC 12.NW.1153). Subcutaneous fat was

removed, tissue cut to small pieces and digested in collagenase P overnight at 37°C 5% CO_2. After overnight digestion, the remaining tissue was homogenized with a Pasteur pipette and endonuclease deoxyribonuclease I (DNase I) 200 Kunitz unit/ml (Roche 10104159001) added for 15 minutes at room temperature. The digested tissue was passed through a 70µm and then 40µm cell strainer, washed with cold 10mM EDTA and resuspended in R10 medium. MNCs were isolated by density gradient centrifugation as described above.

2.1.5 Th2 and TC2 isolation by sorting and cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy adult donors by density gradient centrifugation (Lymphoprep^{TM} density gradient medium). Then T cells were separated from the PBMCs using magnetic-activated cell sorting (CD3 MicroBeads, Miltenyi Biotec) and the CD3+ enriched sample was used for flow cytometric cell sorting. Th2 cells were defined as CD4+ CRTH2+ and TC2 cells were defined as CD8+ CRTH2+. Th2 and TC2 were sorted into 96-well plates separately at 50 cells per well. Then, they were resuspended in a mixed leukocyte reaction (MLR) of irradiated PBMCs taken from three healthy volunteers of diverse ethnic backgrounds with IL-2 (200IU/ml) and PHA (2.5ug/ml). MLR was prepared as the same methods as done for ILC2s. Sorted cells were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 5% Human serum and 2 mM L-glutamine, 10 ml/liter penicillin-streptomycin (Sigma-Aldrich), HEPES Non-essential amino acid, sodium pyruvate and 2-Mercaptoethanol (T cell Media). Then the sorted cells were rested for 5-7days in MLR suspension and thereafter, half of the medium was replaced with equal volumes of ILC2 media containing 200 IU/ml IL-2. They were expanded in the same method as ILC2 over the next 4 to 6 weeks and the growing cells were checked by flow cytometry, to ensure a pure population of CD4+ CRTH2+ and CD8+ CRTH2+ were obtained. Cells with purity more than 98% were used

to conduct experiments. When the purity is less than the mentioned, CRTH2 micro beads (Miltenyi Biotec) separation was used to ensure a pure population. The enriched samples were then further checked to ensure a pure population to be used for experiments. In addition to that, pure populations could be re-fed using MLR and expanded over 4-6 weeks to be used for experiments.

2.1.6 Surface staining of ILC2

For FACS surface staining, the cells were labelled by the following anti–human antibodies: CD3 (SK7; BD), CD19 (SJ25C1; BD), CD123 (FAB301C; R&D Systems), CD11b (DCIS1/18), CD11c (BU15; Abcam), CD8 (RPA-T8), FcεRI (AER-37 ([CRA-1]), CD14 (MϕP9; BD), CD45 (H130), CD56 (B159), CRTH2 (BM16; Miltenyi Biotec), IL-7Rα (A019D5), and live/dead violet (Invitrogen). The samples were acquired using FACSDiva on LSRFortessa flow cytometer. FlowJo software was used for data analysis.

2.2 Dengue virus propagation, quantification and infection

Preparation of samples from dengue clinical samples and generation of dengue virus stocks were performed in the CL-3 laboratory at the MRC Weatherall institute of Molecular Medicine. The cell lines of C6/36 and Vero cell lines were propagated in CL-3 facility and all infection experiments in ILC2, monocyte and DCs were performed. Preparation of infected cells for flow cytometry was performed in CL-3 facility until they were fixed and permeablised. Prior to these experiments, successful inactivation of the virus was demonstrated and validated for fixation and permeablisation of cells (Fix/Perm buffer Invitrogen, 4% PFA, 2% Triton).

2.2.1 Dengue virus generation and propagation

The dengue virus was propagated using the C6/36 mosquito cell line, concentrated and stored in aliquots at −80 °C until used in infection experiments. C6/36 cells were cultured and propagated in T75 close capped flasks at a temperature of 28 °C. When cells grow to reach approximately 90% confluence, subcultures were prepared by vigorous pipetting of the cells with media and were passaged to new flasks. L15 (Leibovitz's L-15) media with 10% Fetal Bovine serum, 2 mM L-glutamine, 10 ml/liter penicillin-streptomycin (Sigma-Aldrich), 1% HEPES (10% L15 media) were used for C6/36 cell culture.

Dengue virus serotype 2 (Strain SL 5-17-04, #NR-49751) was used to generate viral stocks. The viral stocks were aliquoted and stored in -80°C until further use. C6/36 cells (kindly donated by Professor Gavin Screaton and Dr Juthathip Mongkolsapaya) were counted and plated in T-75 close capped flasks (5 x 10^6 cells in 12-15ml of 2% L-15 media per flask). For virus inoculation, C6/36 flasks with 15ml of media which has reached 80% confluence was used and inoculated with the virus with a Multiplicity of Infection (MOI) of 0.001 to 0.01 in 2% L15 cell culture media containing 2% Fetal Bovine serum (FBS), 2 mM L-glutamine, 10 ml/liter penicillin-streptomycin (Sigma-Aldrich), 1% HEPES. At 80% confluence, C6/36 should have approximately 1 x 10 7 C6/36 cells in T75 flask. Then, flasks were incubated at 28 °C. First add 6ml of media containing the virus and incubate for 4 hours. Afterwards, replenish the flasks up to 12-15ml total volume. The virus rich supernatants were collected at day four and the flasks were replenished with 12ml of fresh 2% L-15 media. Usually, the cytopathic effects (CPE) of the virus on C6/36 should be assessed using the light microcopy. Density of the cytopathic effects can be taken as a crude estimate of the viral titre in the supernatant. Collect virus supernatant once 80-90% CPE can be observed. Virus rich supernatants were spun to sediment the cells or debris at 1500g for 10minutes and then the supernatant

was concentrated using a 100kDa molecular weight (MW) cut off spin columns (Vivaspin 20, GE Healthcare) and centrifuged at 3200g for 30minutes and the concentrated virus stocks were aliquoted and stored in -80°C until used in experiments. For certain viral stocks several rounds of concentrations cycles were used to obtain high viral titres. At day six, again the supernatants were collected and concentrated.

2.2.2 Infection of cells with DENV2

ILC2 were infected using dengue serotype 2 virus (Strain SL 5-17-04, #NR-49751) which were generated as mentioned above. Stocks with virus titre of 10^5 – 10^6 FFU/ml were used. ILC2 were inoculated with virus at MOI of 5 for 2 hours in 96 well plates. 2% L15 media (concentrated from supernatants from mock T-75 flasks) was used as the negative/mock control. Dengue antibody blocking was performed by incubating virus supernatant and dengue envelope protein antibody (1µg/ml) for 30 minutes and then performing virus adsorption. Next, the cells were washed twice to remove excess unbound virus and incubated in ILC2 media containing IL-2 (200U/ml) for 48 hours. Infection rate was detected using intracellular envelope protein staining after permeabilizing and fixing cells.

For flow cytometry surface staining, the cells were labelled with the following anti–human antibodies: CRTH2 (BM16; Miltenyi Biotec), IL-7Rα (A019D5), and live/dead violet (Invitrogen) or PE-Texas-Red (Invitrogen).

2.2.3 Staining for dengue infection in ILC2/monocytes/DCs

After the surface staining of cells, intracellular staining of dengue virus infected cells was performed by permeabilising cells for 20 minutes using BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (554714). Then, cells were washed with permeabilization wash buffer (PWB) and incubated in intracellular dengue envelope antibody (4G2 NBP2-

52709) for 1 hour at 37ºC. Cells were washed with PWB twice and incubated in 3% FCS PBS buffer. The samples were acquired using FACSDiva on LSRFortessa flow cytometer. FlowJo software was used for data analysis.

2.2.4 Foci forming assay to determine viral titres of ILC2 supernatants

The concentration of the virus was determined by focus forming assays on Vero-81 cells (kindly donated by Professor Gavin Screaton and Dr J Mongkolsapaya) and expressed as FFU/ml. Vero cells were grown in T-75 vented cap flasks at 37 °C. When cells reach approximately 90% confluence, the cells were trypsinised, centrifuged, counted and propagated to new flasks. For the Foci forming assay (FFA) of supernatants, 50µl of supernatants from mock and infected ILC2 were added on Vero-81 cell monolayers in 96 flat bottom plate in duplicates and incubated at 37 °C with 5% CO2 for 2–3 days. For FFA of virus stocks multiple dilution of stocks were used. Then Carboxymethulcellulose (Sigma #C-5013) 100 ml of overlay was added to each well and incubated at 37 °C for 3 days. After 2–3 days the medium in the wells was discarded and the monolayer was washed five times with PBS. Then the cells were fixed with 4% paraformaldehyde, permeabilized with Triton-X100 and washed again with PBS 3 times. To detect foci, clone-4G2 monoclonal antibody which binds to the fusion loop at the extremity of domain II of envelope protein of flavivirus group (NBP2-52709, Novus Biologicals) was used as the primary antibody and HRP conjugated goat anti-mouse IgG HRP (KPL #074-1806) 1:500 in Ab diluent, as the secondary antibody. The plates were developed using the True-Blue Peroxidase Substrate (KPL #50-78-02). All assays were done in duplicate. The infected foci were counted using EliSpot plate reader. Virus stock titre (FFU/ml) was calculated by number of foci x 10 x 2 divided by the dilution of the virus.

2.3 Gene expression in ILC2

2.3.1 qRT-PCR

mRNA extraction was performed from 10^5 cells/well ILC2, following stimulation for 2 hours using a TurboCapture 96 mRNA kit (Qiagen, 72251) according to manufacturer's instructions. Complementary DNA (cDNA) was prepared from mRNA by reverse transcription PCR (M-MLV, Invitrogen). qPCR performed using Taqman probes and TaqMan gene expression master mix. TaqMan probes used are mentioned below.

GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Hs02786624_g1)

IFITM3 (Hs03057129_s1)

OAS1 (Hs00242943_m1)

ISG15 (Hs01921425_s1)

RSAD2 (Hs00369813_m1)

STAT1 (Hs01013996_m1)

IFNAR1 (Hs01066116_m1)

EGR2 (Hs00166165_m1)

TBET/TBX21 (Hs00894392_m1)

2.4 Western blot

Protein was extracted from 1million ILC2 in each condition using M-PER (Thermo Scientific - 78501) and protein samples were prepared in reducing buffer and boiled for 5 minutes, analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE), and transferred to a polyvinylidene difluoride membrane (Invitrogen). IFITM proteins were detected using mouse anti-human monoclonal IFITM3 antibody 1:1000 (kindly donated by Professor Tao Dong's lab). GAPDH (FF26A/F9 - 649202) was used as the loading control (1:1000).

2.5 Miscellaneous

2.5.1 Differentiation of Monocyte Derived Dendritic cells (DCs)

Monocytes were positively selected from the PBMCs using CD14 magnetic beads (Miltenyi Biotech) using MACS separation columns (Miltenyi Biotech). Then, they were differentiated in to MoDC by adding IL-4 (10ng/ml) and GM-CSF (100ng/ml). On day 3, the half of the media was removed and replenished with cytokines. On day 5 of differentiation the MoDCs were checked for purity by flow cytometry and used for experiments.

2.5.2 Effect of ILC2 supernatant on monocytes

Monocytes were positively selected from the PBMCs using CD14 magnetic beads (Miltenyi Biotech) using MACS separation columns (Miltenyi Biotech). Then, they were treated with supernatants of ILC2 which were activated for 48 hours treated with IL-33 (50mg/ml) and IL-25 (50ng/ml). For the blocking conditions IL-4Ralpha (Human IL-4 R alpha Polyclonal antibody – AB-230-NA - R&D Systems - 10µg/ml) and GM-CSF neutralising reagent (anti-human GM-CSF monoclonal antibody 23B6 - 3480-5N-500 – Mabtech – 5µg/ml) were incubated for 1 hour before adding ILC2 supernatants. After 48 hours monocytes with were stained for expression of MMR (15-2, AF488: 321114). After 48 hours of incubation they were adsorbed with dengue virus at a MOI of 2.5 and unbound virus was washed off and plated in GM-CSF (50ng/ml) and IL-4 (5ng/ml) for another 36-48 hours and intracellular dengue envelope protein was stained by using AF488/PE conjugated anti-E protein antibody (4G2 NBP2-52709). The samples were acquired using FACSDiva on LSRFortessa flow cytometer. FlowJo software was used for data analysis.

2.5.3 Staining for transcription factors of ILC2

Surface staining for markers were performed as mentioned above in ILC2. For intra-nuclear staining for transcription factors (TFs), eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (00-5523-00) was used and manufacturer's instructions were followed.

2.6 ELISA

2.6.1 ELISA of PGD_2 and PGE_2 metabolites

The 11β-prostaglandin $F_{2\alpha}$ (11β-PG $F_{2\alpha}$) and prostaglandin E (PG E) metabolite were measured in urine samples collected from acute dengue patients, which was stored in -80°C. These were measured using 11β-prostaglandin $F_{2\alpha}$ quantitative ELISA (Cayman Chemical, USA) and prostaglandin E metabolite quantitative ELISA (Cayman Chemical, USA). To perform both assays urine samples were diluted at 1:10 ratio using the respective ELISA dilution buffer. As these metabolites were measured in urine and the renal function affects the levels being excreted, the metabolite levels were normalised using urinary creatinine level of each sample. The urinary creatinine levels were measured using the urinary creatinine colorimetric assay (Cayman Chemical, USA) in urine diluted at 1:10 ratio. All assays were performed according to the manufacturer's instructions. I would extend gratitude towards Tehani Silva and Gathsaurie Neelika Malavige for collecting urine specimens from patients having dengue and for performing the above ELISA tests in Sri Lanka.

2.6.2 NS1 ELISA

NS1 ELISA (Novateinbio, NR-R10004) was performed on ILC2 supernatants from mock infected and infected ILC2. Unstimulated ILC2 and activated ILC2, activated for 24 hours with IL-33 (50ng/ml), PGD2 (200nM), LTE4 (100nM), were infected with DENV2 and incubated for 2 hours. Thereafter, the cells were twice washed with ILC2

media and cell were plated in 96 well plates for 48 hours in ILC2 media supplemented with IL-2. Supernatants were collected after 48 hours of incubation. DENV NS1 ELISA was performed according to manufacturer's instruction. Supernatants were diluted 1:20 for the experiment. Quality control was performed with positive and negative control samples. Immune status ratio (ISR) was calculated for each sample condition from the ratio of the mean optical density (OD) obtained from the test sample divided by the mean cut-off value. If ISR value >1 was considered as 'positive' for the presence of DENV NS1 antigen. ISR value <1 was considered as 'negative' for the presence of DENV NS1 antigen.

2.7 Reagents used for stimulation and blocking experiments

Reagent	Target	Source	Concentration
IL-33	ST2	Biolegend	50ng/ml
PGD_2	CRTH2	Enzo Life Science	200nM
LTE4	CysLT1	Cayman Chemicals -20410	100nM
IFN-β	IFNAR1, IFNAR2	R&D Systems - 8499-IF-010	25-250U/ml

Anti-IL-4Ralpha	IL-4Ralpha	R&D Systems - AB-230-NA	10µg/ml
Anti-GM-CSF	GM-CSF	Mabtech - 23B6 - 3480-5N-500	5µg/ml
Anti-IFNAR1	IFNAR1	MMHAR-2	10 µg/ml
Mannan	MMR	M7504	20 µg/ml
Heparin	Heparan sulphate	Stemcell - 07980	100 µg/ml

3 Chapter 3 – *Ex vivo* RNA sequencing of ILC2 and validation of data from in-vitro stimulation

3.1 Introduction

3.1.1 ILC2 in viral infections and their potential role in dengue infection

Tissue-resident lymphocytes, such as innate lymphoid cells (ILC) and "unconventional" T cells (e.g. iNKT, γδ T cells, mucosal associated invariant T cells) have been shown to have critical roles during pathogen challenge(157). It has been found that these lymphocyte families are relatively abundant in peripheral tissues. ILC are effector innate immune cells that rapidly sense environmental stimuli and participate in early immune responses by promptly secreting large amounts of cytokines(157).

As above, ILC2 are found to be activated in viral infections such as Respiratory Syncytial Virus (RSV) and influenza virus infections. In RSV infection, ILC2 act as a significant driver of the type 2 response stimulated by epithelial-derived IL-33 leading to mucus production and airway dysfunction and these effects are partly IL-13 driven. In influenza infection, ILC2 cells accumulate in the lungs of mice and play a role in epithelial regeneration and tissue remodeling role through production of amphiregulin(206). In addition, IL-33 induced activation of ILC2 promotes wound healing through improved epithelial repair(201). ILC2 are shown to mediate type 2 responses and to recruit eosinophils during viral lung infections upon the release of alarmins such as IL-33 by damaged epithelial cells. In a murine model, multiple pathogenic strains of RSV induced proliferation of IL-13 producing ILC2 and exerted activation through a TSLP-dependent mechanism(204). Additionally, a recent paper has shown that ILC2 are increased in COVID-19 patients in parallel with elevated serum type 2 cytokine levels. Also, the authors observed a significantly reduced proportion of patients requiring mechanical ventilation in the severe disease group who had high numbers of NKG2D+ ILC2,

indicating a possible protective role of this cell subset in the response against the virus(236).

MAIT cells are known to be activated in dengue fever(222). Additionally, invariant NKT cells (iNKT) are activated during acute dengue infection. Moreover, during acute dengue infection, monocytic CD1d expression was upregulated, which presents lipid antigens to iNKT cells. The level of iNKT cell activation associates with the disease severity with an altered functional response to *ex vivo* stimulation with α-GalCer (237). The role of ILC2 in viral infection is predominantly investigated in the context of respiratory viruses, and incompletely understood in other viral infections such as dengue fever.

From above, it is evident that ILC2 are abundant in tissues and have effector functions in viral infections and allergic inflammation. In severe dengue infection, having vascular hyperpermeability, mast cell activation and prominent type 2 cytokine responses prompted consideration that allergic inflammation a prominent role in the pathogenesis. Therefore, we investigated the role of ILC2 in dengue viral infection.

3.1.2 Frequency of ILC2 in dengue Fever

First, to determine the role of ILC2 in dengue infection, it had been investigated whether there are differences in the frequency of ILC2 in DF and DHF. I am grateful to Dr Maryam Salimi and Professor Gathsaurie N Malavige for carrying out this work. Patients were enrolled within 2–5 days of symptom onset based on clinical presentation compatible with dengue infection with positive NS1 antigen and/or anti-DENV IgM antibody. Disease severity was classified using SEARO classification upon presentation to the hospital. To identify the frequency of ILC, PBMC from Sri Lankan healthy individuals (HC) (n=14), DF (n=29) and DHF (n=16) patients were stained with common lineage markers, CD45, CRTH2 and c-Kit. Lineage negative cells expressing

haematopoietic and lymphoid markers CD45 and IL-7Rα were selected; and based on the expression of CRTH2 and c-Kit, group 2 ILC (CRTH2+ c-Kit+/−) were recognised**.** ILC2 express CRTH2 and variable levels of c-Kit, while ILC1 express neither of CRTH2 or c-Kit. ILC3 express high levels of the SCF receptor c-Kit (CD117) but do not express the prostaglandin receptor CRTH2 (CD294)(238). Compared to healthy individuals, it was evident that the DHF group had higher percentages of ILC2 (Figure 4a). Although the ILC2 remain a small proportion of circulating lymphocytes, the frequencies observed are far higher than published data in the setting of atopic disease. Given that ILC2 are able to rapidly produce large amounts of type 2 cytokines and amplify T cell responses through MHC and CD1-mediated antigen presentation(164, 239), they may contribute to a type 2 milieu in blood and tissues from dengue infected individuals. Additionally, ILC1 populations were significantly lower than ILC2 cell population in dengue (Figure 4b).

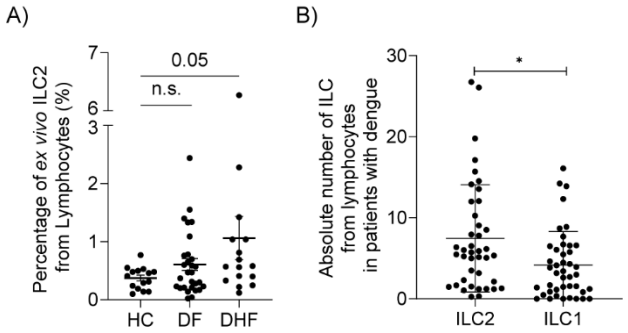


Figure 4: Frequency of Group 2 Innate Lymphoid cells (ILC2) in dengue infection. (**A**) PBMC from healthy controls (HC) (n=14), DF (n=29) and DHF (n=16) patients were stained for lineage markers, CD45, CRTH2, IL-7Rα and c-Kit. Lineage negative cells expressing haematopoietic and lymphoid markers CD45 and IL-7Rα were selected. ILC2 were recognised as CRTH2+, IL-7Rα+ and c-Kit+/-. Percentage of group 2 innate lymphoid cells from lymphocytes in healthy (HC), dengue fever (DF) and dengue hemorrhagic Fever (DHF) was analysed. (**B**) Absolute numbers of ILC2 and ILC1 (from a million of total cells) in all patients with dengue (n=45). ILC1 express neither of

CRTH2 or c-Kit. *P<0.05, **P<0.01, ***P<0.001. Statistical significance was obtained using one-way ANOVA followed by Tukey's multiple comparisons and unpaired T test.

3.2 Hypothesis and aims

On the basis of these preliminary findings, it was hypothesised that ILC2 are phenotypically altered during acute dengue infection. The work of the chapter aimed:

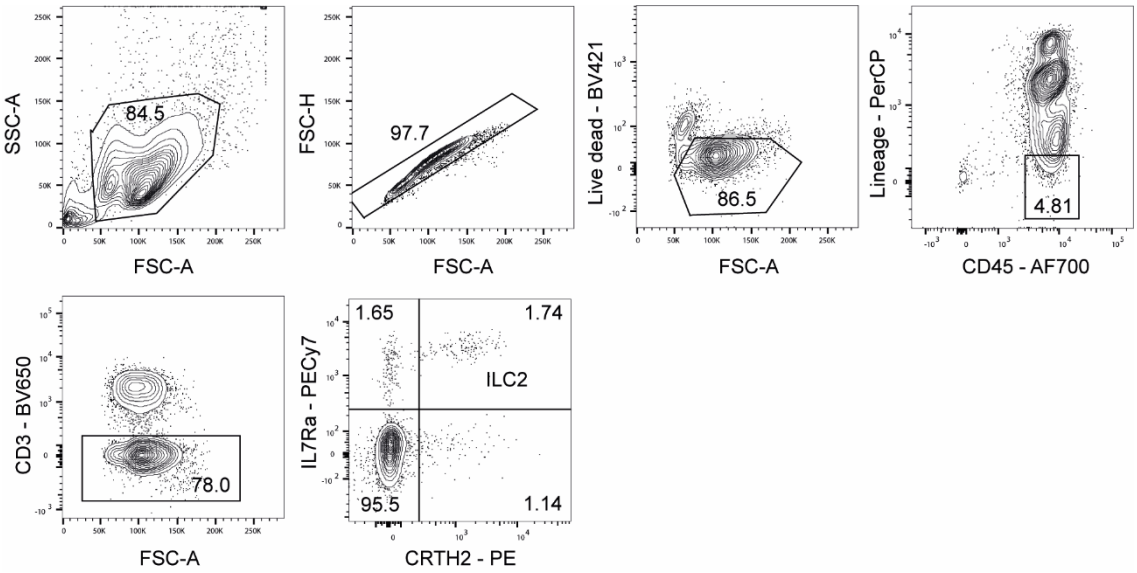
- To investigate whether ILC2 are activated in dengue fever
- To ascertain whether ILC2 are infected in patients with dengue infection
- To determine whether ILC2 show evidence of altered antiviral signaling

3.3 Results

3.3.1 *Ex vivo* RNA sequencing of ILC2 of in healthy individuals and patients with dengue Fever and dengue Hemorrhagic Fever

Having observed that ILC2 frequency is increased in severe dengue, it was imperative to investigate for differential gene expression patterns between healthy, mild and severe dengue infections. To investigate the possible role for ILC2, RNA sequencing was performed of ILC2 isolated from PBMC (Figure 5a) of individuals with dengue fever (DF) and severe dengue (DHF) and healthy donors (HC) of Sri Lankan ethnicity. We have not explored whether these samples were from patients with primary or secondary dengue infection. Blood was taken at 2-7 days of symptom onset in patients having DF and DHF. Donor details are summarised in Figure 5b.

A)



B)

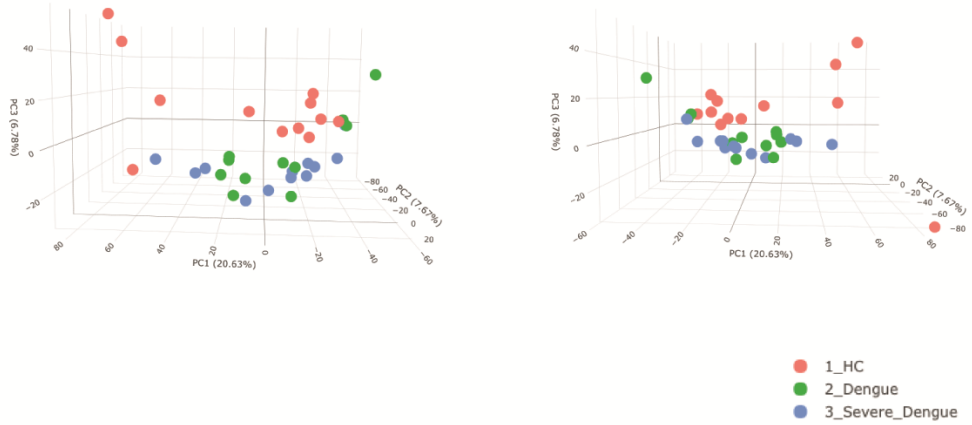
Serial No.	Age	Gender	Days since first symptom	Past Dengue	DF/DHF	Ascites (+/-)	Pleural effusion (+/-)	Bleeding (+/-)
HC1	32	M						
HC2	47	F						
HC3	23	M						
HC4	28	F						
ILCP06	48	F	7	N	DF	-	-	-
ILCP09	22	M	5	N	DHF	+	-	-
ILCP10	28	M	2	N	DF	-	-	-
ILCP13	29	M	-	N	DF	-	-	-
ILCP14	60	F	7	N	DF	-	-	-
ILCP19	37	M	4	N	DHF	+	+	-
ILCP20	27	M	6	Y	DHF	+	+	-
ILCP21	34	F	5	N	DHF	+	+	-

Figure 5: Isolation of blood ILC2 for *ex vivo* RNA sequencing. (A) Representative plot of ILC2 gating of a healthy individual depicted in the figure. Above gating strategy was used for isolation of ILC2 of three clinical groups: Healthy individuals (HC), dengue fever (DF), dengue haemorrhagic fever (DHF). ILC2 are CD45+/CD3-/lineage-/CRTH2+/IL-7Rα+. (B) Summary table of donor characteristics and clinical details.

Firstly, an unsupervised analysis approach was undertaken and principal component analysis (PCA) was performed to assess clustering of genes with respect to the patient groups (Figure 6a,b). I am grateful to Dr Jeongmin Woo, group bioinformatician for guidance and support for performing these analyses.

ILC2 of healthy individuals showed a markedly different gene expression (DEG) profile compared to the patients with DF and DHF. It was evident that healthy control (HC) samples were clustered separately from dengue disease groups while there were no notable differences between the two dengue infected groups, DF and DHF (Figure 6a). A similar trend was also found in cluster of heatmap generated from 281 DEGs, which were differentially expressed within the 3 disease groups (Likelihood ratio test, FDR < 0.05) (Figure 6b)(232).

A)



B)

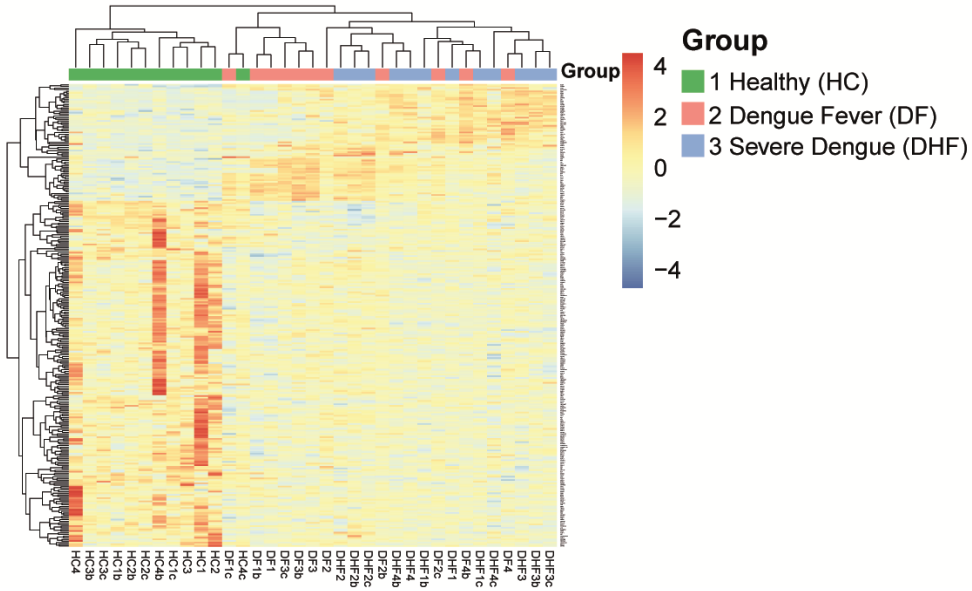


Figure 6: Gene expression profile in healthy and in patients with DF and DHF. (**A**) Principal component analysis (PCA plots) and (**B**) heat map of differentially expressed genes (DEGs) from *ex vivo* ILC2 from three groups: healthy individuals (HC) (n=3), dengue Fever (DF)(n=4) and dengue Hemorrhagic Fever (DHF) (n=4) (Likelihood ratio test, False discovery rate (FDR) < 0.05 used). Samples analysed in duplicate depicted in PCA plot and heat map. Clinically, DF and DHF groups are categorised using SEARO (WHO 2011) classification.

3.3.2 Overall gene expression – UMAP plots/heatmaps

Thereafter, crucial pathways that could be differentially regulated in viral infections were explored. Likelihood ratio test analysing differentially expressed genes across groups revealed 280 genes that were differentially regulated in HC comparing to DF and DHF groups. Among them, 210 genes were downregulated relative to healthy controls and 70 genes upregulated relative to the healthy individual gene expression (Figure 7).

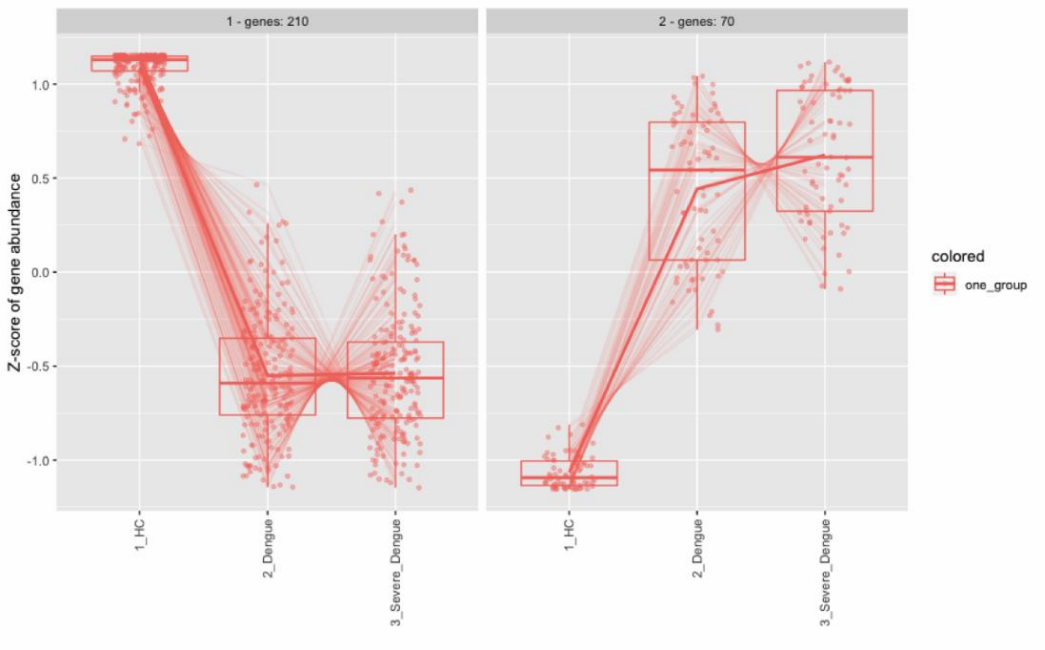


Figure 7: Differentially expressed genes in healthy compared to gene expression profile of DF and DHF. Gene expression of 280 differentially expressed genes within the 3 groups of HC, DF and DHF, according to the likelihood ratio test (LRT). Adjusted p value <0.05. Left panel shows 210 genes which were downregulated in DF and DHF compared to HC. Right panel shows 70 genes which were upregulated in DF and DHF compared to HC. Z score values depict the relative abundance of DEGs in DF and DHF relative to the HC gene expression. Each dot represents an individual gene.

Among the 70 genes that were upregulated in DF and DHF, RNA expression related to defense against viral infections and the type I interferon response were prominent (Figure

8a,b). Other than these, genes controlling viral life cycle, viral genome replication and genes related to innate immune responses were observed to have higher expression values compared to healthy individuals (Figure 8a,b).

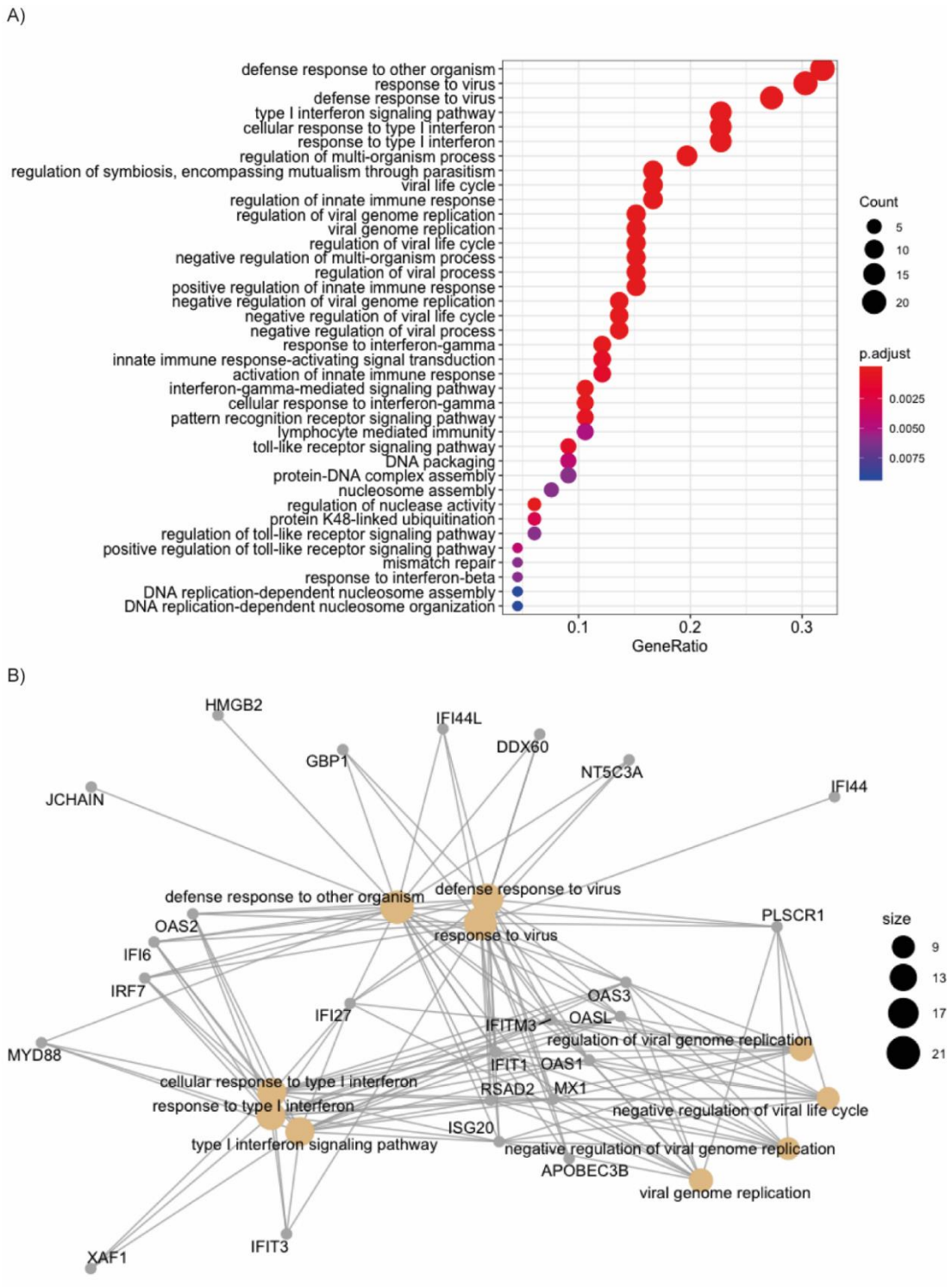


Figure 8: Genes with higher expression in DF and DHF compared to HC. (**A**) Gene

ontology (GO) enrichment analysis comparing highly expressed genes of ILC2 of healthy individuals (HC) group with both DF and DHF. The top GO terms in which the DEGs were enriched according to the gene count are presented (70 genes). The top 40 ranked GO terms according to gene count, gene number within DEG list to gene number within background reference, are visualized as dotplots. 'Gene count' is the number of genes enriched in a GO term. 'Gene ratio' is the percentage of total DEGs in the given GO term. GO= gene ontology. (**B**) Network visualization of genes which are upregulated in dengue infection. Each circle represents a gene, and edges connect all genes that are nearest neighbors based on their co-expression in an aggregate network. Genes are clustered under broad functional categories and are labeled by GO enrichment. Size of each circle depicts the abundance of the gene.

Interestingly, interferon stimulatory genes (ISGs) such as *ISG15*, *OAS* related genes (*OAS1*, *OAS2*, *OAS3*, *OASL*), *IFITM*, *IFIT*, *ISG20*, *RSAD2* and *MX1* were observed to be upregulated in the dengue infection group (Figure 8b). These genes have been shown to have important functions in dengue fever; for example, ISG15 related genes inhibits viral replication by modifying viral or cellular proteins(240). OAS1 restricts viral infection by degrading viral RNA in combination with RNase L, resulting in an inhibition of viral replication. Additionally, polymorphisms of OAS1 have been shown to associate with dengue patients requiring hospitalisation(241). Moreover, *MyD88*, which is a downstream signaling gene of Toll-like receptors of IL-1 pathway, and *IRF7*, which has a role in IFN antiviral innate immune responses, were observed to be prominently expressed (Figure 8b). In addition, *GBP1*, *HMGB1* and *APOBEC3G* were prominent genes depicted in the network analysis (Figure 8b). GBP1 is a gene that exhibits antiviral activity against influenza virus. GBP1 promotes oxidative killing and delivers antimicrobial peptides to autophagolysosomes, providing broad host protection against

different pathogen classes. HMGB1 is a protein released by necrotic cells, which has chemoattractant activity for inflammatory and stem cells. This acts through binding to a receptor for advanced glycation end products. In addition, HMGB1 can be actively secreted by inflammatory cells. HMGB2 is a multifunctional protein which enhances DNA flexibility by looping modifications, thus providing a mechanism to promote activities on various gene promoters by enhancing transcription factor binding(242). HMGB2 can be secreted by THP-1 cells, and promotes proliferation and migration of endothelial cells. APOBEC3G is an inhibitor of retrovirus replication and retrotransposon mobility via deaminase-dependent and independent mechanisms. This exhibits potent antiviral activity against Vif-deficient HIV-1. After the penetration of retroviral nucleocapsids into target cells of infection with initiation of reverse transcription, this gene induces the conversion of cytosine to uracil in the negative-sense single-strand viral DNA, leading to G to A hypermutations in the subsequent plus-strand viral DNA(243). Interestingly, these are known negative regulators of viral life cycle.

Furthermore, 210 genes that were downregulated in dengue disease in ILC2 were related to RNA splicing, processing and translational processes. Additionally, ribosome protein related genes such as *RPL* and *RPS* were shown to be downregulated (Figure 9a,b). These genes are known to be important in the induction of antiviral control mechanisms through reducing the ribosomal subunit genes(244).

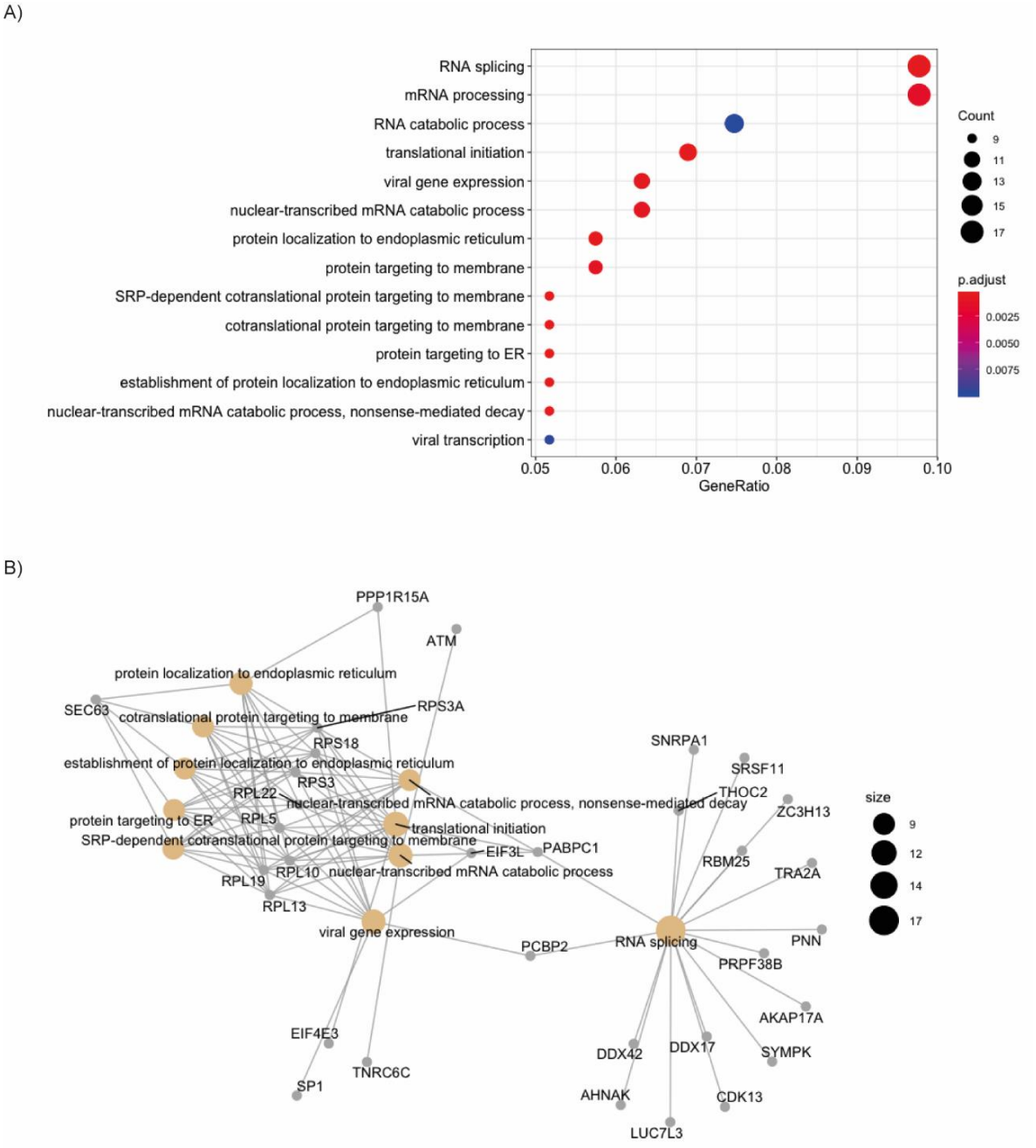


Figure 9: Genes with lower expression in DF and DHF compared to HC. (**A**) Gene ontology (GO) enrichment analysis comparing low expressed genes of ILC2 of healthy individuals (HC) group with DF and DHF group (210 genes). The top 15 ranked GO terms according to gene count, gene number within DEG list to gene number within background reference, are visualized as dotplots. 'Gene count' is the number of genes enriched in a GO term. 'Gene ratio' is the percentage of total DEGs in the given GO term. GO= gene ontology. (**B**) Network visualization of genes which are upregulated in dengue

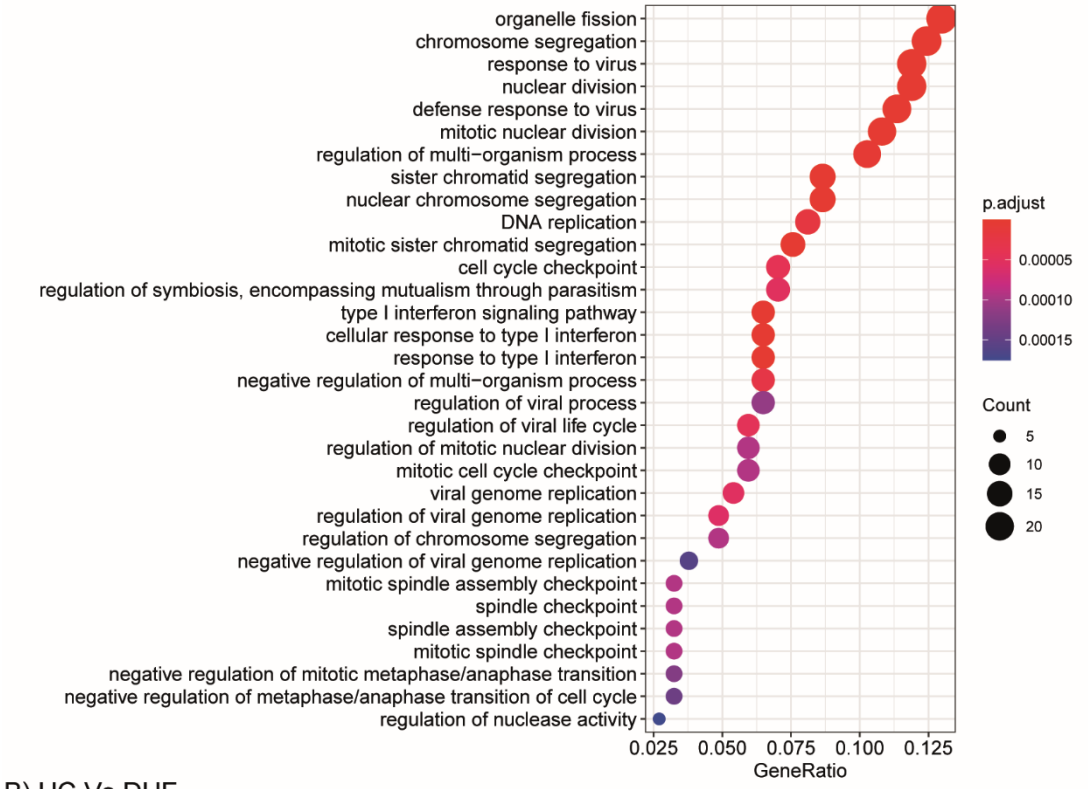
infection. Each circle represents a gene, and edges connect all genes that are nearest neighbors based on their co-expression in an aggregate network. Genes are clustered under broad functional categories and are labeled by GO enrichment. Size of each circle depicts the abundance of the gene.

Subsequently, it was aimed to explore differences in gene expression between DF and DHF groups. To accomplish this a pairwise analysis of the groups was performed, and HC gene expression was compared with DF or DHF (Figure 10a,b). To accomplish this, gene Ontology (GO) enrichment analysis was performed to understand the biological significance of these genes. It was observed that 217 DEGs were upregulated in DF and 162 DEGs were upregulated in DHF compared to HC (FC > 1.5 and FDR < 0.05). Genes related to cell proliferation such as organelle fission and chromosomal segregation had the highest gene ratio showing that proliferation related genes are highly expressed in DF. In addition, data revealed that DEGs which are upregulated in DF were predominantly associated with viral defense with type I interferon response (i.e GO:006033: type I interferon signalling pathway) (Figure 10a). Whereas GO analysis of DEGs upregulated in DHF are mainly associated with cell proliferation and DNA replication, whereas the type I interferon related gene responses are blunted (Figure 10b). The type I interferon related individual gene expression is discussed in the next section (Figure 10c).

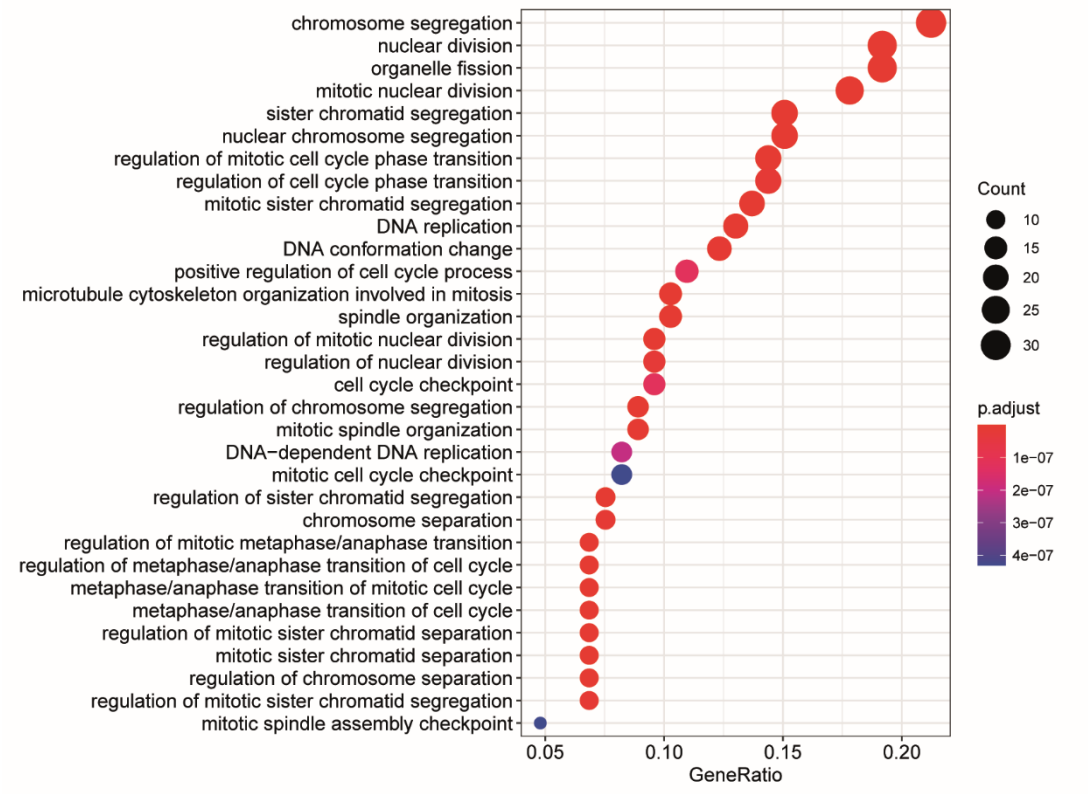
Next, it was investigated whether ILC2 are activated in dengue fever. To accomplish this, expression of genes related to activation of ILC2 in HC, DF and DHF were examined. It was found that ILC2 showed evidence of being highly activated. Genes encoding the key effector receptors *PTGDR2* (CRTH2) and *CD127* appeared to be down regulated in DF (Figure 11a), which is known to occur following ILC2 activation. Also, *CD38*, a surface marker that is known to upregulate with cellular activation and through interferon

stimulation(245, 246), was significantly upregulated in DF and DHF (Figure 11a). Additionally, *CD25 (IL12RB)* showed a trend of upregulation in dengue infection (Figure 11a). These findings indicated that *ex vivo* ILC2 are activated in dengue disease.

A) HC Vs DF -
GO analysis of 217 Genes highly expressed in dengue fever



B) HC Vs DHF -
GO analysis of 162 Genes highly expressed in dengue hemorrhagic fever



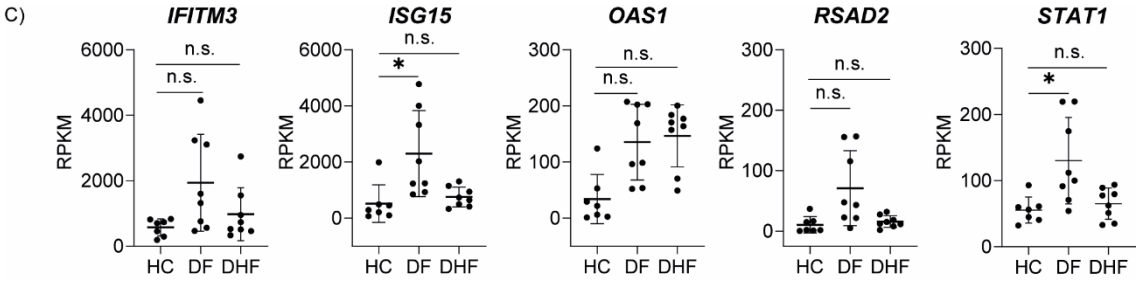


Figure 10: *In vivo* ILC2 show impaired type I IFN signature in severe Dengue. *Ex vivo* ILC2 from three groups: healthy individuals (HC) (n=3), dengue Fever (DF)(n=4) and dengue Hemorrhagic Fever (DHF) (n=4). (**A**) Gene ontology (GO) enrichment analysis comparing highly expressed genes of ILC2 of healthy individuals (HC) group with DF group (217 genes). (**B**) Gene ontology enrichment analysis comparing highly expressed genes of ILC2 of HC group with DHF group (162 genes). The top ranked GO terms according to gene count, gene number within DEG list to gene number within background reference, are visualized as dotplots. 'Gene count' is the number of genes enriched in a GO term. 'Gene ratio' is the percentage of total DEGs in the given GO term. (**C**) Gene expression of type I interferon related genes (*IFITM3, OAS1, ISG15, RSAD2, STAT1*) of human blood derived ILC2 in HC, DF and DHF, determined by RNA sequencing and represented in RPKM values. Statistical analysis performed using adjusted p-value from the DEG result. *P <0.05, n.s. not significant.

3.3.3 Type I interferon related gene expression in ILC2 in dengue associated disease

The innate immune response is primarily responsible for the control of viral spread during the early stages of infection(100). The type I IFN response represents a principal effector mechanism of innate immunity and contributes to the control of dengue viral replication and to a rapid development of adaptive immune responses that can contribute to elimination of the virus(247, 248). As above, type I interferon responses are found to be

prominent in DF and this response was not prominently observed in severe dengue (Figure 10a,b). Additionally, individual genes with anti-viral effector functions in the group of interferon stimulatory genes (ISGs) were analysed. ISGs consist of series of proteins that have variety of antiviral effector functions from enhancing innate pathogen sensing capabilities to suppress viral life cycle at different stages(249). An upregulation of genes such as *ISG15*, *IFITM3*, *OAS1*, *RSAD2* and *STAT1* in ILC2 derived from individuals with DF was noted but not in those from DHF (Figure 10c). Each of these genes are known to have important antiviral effector functions in dengue fever. The STAT1 dependent pathway acts early and plays a role in controlling initial viral replication, whereas STAT1 independent pathway functions later and is responsible for complete viral clearance in dengue infection(250). These intrinsic findings from ILC2 are consistent with results from two other studies where it was observed that the expression of type I IFN related genes are clearly up regulated in peripheral blood mononuclear cells (PBMCs) derived from individuals with DF and reduced in patients with DSS(251, 252).

3.3.4 Dengue virus detection in *ex vivo* ILC2 from patients with DF and DHF

It was aimed to explore whether *ex vivo* ILC2 show evidence of dengue infection. For this, raw RNA sequencing data from the *ex vivo* sorted ILC2 were aligned against human (hg38) and dengue virus genomes (including all serotypes 1-4) simultaneously to explore whether dengue viral sequences were detectable in ILC2 sequence data. Interestingly, ILC2 from 3 patients (two DF, one DHF) showed detectable dengue sequence of DENV serotype 2. Dengue serotype 2 was the predominantly detected serotype in Sri Lanka when the samples were obtained. The reads were related to the proteins of the non-structural regions of the dengue viral genome. Most of the reads originated from the region of 600bp-9000bp of DENV genome, corresponding to NS4A, protein-2K, NS4B,

RNA-dependent RNA polymerase NS5 (Figure 11b). These data show that dengue virus sequence could be detected in host ILC2 and also that ILC2 are infected *in vivo*.

In addition, it was showed that *ex vivo* ILC2 from dengue patient samples show dengue virus infection through staining for intracellular dengue envelope protein. To demonstrate this, PBMC from individuals with dengue infection (DF, DHF) and healthy controls were stained with surface antibodies to identify ILC2 and with intracellular dengue viral E protein to detect infection. Dengue viral staining among ILC2 was variable from 2-87% (Figure 11c) and it was significantly higher in dengue patient samples (Figure 11c,d), reaching 75-85% in certain dengue patients.

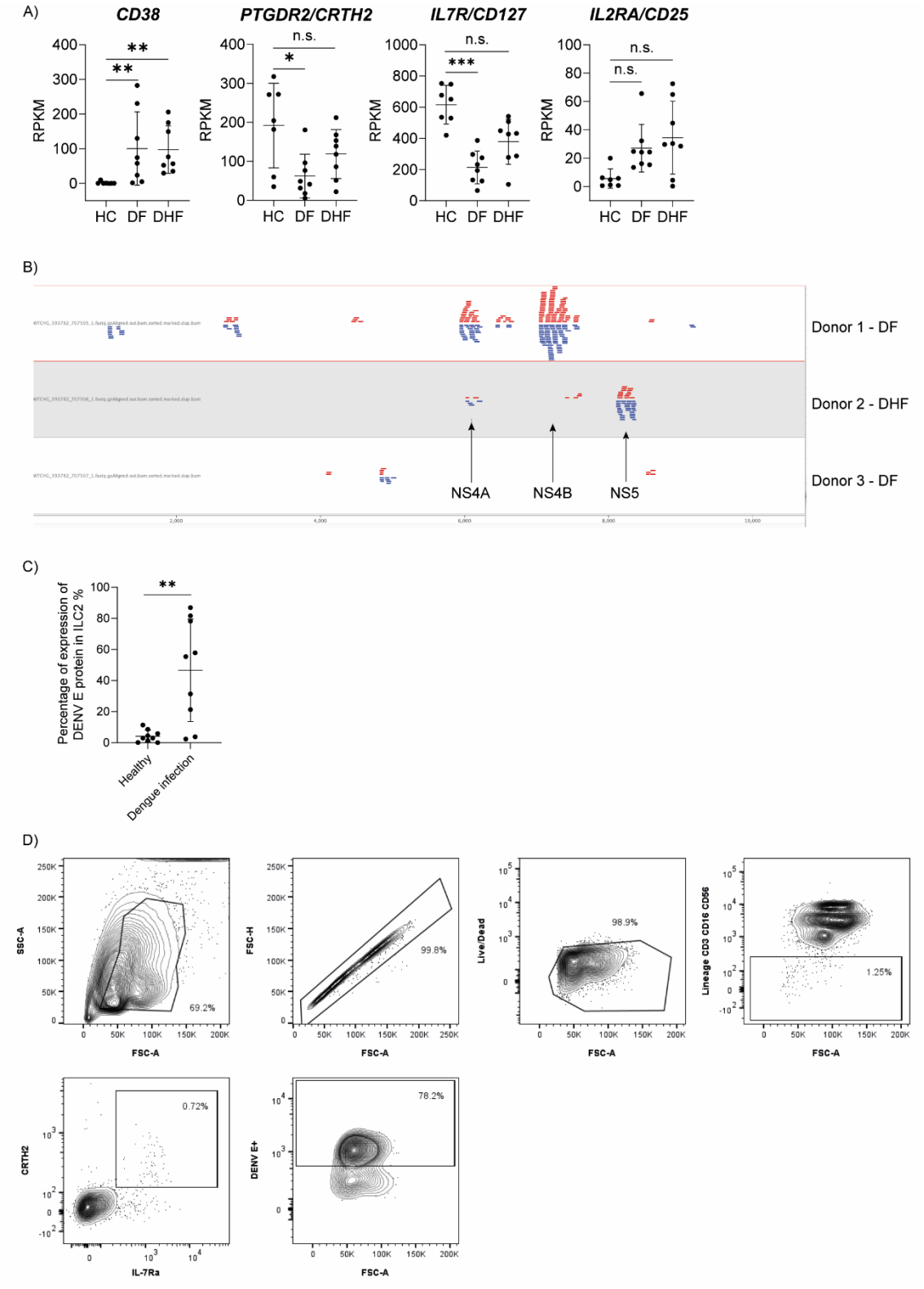


Figure 11: *In vivo* ILC2 are activated and infected in dengue infection. (**A**) Activation related gene expression of *CD38, PTGDR2 (CRTH2), CD127(IL-7Rα), IL-2RA (CD25)* of human blood derived ILC2 of HC, DF and DHF, determined by RNA Sequencing and

represented in RPKM values. Statistical analysis performed using adjusted p-value from the DEG result. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. RNA sequencing data from ILC2 from three groups: healthy individuals (HC) (n=3), dengue Fever (DF)(n=4) and dengue Hemorrhagic Fever (DHF) (n=4). (**B**) Mapping figure from genome browser depicting matching genes of dengue genome to the raw RNA sequencing data from ILC2 in patients having with Dengue fever (DF, n=2) and Dengue Hemorrhagic Fever (DHF, n=1). Corresponding proteins for the base pair ranges were depicted. 6376~6756bp: Non-Structural protein NS4A, 6757~6825bp: protein 2K, 6826~7569bp: Non-Structural protein NS4B, 7570~10269bp: RNA-dependent RNA polymerase NS5. (**C**) For *ex vivo* staining of ILC2 for dengue infection, frozen PBMC from HC (n=9) and patients with acute dengue infection (n=9) from Sri Lankan ethnicity were used. Samples were thawed and stained for ILC2 (Live/CD45+/CD3-/CD56-/lineage-/CRTH2+/IL-7Rα+) and intracellular dengue virus envelope protein. (**D**) Representative flow cytometry plot of DENV E protein staining from a patient with dengue infection is shown.

3.3.5 Type III interferon gene expression in dengue fever in RNA sequencing data

It is unclear why patients develop different levels of disease severity upon dengue infection. It has been hypothesised that IFN production could play a role in reducing the severity of disease. Type III IFN is the main IFN produced in the epithelia and includes 4 types (IFN- λ1, IFN- λ2, IFN- λ3, and IFN- λ4) (253). IFN-λ binds to a receptor complex containing two subunits, IFN-λ receptor 1 (IFN-λ R1) and interleukin (IL)-10R2, and, like type I IFN, mediates antiviral activity through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway and induction of IFN-stimulated genes (ISGs)(254). In contrast, the signal transduction of type I IFN depends on the IFNAR complex, which is composed of IFNAR 1 & IFNAR2.

It has been shown that DENV infection can induce IFN-λ1 production in dendritic cells (DCs) and lung epithelial cells, and can induce CCR7 expression and DC migration. IFN-λ1 has been shown to inhibit DENV2 replication in an epithelial cell line(254). Additionally, stimulated keratinocytes produce high levels of IFN-λ1, but only low levels of IFN-α, IFN-β, and IFN-γ, suggesting that IFN-λ is the major IFN of human epidermal cells produced in direct response to viral stimuli(255). Another study demonstrated productive DENV-2 infection of HaCaT cells and was able to establish an antiviral response through production of both type I and type III interferons (IFN-β and IFN-λ). The production of IFN from HaCaT cells correlated with upregulation of IFN-inducible transmembrane protein-3 (IFITM3) and viperin (RSAD2) in bystander, uninfected cells(256).

Importantly, in mice infected with Influenza, IFN-λ emerges earlier at the epithelial barriers than type I IFN, and shows potent protective function during the early phase of the infection without activating inflammation, when type I IFN levels are still scarce. If infection progresses, type I IFN come into play to enhance viral resistance and induce pro-inflammatory responses essential for confronting infection but causing immunopathology. IFN-λ treatment induces expression of antiviral genes but not pro-inflammatory cytokines in neutrophils, while type I IFN induces the expression of both types of genes and are involved in causing inflammation and immunopathology(257). Pott *et al* demonstrated that mice lacking a functional IFN-λ receptor complex were less able to control oral rotavirus infection and only systemic administration of IFN-λ, but not IFN-α, was able to suppress rotavirus replication in the intestinal epithelium (258). This unique role of IFN- λ has not been elucidated in other epithelial surfaces. It appears that pDCs, B cells, and perhaps other DC subsets, are sensitive to IFN-λ, whereas monocytes, T and NK cells are not (259). In hepatitis C infection, another flavivirus infection,

polymorphism near the *IL28B* locus was associated with enhanced spontaneous HCV clearance (260).

As above, IFN-λ has been shown to act through specific receptor consisting of IFNLR1 and IL10RB. High levels of IFNLR expression, and STAT1 phosphorylation in response to IFN-λ treatment, are consistently reported for pDCs which are considered to be "professional" type I IFN-producing cells and produce 10- to 100-fold more IFN-α than other cell types in response to enveloped viruses(261). However, data on IFN-λ responsiveness of monocytes and MoDCs are conflicting. Efforts have been made to determine whether other immune cells express the IFNLR and, if so, the manner of their response to IFN-λ.

Tissue resident innate lymphoid cells in the skin could be a cell type that is exposed to dengue virus at an early stage. Also, it is evident that IFN-λ related pathway genes (*IFNLR1, IL10RB, STAT1*) are expressed in ILC2 (Figure 12a). Stimulation of ILC2 with IFN-λ did not induce significant *IFNLR1* expression, which is the receptor complex for the IFN- λ activation. However, IFN-α stimulation induced *IFNLR1* expression in ILC2 (Figure 12b). Moreover, IFN-α stimulation, but not IFN-λ stimulation induced *OAS1* expression in ILC2 (Figure 12c).

Additionally, *SOCS1*, a prominent negative regulatory factor of the IFN signaling pathway, which acts by binding to the IFNAR1-Tyk2 complex and directly inhibiting kinase activity, was highly expressed in DHF compared to DF(Figure 12d). Altogether, this points to relevance of the IFN pathway in ILC2 during dengue infection, and signifies that further exploration is necessary to determine whether ILC2 could functionally respond to IFN signaling. This is addressed in subsequent work of the thesis.

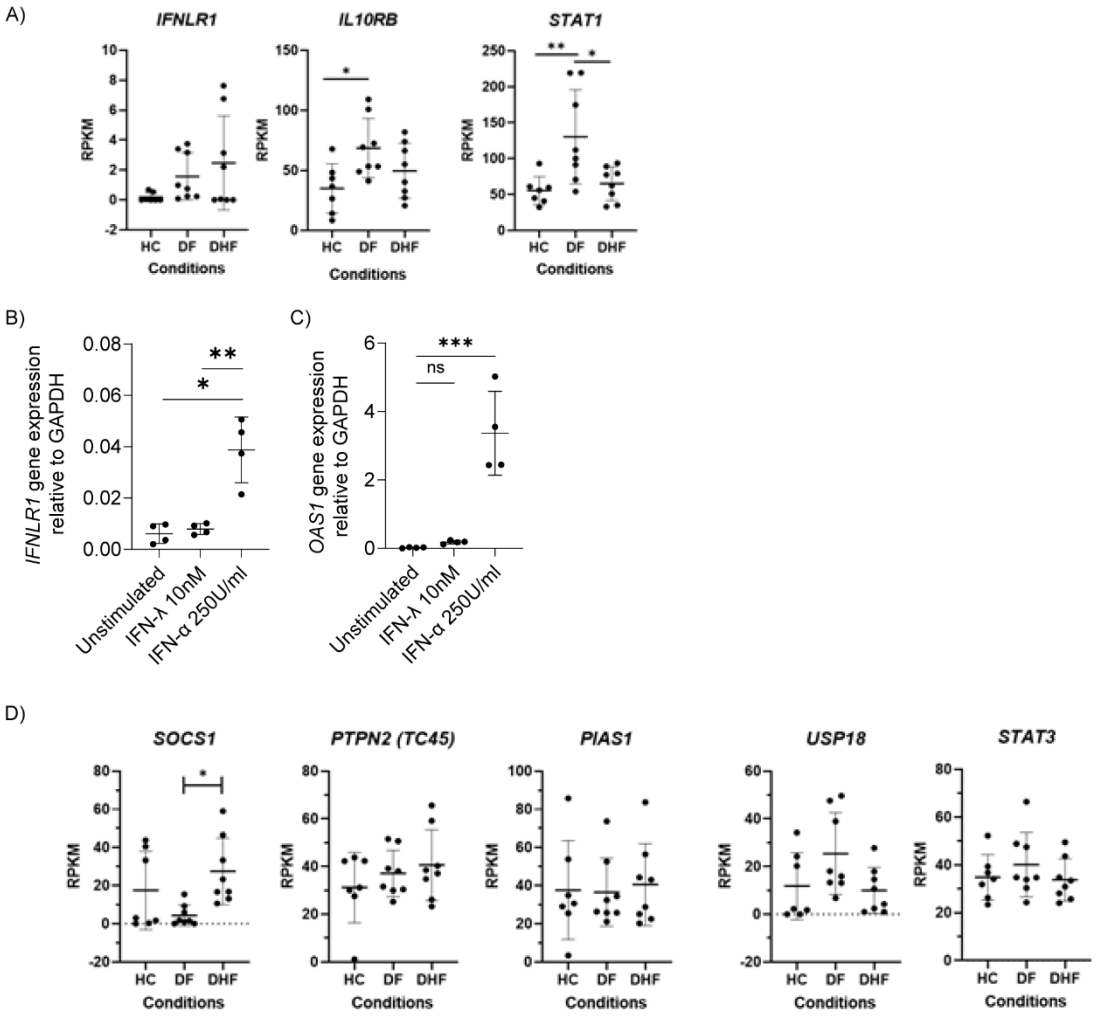


Figure 12: Differential gene expression of type III IFN and negative IFN regulators genes in HC, DF and DHF. RNA sequencing data of ILC2 from three groups: healthy individuals (HC) (n=3), dengue Fever (DF)(n=4) and dengue Hemorrhagic Fever (DHF) (n=4). (**A**) Type III interferon related gene expression (*IFNLR1, IL10RB, STAT1)* of human blood derived ILC2 of HC, DF and DHF, determined by RNA Sequencing and represented in RPKM values. Statistical analysis performed using p-value from the DEG result. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**B**) Real-time PCR analysis of *IFNLR1, OAS1* gene expression by ILC2 following stimulation with IFN- λ (200nM) or (**C**) IFN-β (250U/ml) for 2 hours. n=4, one-way ANOVA with Tukey's multiple comparison test. Gene expression normalised to *GAPDH*. (**D**) Negative regulatory IFN

genes (*SOCS1*, *PTPN2*, *PIAS1*, *USP18*, *STAT3*) of human blood derived ILC2 of HC, DF and DHF, determined by RNA Sequencing and represented in RPKM values. Statistical analysis performed using p-value from the DEG result. *<0.05, **P<0.01, ***P<0.001. Graphs without a stat bar showed no significant differences.

3.3.6 Activation markers of ILC2 *in vitro*

Next, ILC2 activation marker protein expression was characterised and aimed to validate the observation of ILC2 activation in the RNA sequencing data. CD25 was a reliable activation marker of ILC2, which upregulated with PGD_2 mediated activation (Figure 13a). IFN-β mediated activation did not upregulate CD25 expression in ILC2. However, CD38 expression was upregulated predominantly with IFN-β mediated activation (Figure 13b) suggesting that CD38 would be a reliable surface receptor for type I interferon mediated activation in ILC2. PGD_2 did not significantly upregulate CD38 expression in ILC2 (Figure 13b).

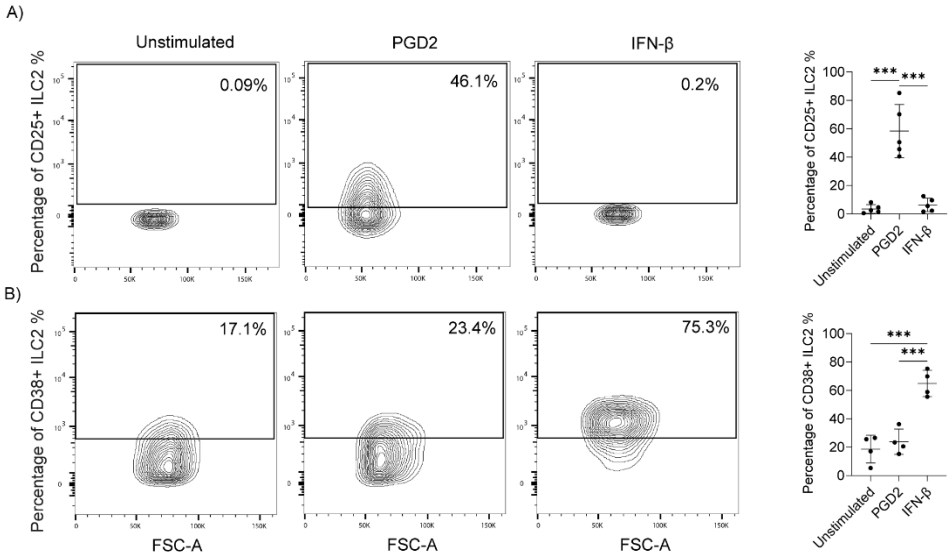


Figure 13: Activation markers of ILC2 *in vitro*. (**A**) ILC2 were activated for 24 hours with PGD_2 (250nM) and IFN-β (250U/ml). CD25 surface stain and (**B**) CD38 surface stain was performed and analyzed by flow cytometry. Left panel depicts flow cytometry

plots representative of single donor and right figures depicts compiled results from 4 donors. Experiment repeated thrice to validate. One-way ANOVA followed by Tukey's multiple comparisons was performed to determine statistical significance. Error bars shows mean ± SD. *<0.05, **P<0.01, ***P<0.001. Graphs which were not indicated with a stat bar showed no significant differences.

3.3.7 Anti-viral gene expression of ILC2 *in vitro*

To further investigate whether ILC2 are responsive to type I interferons, *in vitro* expanded ILC2 were treated with exogenous IFN-β (250U/ml) and it was observed that anti-viral genes *ISG15, IFITM3, OAS1, RSAD2* and *STAT1* were significantly upregulated in ILC2 (Figure 14). PGD_2 activation did not influence expression of anti-viral gene expression (Figure 14).

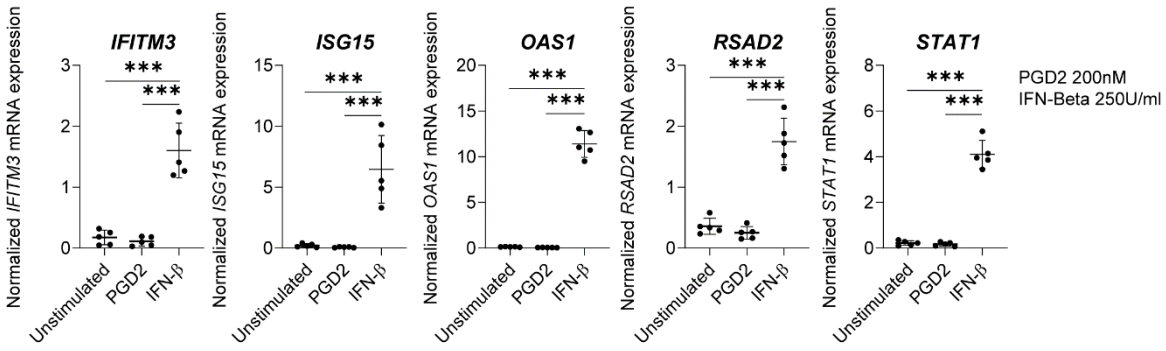


Figure 14: Anti-viral gene expression in ILC2 with type I interferon stimulation. Real-time PCR analysis of *IFITM3, OAS1, ISG15, RSAD2, STAT1* gene expression by ILC2 following stimulation with PGD_2 (200nM) and IFN-β (250U/ml) for 2 hours. (n=4-6, one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments). Gene expression normalised to *GAPDH*.

3.3.8 Anti-viral protein expression in ILC2 and its regulation with PGD_2

Having shown that type I interferons establish an anti-viral gene expression state in ILC2, it was sought to determine regulatory factors of type I interferon responses in ILC2. As

above, PGD_2 is a lipid mediator predominantly produced by mast cells, and also produced endogenously by Tc2 and ILC2 following activation (126, 188). Therefore, it was investigated whether PGD_2 activation modifies the anti-viral gene expression and protein responses of ILC2.

Stimulation of ILC2 with PGD_2 significantly down-regulated ILC2 type I interferon receptor, *IFNAR1* expression (Figure 15a). *IFNAR1* expression was significantly upregulated with IFN-β treatment (Figure 15a). IFNAR1 is a membrane protein that forms one of the two chains of a receptor for interferons alpha and beta. Binding and activation of this receptor stimulates Janus protein kinases, which in turn phosphorylate several proteins, including STAT1(262). Activation via PGD_2 also suppressed other anti-viral gene expression such as *OAS1* (Figure 15b) and *IFITM3* (Figure 15c) in a dose dependent manner. IFITM proteins are known mediators of innate immunity that inhibit viral infection in part by blocking viral entry and have shown to restrict replication of multiple viruses including dengue (263, 264). In addition, it has been demonstrated in an experimental model involving K562 human myelogenous leukemia cells that IFITM proteins are able to restrict both direct and ADE-mediated infection(265). These proteins are strongly upregulated by Type I and II interferons(266). IFITM proteins are key mediators of innate immunity that inhibit viral infection by blocking viral entry and replication(264). Additionally, at the protein level PGD_2 downregulated expression of IFN-β induced IFITM3 in ILC2 (Figure 15d). With these results we propose that PGD_2 could suppress anti-viral mechanisms in a setting of dengue infection.

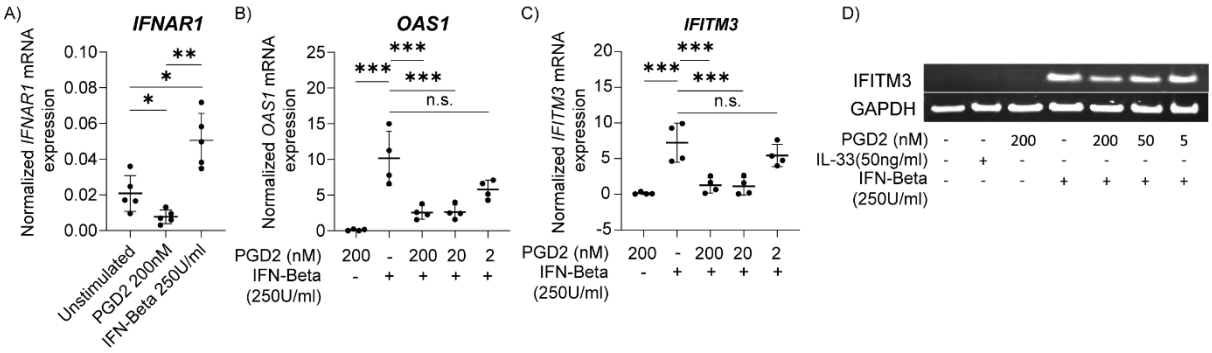


Figure 15: ILC2 activation through PGD_2 suppresses the antiviral state of ILC2. (**A**) Real-time PCR analysis of *IFNAR1* gene expression by ILC2 following 2 hours of stimulation with PGD_2 (200nM) and IFN-β (250IU/ml). (n=5, one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. Gene expression normalised to *GAPDH*. Real-time PCR analysis of (**B**) *OAS1* and (**C**) *IFITM3* gene expression by ILC2 following stimulation with PGD_2 (2, 20, 200nM) for 1 hour and then treated with IFN-β (250IU/ml). (n=3, one-way ANOVA with Tukey's comparison test, data representative of 3 independent experiments). Gene expression normalised to *GAPDH*. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**D**) Western blot of IFITM3 protein expression of ILC2 treated with PGD_2 (2-200nM) and subsequently added IFN-β (250IU/ml). Experiment performed twice with 2 donor samples.

3.3.9 Type I IFN related proliferation changes in ILC2 in dengue infection

Additionally, the effect of type I interferon signaling on ILC2 proliferation was investigated. It has been shown that dengue virus infected monocyte derived dendritic cells (moDCs) secrete IFN-α(267). Type I interferons reduce proliferation and type 2 cytokine secretion in ILC2 and increases apoptosis. Suppression of ILC2 activity occurred in human and mouse ILC2 and occur depending on transcriptional factor ISGF3(184). To demonstrate this in the context of dengue infection, moDCs were

infected with dengue virus and IFN-α rich supernatant was collected (discussed later in Figure 30a,e). This supernatant was used to analyse the type I interferon related proliferative and survival effects in ILC2. Supernatants from DENV infected moDC reduced proliferation of ILC2 and increased cell death of ILC2 significantly (Figure 16a-c). Additionally, blockade of type I interferon receptor rescued the reduction of proliferation of ILC2 and reduces the cell death (Figure 16b, c).

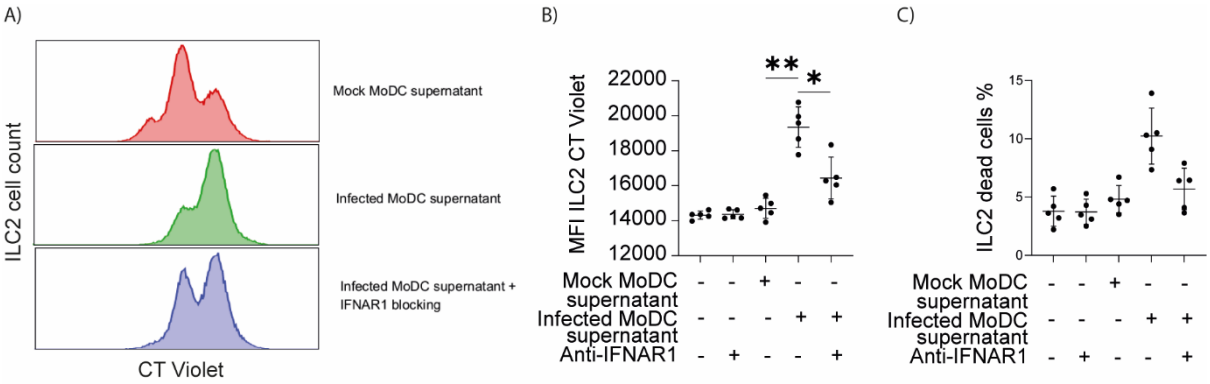


Figure 16: ILC2 proliferation and survival with IFN-β stimulation. (**A**) MoDC were infected with dengue virus at a MOI of 2.5 and supernatant was collected from mock (control / uninfected) and infected MoDCs after 48 hours. ILC2 were treated with supernatants from mock moDC or DENV infected moDC and incubated for 4 days. Next, ILC2 proliferation and cell death (Live/dead stain – PE Texas Red) was assessed. ILC2 were tagged with CellTrace^{TM} (CT) violet to detect proliferation prior to the experiment. Inhibitory conditions: anti-IFNAR1 (MMHAR-2 clone - 10µg/ml) was added and incubated for 1 hour before adding the supernatant. Anti-IFNAR1 was replenished at same concentration at 48 hours. Representative plot of ILC2 proliferation depicted. X axis depicts proliferation and y axis depicts the ILC2 cell count. Single representative figure shown out of 5 donors. Experiment repeat thrice. (**B**) Compiled data from 5 donors showing ILC2 proliferation with CT violet staining after 4 days of incubation. (**C**) Cell death of ILC2 after 4 days of incubation with MoDC supernatants. n=5, *<0.05,

**P<0.01, ***P<0.001. one-way ANOVA followed by Tukey's multiple comparison test, data representative of 3 independent experiments. Experiment repeated thrice for validation. Data depicted as mean ± SD.

3.3.10 Cytotoxicity related gene expression in ILC2

Cytotoxic lymphocytes eliminate virally infected host cells via contact-dependent and contact-independent mechanisms. Upon specific interaction with the target cell, the cytotoxic granules are polarised towards the area of contact between killer cell and target cell, and the granule contents are secreted into the intercellular space(268). It has been shown that granzymes A, B, and K have been found at high levels in the plasma of DENV-infected patients (269). Moreover, it was demonstrated that dengue-specific CD4+ T cells express CXCR3+ and mediate cytolytic activity (270). Further studies have confirmed that T cell activation leads to the release of granzyme and perforin (271), thus triggering the apoptosis of target cells. This denotes that granzymes may play a significant role in killing viral infected cells. Recently, it has been demonstrated that T cell activation triggered by DENV infection leads to the secretion of GZMA and Perforin by CD4 T cells, although in this study an increase in granzyme production in CD8 cells was not observed(272). It was demonstrated that DENV-NS3 specific T cells of those with past mild/subclinical dengue infection were more likely to produce granzyme B and those who were hospitalized due to dengue were more likely to have DENV-NS3-specific T cells that produce TNF-α and IFN-γ or TNF-α alone(273).

Although, ILC2 are considered non-cytotoxic cells, a recent study described that increased ILC2 activation may change their phenotype to a more natural killer (NK)–like ILC, with upregulation of the NK receptor NKp30 as well as granzyme B, but not perforin production(274). Additionally, stimulation via NKp30 expressed on ILC2 induces the production of IL-5 and IL-13 in ILC2(275). Virus-infected cells often induce or increase

the expression of ligands at their surface, allowing for recognition by NK cell activating receptors, including NKG2D, DNAM-1, CD94-NKG2C; the NCR receptors NKp46, NKp30, NKp44. NKG2D recognises ligands including MICA, MICB, and ULBP1-6 (276).

MICB is an inducible activating ligand for the NKG2D type II receptor on natural killer (NK) and CD8+ T cells. Ligation of NKG2D by MICB stimulates anti-viral effector functions in NK cells including cytokine expression and the cytolytic response and function and thereby MIC function as signals of cellular stress. Interestingly, polymorphisms within *MICB* gene showed genome-wide significant association with DSS(277). NKG2D is expressed on most natural killer cells, some γδ T cells and some CD8 αβ T cells. NKG2D ligands are absent in most cells and tissues under steady state but can be induced by viral and bacterial infections and other cell stress (278). NK-activating receptor NKp44 is involved in virally-mediated NK activation through direct interaction with the flavivirus envelope protein. Recombinant NKp44 directly binds to purified domain III of envelope protein of the dengue virus. Functionally, interaction of NK cells with infective and inactivated flaviviruses results in NKp44-mediated NK cell degranulation(279). This may be a mechanism for virus induced cytolytic activity of infected cells.

From *ex vivo* RNA sequencing data of ILC2, it appears that gene expression consistent with acquiring cytotoxic functionality was evident. ILC2 expressed significantly higher levels of granzymes (A and B) and perforins than healthy controls (Figure 17a). ILC2 have other cytolysis related genes such as *NKG7* and granulysin at higher levels in dengue infection than in healthy controls (Figure 17a). In addition, *MICB* expression is higher in DF and DHF patients than healthy controls although the levels were much lower (Figure 17a). This signifies that not only do ILC2 express cytolytic activity related genes but

could potentially induce cytolytic activity through MICB. Therefore, it was hypothesised that ILC2 may acquire capacity for cytolytic activity.

To test whether ILC2 can produce granzymes ILC2 were activated PGD_2 for 24 hours and an intracellular stain for GZMB was performed. ILC2 significantly expressed GZMB after activation PGD_2 than unstimulated ILC2 (Figure 17b). Thereafter, whether dengue infection induce GZMB expression was investigated. ILC2 were infected with dengue virus (MOI of 5) and an intracellular stain for GZMB was performed. ILC2 infection data are presented in the next chapter in detail. Mock (uninfected) ILC2 had low staining for GZMB and this did not significantly increase with dengue infection (Figure 17c).

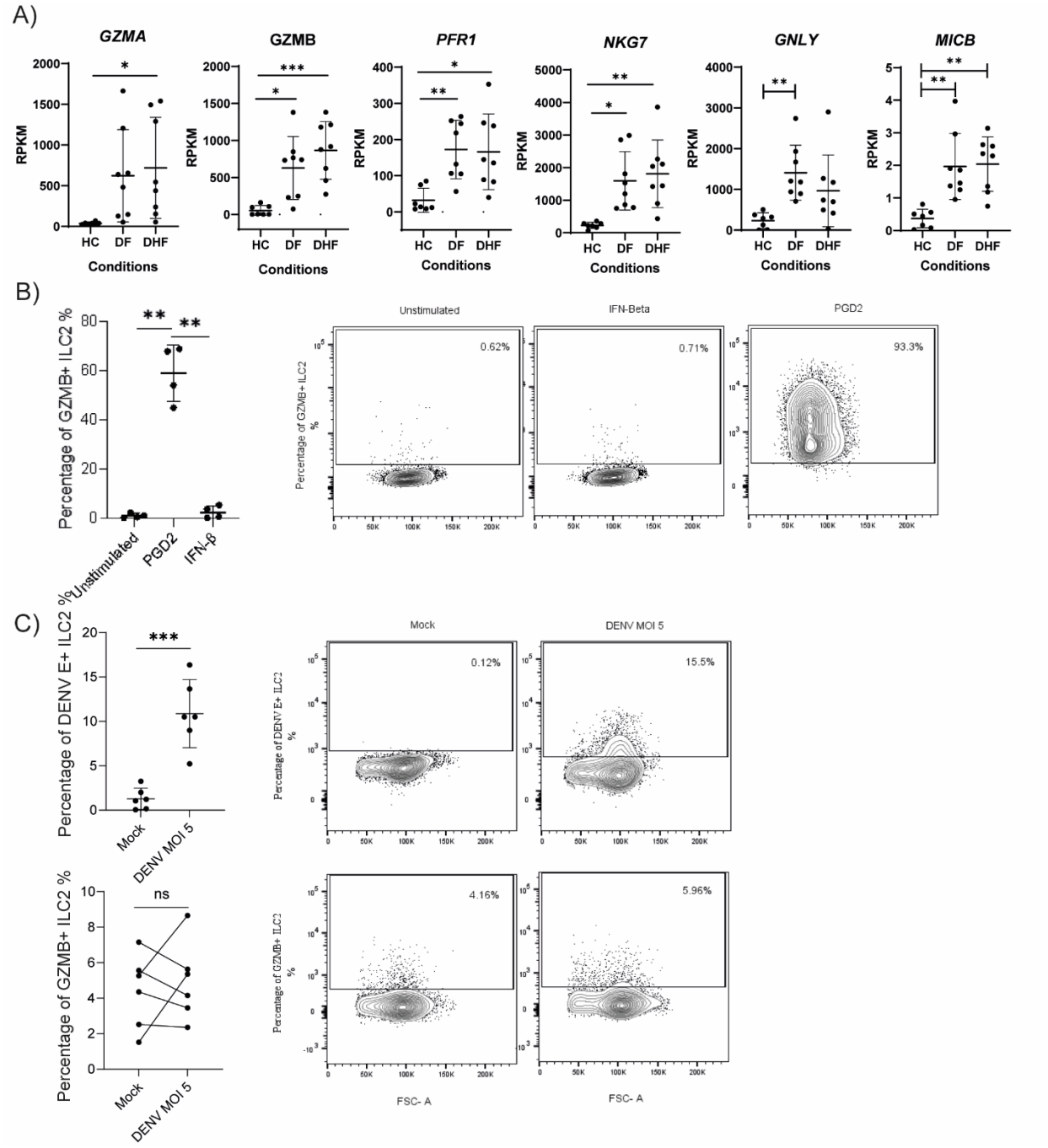


Figure 17: Expression of cytolysis related genes in ILC2. (**A**) RNA sequencing data from ILC2 from three groups: healthy individuals (HC) (n=3), dengue Fever (DF)(n=4) and dengue Hemorrhagic Fever (DHF) (n=4). Differential gene expression of *GZMA, GZMB, NKG7, PFR1*, *GNLY* (Granulysin) in healthy (HC), dengue fever (DF), dengue hemorrhagic fever (DHF). Gene expression levels are shown in RPKM values. One-way ANOVA followed by Tukey's multiple comparisons was performed to determine

statistical significance. P value *<0.05, **P<0.01, ***P<0.001. (B) *Ex vivo* sorted and expanded ILC2 were stimulated with PGD_2 (200nM) and IFN-β (250U/ml) and intracellular GZMB level was stained after 24 hours. (**C**) *Ex vivo* sorted and expanded unstimulated ILC2 were incubated with dengue virus (MOI 5) for 2 hours and the excess virus was washed off and incubated with media for 48 hours. Intracellular DENV envelope protein and GZMB was stained. Experiment performed twice (n=6).

3.4 Discussion

It is known that ILC2 undergo activation and produce a type 2 response during respiratory syncytial virus infection and possibly have a repair role in influenza infection(280, 281). In COVID-19 infection, an increase in ILC2 numbers has been detected in patients. Furthermore a higher level of IL-33 was detected in COVID-19 patients, consistent with ILC2 activation and comparatively higher levels of type 2 cytokines(236). In this study we extend the impact of ILC2 to the pathogenesis of a non-respiratory virus. Usually, dengue infection is associated with depletion of T cells and leukocytes likely to be due to virus induced bone marrow suppression or sequestration in the peripheral lymphoid organs(282). We showed that ILC2 are increased in number in severe dengue. In addition, recent data suggested that there could be an association of ILC2 with severe dengue. It has been shown that *ex vivo* stimulated human ILC2 secreted significantly higher IL-4 and IFN-γ levels, also has a trend of secreting higher IL-13 in DHF than DF patient samples(283). Another study showed that ILC produce IL-4/IL-13 or GATA3+ more frequently in dengue with warning signs compared to dengue without warning signs(284).

In addition, we show that the innate type 2 pathway impacts type I IFN mediated control of dengue viral replication within ILC2. Plasmacytoid DC (pDC) and myeloid DC are thought to be a major source of type I interferons in dengue disease, and a blunted blood pDC response to dengue virus infection has been associated with severe disease with a higher viremia level, indicating a type I interferon mediated protection (98, 285). IFN-α levels are higher in patients with DF than DHF. Secondary dengue, which was associated with higher morbidity and mortality, had lower IFN-α levels than primary dengue(98, 285). With gene ontology enrichment analysis, it was here shown that genes related to the type I interferon pathway were highly expressed in ILC2 of individuals with DF,

while in severe disease this response was blunted. From our *in vitro* data, type I IFN secreted by infected moDC could control ILC2 proliferation and increase cell death of ILC2. This could be an important mechanism where sequential infection of DC could lead to IFN- α mediated protection. Although moDCs produce IFN-α following dengue viral infection, dengue virus impairs IFN I signaling in DCs (99, 248) compared to other viruses, leading to less efficient priming of dengue specific adaptive immune responses(286). Dengue virus therefore utilizes a number of mechanisms to subvert the type I IFN response(103). DENV NS2B3 protease complex inhibits type I IFN in human moDCs. Thus, DENV inhibits type IFN I production in moDCs through the action of its viral protease NS2B/NS3 that degrades STING(103). STING is a protein that act as a DNA sensor and a signaling adaptor protein that mediate type I IFN responses through activating IRF3/STAT6. This signifies that moDC infection in dengue viral infection can lead to a blunted interferon response due to dengue viral defense mechanisms. If the initial interferon response is strong, it will likely impair ILC2 activation and proliferation.

Interstingly, PGD_2 downregulated type I interferon induced responses in ILC2, suggesting that type 2 pathways could be an under-appreciated escape mechanism to evade type I interferon mediated protection during dengue infection. PGD_2 acts through both DP1 or DP2/CRTH2 receptors and in certain tissues DP1 has anti-inflammatory functions while DP2/CRTH2 has pro-inflammatory functions. Werder *et al* showed using RSV-infected airway epithelial cell cultures, that DP1 activation up-regulated IFN-λ production, which in turn increased IFN-stimulated gene expression accelerating viral clearance(287). Therefore, CRTH2 mediated activation could be a potential mechanism to suppress protective type I interferon responses and exacerbate viral replication in ILC2 during dengue virus infection.

We also detected the dengue serotype 2 sequence in the raw RNA sequencing data of ILC2. This was a surprise, but would provide evidence supporting *in vivo* ILC2 infection by dengue virus. At the outset of infection, after their initial delivery of viral RNA to the cytoplasm, positive-strand RNA virus genomes are used as templates for viral protein synthesis. There, the viral RNA acts as an mRNA and serves as a template for synthesizing complementary negative-strand RNA, which then becomes the template for new positive-strand genomic RNAs and subgenomic mRNAs. Dengue virus and other Flaviviruses lack a 3′poly(A) tail to facilitate this process. But, it has been shown that the dengue virus 3′UTR could act in an analogous manner to a 3′ poly(A) tail to increase translation(288). Therefore, it is it evident that 3′ poly-A tail may not be essential for flaviviral RNA to be competitive in a cellular environment with an excess of polyadenylated mRNA. The dengue virus sequence was detectable in 2 patients infected with dengue. We may have missed the infected cells in other dengue patients during sorting as we have used a low number of cells to generate sequencing data. Furthermore, *ex vivo* ILC2 from certain donors had dengue infection staining of ILC2 reaching 70-80%. Although, detection of dengue gene expression within ILC2 was unexpected in the RNA sequencing this was confirmed by *ex vivo* staining in ILC2 for dengue infection. Further, the mechanisms and regulation of ILC2 infection by dengue virus is investigated in subsequent chapters.

Further, it was observed that of ILC2 express granzymes in the context of dengue viral infection. *Ex vivo* RNA sequencing data revealed a higher expression of cytolytic related genes in ILC2 during dengue infection with a prominent expression of granzymes. Recently, it has suggested that ILC2 activation may alter the ILC2 phenotype to a more natural killer cell like ILC, with upregulation of the NKp30 and granzyme B, but without upregulating perforin expression(274). Similarly, it was demonstrated that ILC2

expressed GZMB protein on activation. However, granzyme production was unaffected by dengue viral infection and the cytolytic activity was hypothesised to be related to activation of ILC2 rather than infection. We attempted to investigate whether ILC2 show cytolytic function using a K562-ILC2 co-culture system but we did not observe any cytolytic activity with ILC2 activation. This opened up an interesting avenue to investigate whether these innate cells show antigen independent cytotoxicity to virally infected cells. This area needs further exploration to see whether ILC2 can acquire antigen independent killling properties with activation.

In summary, we showed that ILC2 are activated in dengue fever and show impaired type I interferon response in severe dengue disease. Furthermore, we detected ILC2 infection in patients having dengue infection, suggesting that ILC2 are infected and potentially assist viral dissemination. Additionally, cytotoxicity-related genes and proteins were detected in dengue infected ILC2.

4 Chapter 4: Mechanisms underlying dengue virus infection of ILC2

4.1 Introduction

4.1.1 Infection of immune cells in dengue infection

The mosquito bite is the earliest event that leads to dengue infection. The mosquito penetrates the outer layers of skin and releases viruses with salivary gland products to the vascular and extravascular spaces at the bite site. Probing of the skin leads to inoculation of the virus in to extravascular spaces containing innate immune cells. Interestingly, it has been shown that mosquito salivary gland extracts (SGE) contain mediators that enhance viraemia. Inoculation of SGE from uninfected mosquitos increased viraemia from 24 hours before virus inoculation through to 12 hours after the virus inoculation(289). Potentially, this could enhance viral infection in innate cells in the skin.

In *ex vivo* skin models, dengue virus replicated primarily in the epidermis. Dengue viral replication was first evident in the cells at the base of the epidermis, largely in basal keratinocytes, at 6 hours post-inoculation, and by 48 hours, the epidermis had six times more infected cells than the dermis. At 48 hours, dengue viral replication is detected in Langerhans cells, dermal DCs, macrophages, fibroblasts and mast cells(290). It is well described that type I IFN restricts DENV replication in human skin cells including fibroblasts, DCs, and macrophages(291, 292). However, IFN-α secretion was found to be transient and appeared to peak around 6 hours and reduced to baseline at 12 hours post-infection(290). This could be due to suppression of IFN production or due to the virus circumventing or inhibiting IFN pathway. Furthermore, secretion of IL-1β and CCL20 remarkably increased influx of myeloid cells to the dermis and thereby worsened dengue replication sites in the skin(290).

Dengue can infect a variety of cell types, partly due to the fact that the virus uses a range of receptors for attachment. Although dengue infection of B cells, monocytes and DC is well documented, there is debate as to whether T cells can be infected *in vivo*. Bulk assays suggest that a proportion of $CD2^{+}$ cells can be infected, but single cell data analyses suggest that T cells may be infected (77, 293). Recently, it has been observed that T lymphocytes are permissive for infection by all dengue virus serotypes. CD8 cell populations were more susceptible to infection compared to CD4 purified cell populations. Also, it has been demonstrated that heparan sulfate (HS) moiety participates in DV binding to human T cells(272). It has been shown that activated T cells were resistant to infection after polyclonal stimulation; CD69+ T cells were less infected than CD69- T cells(272). It is unclear whether innate lymphocytes that mirror T helper cell subsets are permissive to dengue infection. Therefore, we aimed to further investigate whether ILC2 are permissive to dengue virus infection and define the underlying mechanisms.

4.1.2 Factors enhancing viral replication in dengue infection

There are many factors that could enhance viral infection in host cells. The most discussed factor leading to this is antibody dependent enhancement (ADE), which is a well-known phenomenon that occurs with secondary dengue infection, as described above. While ADE increases the entry and "burst size" of infected cells, additional factors enhance infected cell mass(294). In *in vitro* experiments to demonstrate ADE, authors used dengue 4G2 monoclonal antibody (MAb), or 3H5 MAb which were serially diluted from 1/10 to 1/163,840 in a volume of 50 µl to demonstrate ADE in monocytes and macrophage(295). In ADE, dengue virus facilitates higher virus production once the concentration of the antibody falls below the neutralization threshold through DENV immune-complexes which would increase virus production per infected cell (295).

However, immediately following dengue infection the body mounts serotype specific IgM and IgG responses and the high concentrations of antibodies provide heterotypic immunity, even for the other 3 dengue serotypes. This may last for several months and then the antibody levels gradually reduce(47, 59). Furthermore, ADE has been demonstrated in cells which have Fc receptors which facilitate internalisation of virus-bound IgG and subsequent productive infection(296). Mechanistically, FcγRIIa and FcγRIIIa have been shown to promote ADE during DENV infection, whereas FcγRIIb acts as a negative regulator for this process(297). After internalisation of the virus-IgG complex into the cell, antibody–virus complexes are sequestered to phagolysosomes, where low pH conditions trigger conformational changes to of the E protein, promoting viral fusion and infection. This may contribute to viraemia and viral dissemination. However, it would be interesting to explore mechanisms other than ADE, where viral replication could be enhanced. As above, severe dengue can occur still with primary dengue infection and secondary dengue infection does not always progress to DHF. Therefore, it would be vital to study other mechanisms that can lead to enhanced viral replication.

Other than ADE, several mechanisms could increase viral replication in the host. More pathogenic DENV strains/serotypes is one other explanation. As an example, DENV serotype 2 is known to evade protective responses and is known to cause higher morbidity and mortality in dengue. Additionally, more pathogenic genotypes may evade host adaptive, cross-reactive immune responses(298). Increased virulence of Asian DENV-2 in human infections has been linked to its enhanced replication *in vitro(47, 298)*. Mutations at certain positions of the viral genome of the virus may affect its virulence. This could either alter replication ability or could reduce neutralisation by the host humoral (antibody-mediated) response.

Additionally, the baseline immune phenotype of individuals has been shown to correlate with severe disease in dengue infection. Individuals who had CD38+CD57+ CD8 T cell and CD38+CXCR5+ B cell phenotypes correlated with higher hospitalisation in dengue. Additionally, higher frequency of CD57+ cytotoxic CD4 T cells was present in patients with warning signs of dengue compared to those without warning signs(299). Patients with such phenotypes may be predisposed to have severe disease correlating with higher viral loads.

Additionally, many cytokines have been shown to affect viral replication in viral infections. As mentioned above, IFN-β is secreted from the cell acts in autocrine and paracrine manners to induce antiviral responses and contribute to early defence(300). Also, type 2 cytokines such as IL-4 and IL-13 are found to increase infectivity in monocytes and macrophages by increasing expression of receptors facilitating dengue virus entry(87). Additionally, in hepatitis B, cytomegalovirus and HIV-1 viral infections TNF-α has shown to reduce viral replication facilitating viral clearance(301). However, in dengue infection, TNF-α stimulation in monocyte-derived macrophages showed an insignificant effect on viral replication(302). TNF-α contributed to vascular leakage and was found to be elevated in dengue infection (40). In an acute respiratory failure model, LTB_4 enhanced TNF-α production. LTB_4 acts synergistically with IL-4 to activate and proliferate B cells(303). As mentioned before, mast cell products including leukotriene such as LTB4 (304), histamine, chymase and tryptase are increased in dengue infection although a direct effect of increasing viral replication, to our knowledge, has not being examined. Therefore, a link may exist between viral replication, lipid mediators and cytokine storm in dengue. Lipid mediators are prime candidates that can provoke hyperimmune responses and have been overlooked in the study of dengue infection in comparison to cytokine storm. The metabolites of lipid mediators are labile and evoke

challenges for detection compared to cytokines which can be easily measured. Fortunately, there are clinically approved safe drugs that can block this pathway. Therefore, further understanding of these molecules is necessary in the context of viral replication and clearance.

4.2 Hypothesis and aims

On the basis of these preliminary findings, the work of this chapter aimed to test the hypothesis that ILC2 could be infected by dengue virus *in vitro*. Specific aims included:

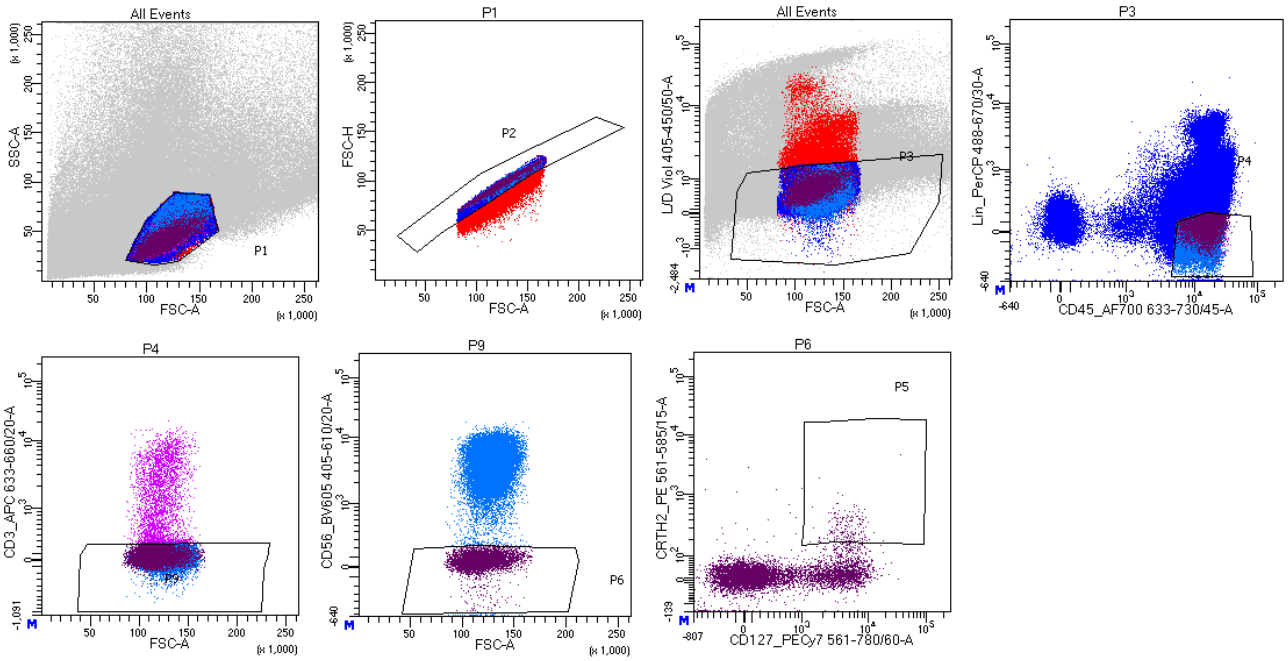
- To ascertain whether ILC2 are infected with dengue virus
- To determine whether ILC2 respond to type I interferons
- To explore PGD_2 metabolite levels in dengue infection
- To ascertain whether Th2 and Tc2 cells are infected with dengue virus

4.3 Results

4.3.1 ILC2 sorting and expansion

It is challenging to study ILC2 *ex vivo* as ILC2 exists in very low numbers in peripheral blood, however this can be overcome by *in vitro* expansion of ILC2. To determine whether ILC2 can be infected *in vitro*, ILC2 were sorted and expanded as described in the methods section. Human ILC2 were identified by lack of common lineage markers and high expression of CD45, CD127 (IL-7Rα) and CRTH2 (Figure 18a). Expansion of ILC2 to significant numbers takes up to 6-8 weeks. Subsequently, they were checked for purity and >98% pure lines were used in the current experiments (Figure 18b).

A)



B)

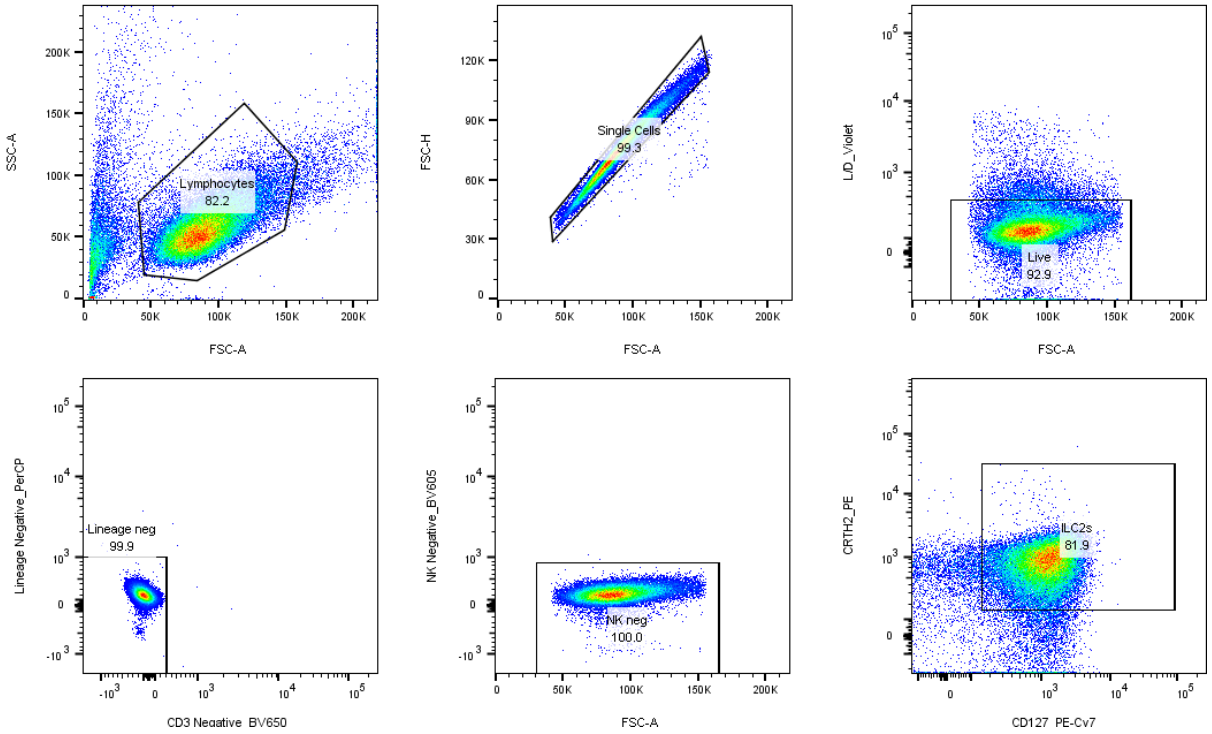


Figure 18: Representative figures of the gating strategy for identification of human ILC2. (**A**) Lineage (CD3, CD19, CD123, CD11b, CD11c, CD8, CD4, FcεRI, CD14, CD16, CD56) negative, CD45+, CRTH2+, IL-7Rα+ ILC2 were sorted from healthy peripheral blood mononuclear cells. (**B**) The purity of isolated ILC2 was confirmed by flow cytometry following *in vitro* expansion for six weeks.

4.3.2 Infection of ILC2 and optimisation of protocol

Given the apparent activation of ILC2 isolated from dengue patients and the *ex vivo* RNAseq data, it was aimed to determine whether the ILC2 could be directly infected and activated by the dengue virus and/or whether the exogenous activation facilitates dengue infection.

First, it was sought to infect ILC2 with dengue virus to observe whether they are permissive to dengue virus infection *in vitro*. ILC2 were infected using different Multiplicity of Infections (MOIs) to determine the optimum MOI to perform

experiments. ILC2 from healthy donors were incubated in virus-containing supernatant for 2 hours to allow virus adsorption. Subsequently, the unbound virus was washed off and the cells were resuspended in ILC2 media containing IL-2. The cells were incubated for 24-48 hours and intracellular dengue Envelope (E) protein was detected using Flow Cytometry. When MOI was increased from 0.1 to 5 the ILC2 infection rate gradually increased in a dose dependent manner (Figure 19a,b). With a MOI of 5 the highest infection rate was observed in unstimulated ILC2 at around 15-25% of total ILC2. Then, the effect of ILC2 activation on infection and cell death was examined. Highest infection of ILC2 was at 24 hours of activation. Activation for 48 hours did not increase ILC2 infection compared to 24-hour activation. With MOI of 5 highest infection was observed and the infection rate did not significantly increase when MOI of 10 was used (Figure 19c). ILC2 cell death usually increased with activation and further increased with MOI of DENV infection (Figure 19d). In fact, greater ILC2 cell death was observed after 48 hours of incubation. ILC2 cell death was highest when MOI 10 was used, and when 48 hours of activation was used concurrently with dengue infection the death rates reached 50% or more (Figure 19d). In contrast, cell death was approximately 20-25% at 24 hours of activation and dengue virus infection. In the optimisation experiments performed, ILC2 that had been cultured with IL-33 and PGD_2 for 24 hours with a MOI of 5 were most susceptible to infection (Figure 19b,c). It was observed that the infection rate was best detected at 48 hours post infection when staining for intracellular envelope protein by flow cytometry. Post- infection 24 hours time point had lesser infection detected than the post-infection 48 hours time point (Figure 19e). Comparatively, post-infection 0hr time point had very low infection rates suggesting that viral replication progress within ILC2(Figure 19e). Foci forming assay was used to examine production of infectious particles and indeed viral titres in the supernatant of infected ILC2 collected 24, 48,72

hours after infection similarly showed that ILC2 secreted significantly more infectious viral particles at 48-hour time point than 24 hour or 72 hour time point (Figure 19f). Therefore, 48-hour post-infection time point was considered for future experiments to perform flow cytometry to assess dengue viral infection.

Analysis of the RNA sequencing data revealed significantly higher gene expression of *FCGR3A* in ILC2 derived from individuals with DF and DHF compared to healthy individuals (Figure 19g). Therefore, it was hypothesised that dengue 4G2 antibodies could enhance viral replication in ILC2 through Fc receptor mediated antibody dependent enhancement. It has been demonstrated by others that mast cells contribute to ADE; using convalescent dengue sera in 1:000 and 1:10,000 dilutions and blocking FCRγII; it was shown that blocking Fc receptor can reduce ADE and also CCL5 production(113).

To test this in our proposed pathway, ILC2 were infected with DEN2 virus (MOI 5) in the presence or absence of 4G2 antibody (1µg/ml). Virus and antibody were pre-incubated for 2 hours prior to infection of ILC2. No intracellular dengue envelope protein staining was detected when cells were exposed to heat-inactivated DENV2 (Figure 19h). The presence of 4G2 antibody significantly reduced detection of intracellular envelope protein, which could indicate that the infection was inhibited in the presence of dengue antibodies (Figure 19h). Despite using a concentration of 4G2 antibody that has been reported to enhance infection in other cell types(113), an enhancement of dengue infection in ILC2 was not observed. Given the increase in ILC2 infection in the setting of broad activation, the specific mediators were next investigated.

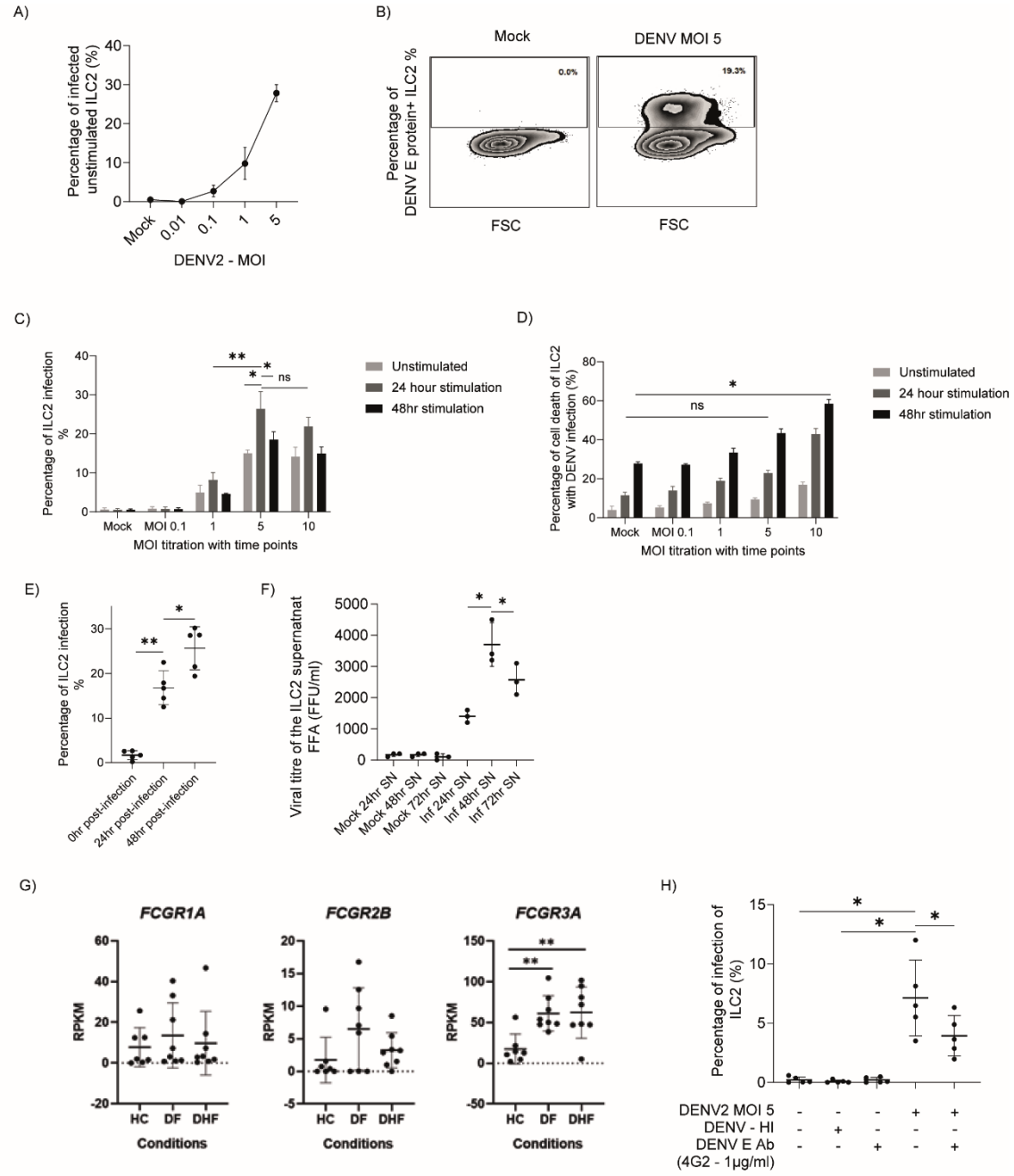


Figure 19: Optimisation of multiplicity of Infection in ILC2 (n=4). (**A**) Unstimulated ILC2 were exposed to live DENV2 virus at serial MOI from 0, 0.01, 0.1, 1 and 5 for 2 hours. Then, unbound virus was removed and the cells were plated in fresh ILC2 media containing IL-2 for 48 hours. Intracellular DENV E protein was analysed by flow cytometry. (n=5, data representative of 2 independent experiments). (**B**) Representative flow cytometry panel showing DENV E protein staining in mock and DENV infected (MOI 5) ILC2. Then, ILC2 were activated with IL-33 (25ng/ml) & PGD_2 (100nM) for

24 hours or 48 hours and dengue virus was inoculated for 2 hours. The excess virus was washed and cells were incubated for 48 hours in ILC2 media containing IL-2. (**C**) Infectivity was checked by staining of intracellular Flavivirus envelope (NBP2-52709AF488) protein by flow cytometry. (n=5, data representative of 2 independent experiments). (**D**) Cell death was checked by live dead stain violet. (**E**) Dengue infection was assessed at 0 hour, 24 hours and 48 hours after infection through staining for intracellular Flavivirus envelope protein. (n=5, data representative of 2 independent experiments). (**F**) Foci forming assay (FFA) was performed on the supernatants (SN) of mock and infected (Inf) ILC2 collected at 24, 48, 72 hours post-infection. Each focus represents an infected Vero cell. The number of foci is calculated in each condition. (n=3, data representative of 2 independent experiments). (**G**) Gene expression of Fc receptors, *FCGR1A, FCGR2B, FCGR3A* of human blood derived ILC2 of HC, DF and DHF, determined by RNA Sequencing and represented in RPKM values. Statistical analysis performed using p-value from the DEG result. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**H**) ILC2 were exposed to live and heat inactivated DENV2 virus at a MOI of 5 for 2 hours. In the blocking condition, dengue envelope protein (clone – 4G2 - 1µg/ml) was mixed with dengue virus (MOI 5) and incubated for 1 hour and was used to infect ILC2. Then, unbound virus was removed and the cells were plated in fresh ILC2 media containing IL-2 for 48 hours. Thereafter, intracellular DENV E protein (Clone – 4G2) was checked by flow cytometry. (n=4, one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments).

4.3.3 Impact of differential ILC2 activation of infection of ILC2 with dengue virus

Immune responses to dengue virus initiate from mosquito bite to the skin. It is likely that the mosquito bite and subsequent scratching can produce a localised inflammation leading to a form of skin injury. There have been observations to indicate that skin

inflammation induces an altered phenotype of ILC2 making these cells more responsive to cytokines in the environment(305). IL-33 is a pro-inflammatory cytokine that can be produced by keratinocytes(306). IL-33 is constitutively expressed in the nucleus of endothelial cells from both large and small blood vessels in most tissues. Abundant nuclear expression of IL-33 has been detected in keratinocytes of the skin, where pathogens, allergens and other environmental agents are frequently encountered. IL-33 has been also detected in the microvasculature of many tissues, including liver, skeletal muscle, kidney (peritubular capillaries) and prostate.

It has been shown that DENV2 infected mice treated with recombinant IL-33 developed markedly more severe disease compared with untreated mice as assessed by mortality, granulocytosis, liver damage and pro-inflammatory cytokine production(307). Additionally, IL-33 plays a disease-exacerbating role during experimental dengue infection in immunocompetent mice(307). Stimulation of ILC2 isolated from healthy human skin with IL-33, induced production of IL-5, IL-6 and IL-13 and this cytokine production was further increased by addition of IL-25 and TSLP(164). It has been shown that ILC2 enrichment occurs in lesional AD skin compared to healthy skin. ILC2 isolated from both healthy and AD lesional skin expressed CRTH2 and CD161(308).

However, it is not yet clear how IL-33 may be released from the nucleus to exert its cytokine activities towards target cells expressing the ST2 receptor(309). Dengue infected patients had higher serum sST2 levels than other febrile illnesses at the end of the febrile stage and at defervescence. Patients with secondary dengue infections had higher serum sST2 levels compared with patients with primary dengue infections(310). Higher levels of soluble ST2 (sST2) were associated with severe cases of pediatric dengue and is positively correlated with IL-6 and IL-8 levels. However, IL-33 levels remained unchanged between infected and uninfected individuals(311). Unlike,

membrane bound ST2, sST2 binds to IL-33 and antagonises signaling. Apparent activation of ILC2 in dengue could indicate that IL-33 acts on ILC2; and sST2 may have increased as a protective mechanism to prevent signaling. Alternatively, ILC2 may be activated from other activating mediators such as PGD_2 or LTE_4.

PGD_2 and LTE_4 are lipid mediators predominantly released by mast cells and activate ILC2 promoting type 2 cytokine production(127, 137). As above, PGD_2 elicits many pro-inflammatory responses in leukocytes including chemotaxis of eosinophils, basophils, and Th2 cells (128) and cytokine production by Th2 and ILC2 (131). Additionally, these responses can be amplified through cysteinyl leukotrienes(137). Recently, it has been shown that tape stripping in skin, lead to intestinal mast cell expansion, which is associated with increasing vascular endothelial permeability. Further, intestinal mast cell expansion is dependent on IL-4, which is produced by ILC2. This suggests that superficial skin injury could lead to mast cell activation through ILC2 effector function(312). Thus, taken together it is possible that skin inflammation occurring from a mosquito bite or viral infection may poise ILC2 to produce type 2 cytokines.

Since activation of ILC2 could conceivably occur in the type-2 inducing environment considered a risk factor of severe dengue, it was investigated whether ILC2 activation state could affect the ILC2 infection rate. With virus inoculation at a MOI of 5, 15-20% infectivity was observed in 'unstimulated' ILC2. Here, ILC2 are cultured with only IL-2, which is the cytokine that is used to maintain and expand ILC2. However, when the cells were activated with IL-33 (50ng/ml) or PGD_2 (200nM) or LTE_4 (100nM) the infection rate increased up to nearly 30-60% (Figure 20a). Also, IL-33 and PGD_2 significantly increased infectivity of ILC2 and acted together to further increase infectivity of ILC2. Additionally, the effect of other cytokines such as IL-12, IL-18, TNF-α, IL-1β and IL-4 on ILC2 infection was assessed. These cytokines have shown to be

elevated in patients with dengue infection. However, these did not show any enhancement of ILC2 infection (Figure 20a). Additionally, qualitative NS1 protein ELISA revealed that supernatants of infected ILC2 were 'positive' for NS1 protein (Figure 20b) which suggest that infected ILC2 secrete NS1 protein. Supernatants of DENV infected unstimulated and ILC2 activated with IL-33, PGD_2 and LTE_4 were positive for NS1 protein (Figure 20b). Dose response curves were performed (data not shown) for all cytokines. For IL-33, PGD_2, LTE_4, IL-12 and IL-18, optimum concentrations that mediate highest infection rates were shown. Titration of TNF-α, IL-1β and IL-4 were performed, but due to negative response highest concentration used was depicted in the figure.

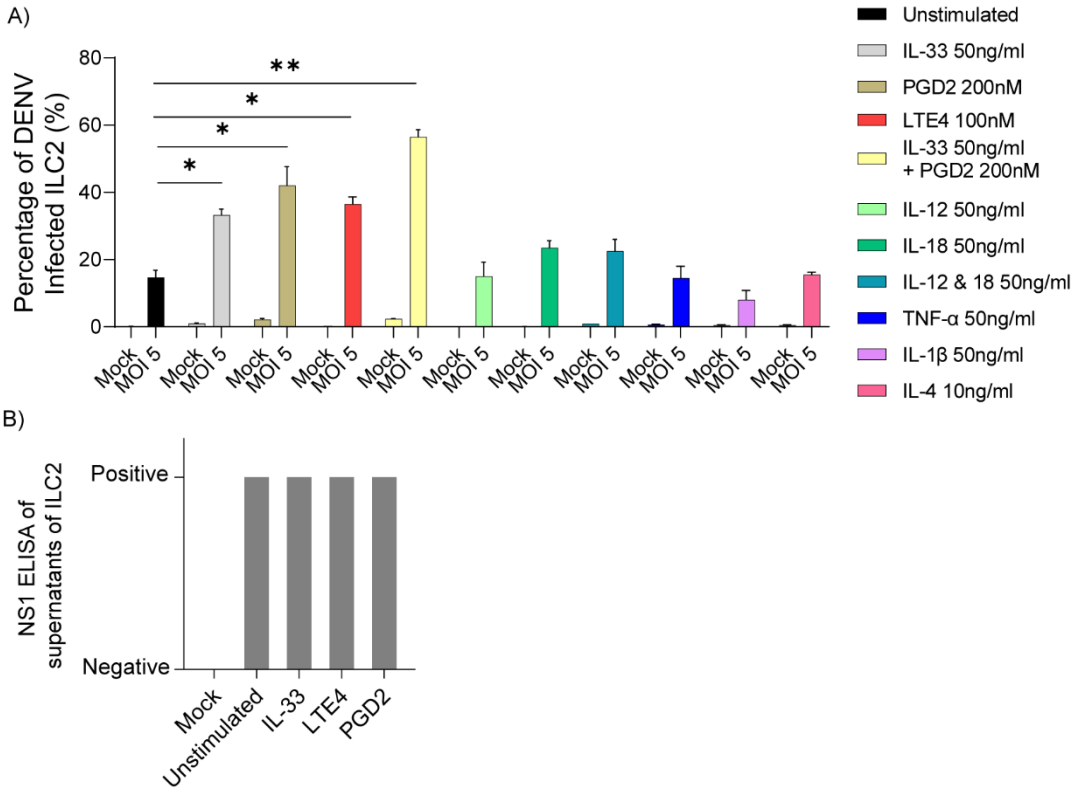


Figure 20: Dengue virus infection in activated ILC2 with different cytokine stimulation conditions. (**A**) ILC2 stimulated with IL-33 (50ng/ml) and/or PGD_2 (200nM), LTE_4 (100nM), IL-12 and/or IL-18 50ng/ml, TNF-α (10ng/ml), IL-1β (10ng/ml), IL-4 (50ng/ml) for 24 hours and virus inoculation was performed, and

infectivity was checked after 48 hours of virus inoculation. Intracellular dengue envelope protein was tested after 48 hours of virus inoculation. *<0.05, **P<0.01, ***P<0.001. Two-way ANOVA followed by Turkey multiple comparisons was performed to determine statistical significance. Mean ± SD. Graphs which were not indicated with a stat bar showed no significant differences. (n=5, data from 3 experiments). (**B**) NS1 ELISA was performed on supernatants of mock and infected ILC2 which were unstimulated or stimulated with IL-33 (50ng/ml), PGD_2 (200nM) and LTE_4 (100nM). (n=6, data from 3 independent experiments).

The predominant effects were mediated through IL-33, PGD_2 and LTE_4 and so these were further investigated. As above, alarmins such as IL-33 and lipid mediators such as PGD_2 and LTE_4 activate ILC2 (127, 137, 164). IL-33, PGD_2 and LTE_4 stimulation increased viral infection of ILC2 in a concentration dependent manner (Figure 21a). PGD_2 significantly increased infectivity of ILC2 and acted together with IL-33 to increase infectivity of ILC2 compared to dengue virus infection in unstimulated ILC2 (Figure 21b). ILC2 activation status may therefore influence dengue viral infection, as stimulation via PGD_2, LTE_4 and IL-33 were observed to increase infectivity.

Next, it was investigated whether ILC2 could support replication-competent dengue virus production. Supernatants from mock and dengue infected ILC2 were collected and Vero cell-based foci-forming assay (FFA) performed to quantify virus titre in the ILC2 cell supernatant. ILC2 underwent productive infection and released infectious viral particles into the supernatant (Figure 21c). Interestingly, activation of ILC2, particularly through PGD_2, induced secretion of significantly greater infectious viral particles compared to unstimulated ILC2 supernatants (Figure 21c). IL-33 mediated activation did not lead to a significantly higher infection and secretion of infectious viral particles although the trend was higher. Combined IL-33 and PGD_2 mediated stimulation led to a further increase in

dengue viral infection and productive infection in ILC2 (Figure 21b,c). Similar significant increase in productive dengue viral infection was noted with infection of LTE_4 activated ILC2. This suggests that activated ILC2 may contribute to increased viral replication.

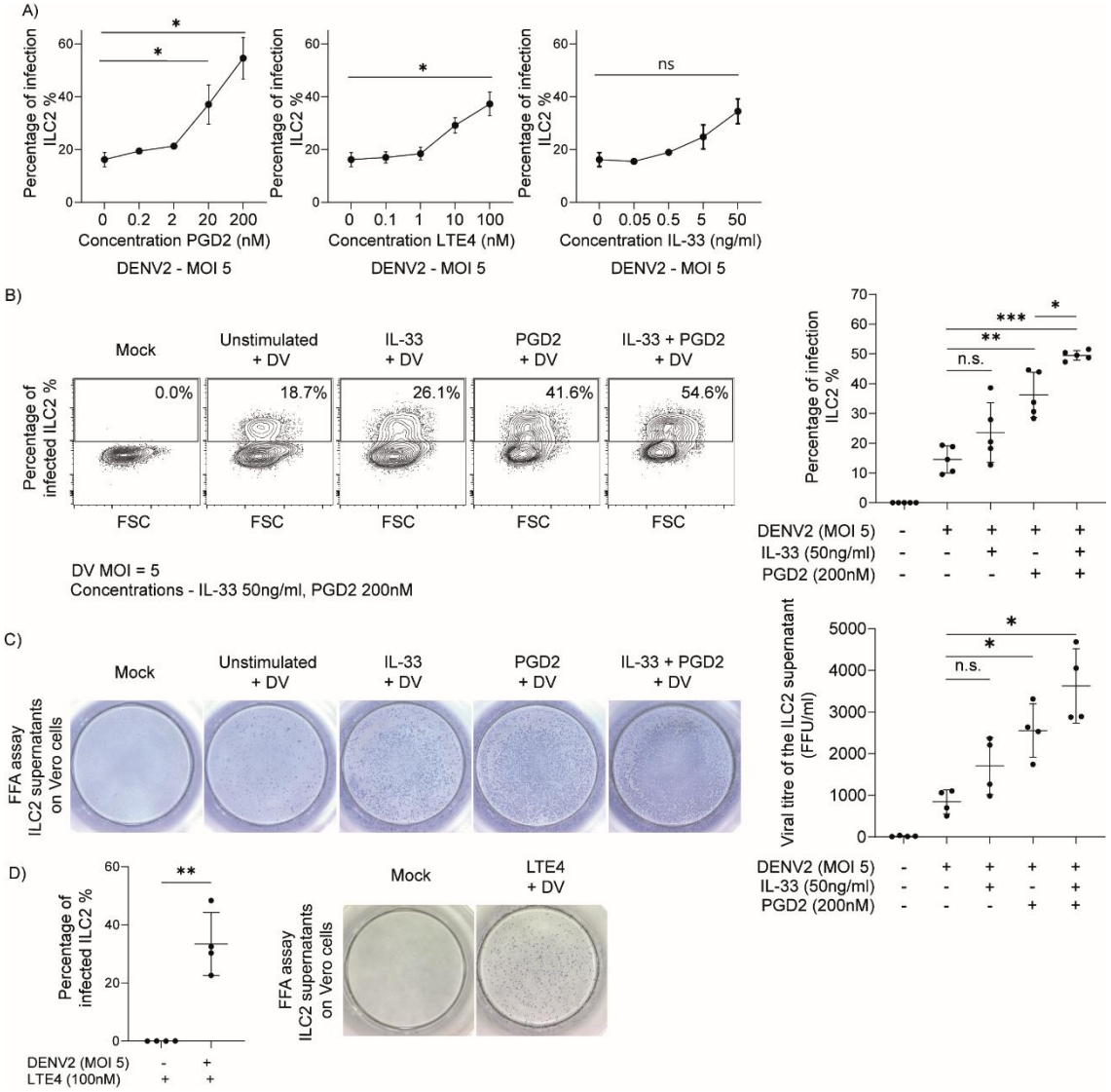


Figure 21: Activated ILC2 are more permissive to infection and secrete infectious viral particles. (**A**) ILC2 were activated through serial concentrations of IL-33 (0-50ng/ml), PGD_2 (0-200nM) and LTE4 (0-100nM) for 24 hours. Then cells were incubated with dengue virus (MOI 5) for 2 hours. Unbound virus was removed and the cells were plated in fresh ILC2 media containing IL-2 for 48 hours. Thereafter,

intracellular DENV E protein was analyzed by flow cytometry. (**B**) Activation of ILC2 with fixed concentrations of PGD_2 (200nM), IL-33 (50ng/ml) was performed for 24 hours, followed by infection with dengue virus and then incubated for 48 hours. Intracellular DENV E protein was analysed by flow cytometry (y-axis). (n=5, Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments. *P <0.05, ** <0.01, *** <0.001, n.s. not significant.) (**C**) Supernatants of infected ILC2 of unstimulated and activated conditions (activated with IL-33 (50ng/ml) and/or PGD_2 (200nM)) infected ILC2 (with MOI 5) were added on Vero cells and a Foci forming assay (FFA) was performed. Each focus is representative of an infected Vero cell. (n=4, Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments. *P <0.05, ** <0.01, *** <0.001, n.s. not significant). (**D**) With LTE_4 (100nM) 24 hour ILC2 activation and infection was performed and FFA was performed on the ILC2 supernatant collected 48 hours post-infection. All above data from 3 independent experiments (n=4-5).

Further, it was aimed to determine whether skin and blood ILC2 are differentially infected. ILC2 from healthy human skin and blood samples were isolated (Methods) and expanded over 6-8 weeks and exposed to the same infection protocol with dengue virus at a MOI of 5. Both unstimulated and activated skin ILC2 were permissive to dengue virus infection. A significant difference of infectivity was not observed between skin and blood ILC2 (Figure 22a). This could be due to the fact that the differences in blood and skin ILC2 may have been diminished after the prolonged period of culture, which is required to expand ILC2 to sufficient numbers to perform functional analyses.

Additionally, it was investigated whether ILC2 secrete type 2 cytokines with dengue infection. However, there was no difference in type 2 cytokine levels of IL-13, IL-5, GM-

CSF between mock and infected ILC2 (Figure 22b-d). Additionally, no difference was noted in the proliferation of ILC2 with or without infection (Figure 22e).

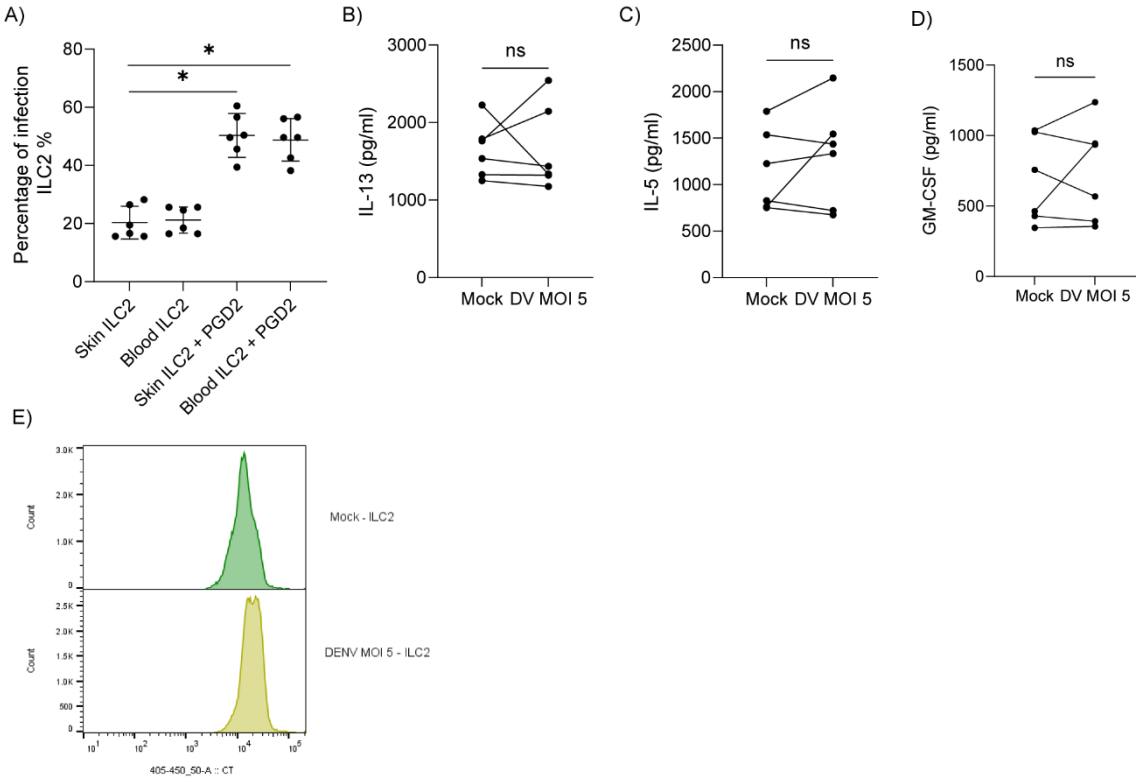


Figure 22: Skin and blood ILC2 infection and type 2 cytokine production. (**A**) ILC2 from skin and blood were sorted and expanded over 6-8 weeks to perform functional analyses. Unstimulated skin and blood ILC2 and activated ILC2 (with PGD_2 200nM for 24 hours prior to infection) were infected with DENV MOI 5 and infectivity was examined after 48 hours Data from 3 independent experiments (n=6).. Mock and infected ILC2 (DENV MOI 5) were incubated for 48 hours and supernatant collected and ELISA was performed on (**B**) IL-13, (**C**) IL-5 and (**D**) GM-CSF. All above data from 2 independent experiments (n=5). (**E**) Mock and infected ILC2 (DENV MOI 5) were stained with CellTrace^{TM} violet and incubated for 96 hours and proliferation was assessed

with flow cytometry (Representative figure out of 4 donors, from 2 independent experiments).

4.3.4 Dengue virus infection of ILC2 with type I interferon stimulation

Next, control of ILC2 infection in the context of dengue viral infection was investigated. Exogenous IFN-β (25-250U/ml) reduced DENV infection significantly in a concentration dependent manner (Figure 23a). Additionally, exogenous IFN-β stimulation led to release of reduced infectious viral particles than unstimulated infected ILC2 (Figure 23b-c). This signifies that exogenous IFN-β reduced productive dengue viral infection in ILC2. Additionally, inhibiting type I interferon receptor did not have any effect on infectivity of ILC2 (Figure 23d) and IFN-β levels in ILC2 supernatant after dengue viral infection or with activation through PGD_2 was undetectable (Figure 23e). This signifies that ILC2 do not intrinsically produce IFN-β with activation or infection but readily respond to exogenous IFN-β.

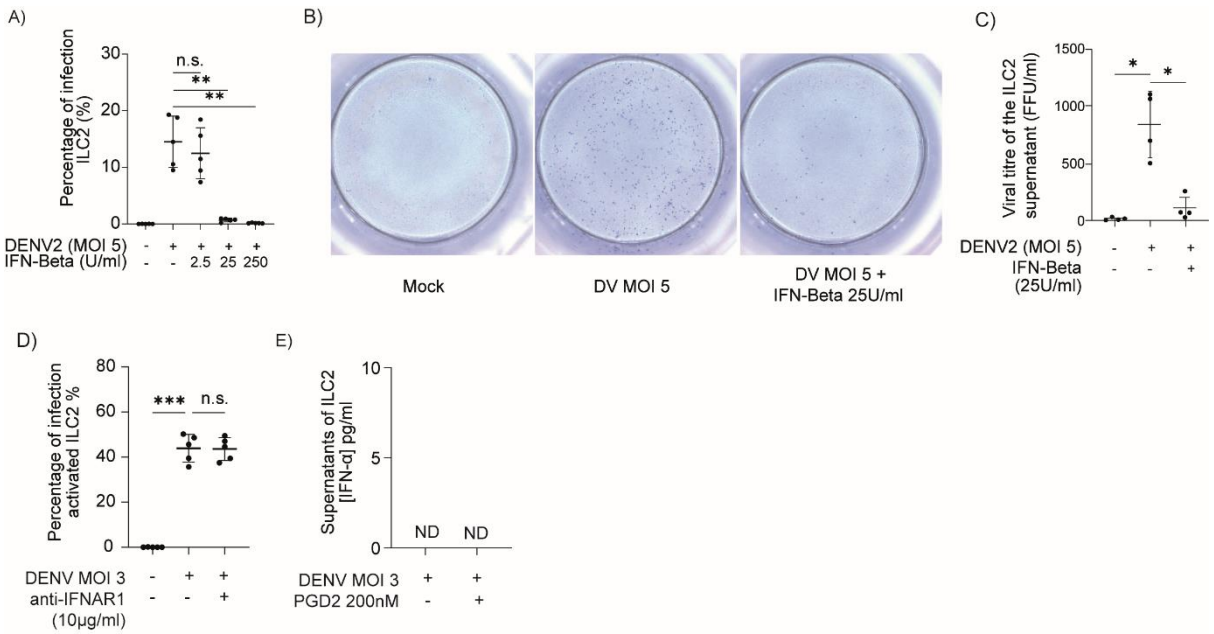


Figure 23: Exogenous IFN-β controls dengue viral infection in ILC2. (**A**) ILC2 were stimulated with IFN-β (2.5-250U/ml), infected with dengue virus (MOI 5) and then

incubated for 48 hours. Thereafter, intracellular DENV E protein (Clone – 4G2) was analyzed by flow cytometry. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments (n=5). *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**B**) Unstimulated and 24-hour IFN-β (250U/ml) treated ILC2 were infected with DENV (with MOI 5) and the supernatants were harvested after 48 hours were added on Vero cells and Foci forming assay was performed. Each focus is representative of an infected Vero cell. Representative figure out of 5 donors. (**C**) Viral titres of ILC2 supernatants in unstimulated and IFN-β treated ILC2 were represented in multiple donors. (n=4, data representative of 2 independent experiments). Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test. (n=4). *P <0.05, ** <0.01, ***<0.001, n.s. not significant. (**D**) Activated ILC2 (activated with PGD_2 for 24 hours) were infected MOI 3 and incubated in ILC2 media containing IL-2 for 48 hours. In blocking conditions anti-IFNAR1 (10µg/ml) added after wash step and incubated for 48 hours. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 2 independent experiments (n=5). *P <0.05, ** <0.01, ***<0.001, n.s. not significant. (**E**) ELISA for IFN-α was performed on supernatants of infected unstimulated and infected activated ILC2 (n=5, from 2 independent experiments).

4.3.5 Dengue viral infection of ILC2 in the presence of CRTH2 antagonism

Subsequently, it was sought to determine whether infection control could be achieved through inhibiting PGD_2 mediated activation. This was accomplished with the use of CRTH2 antagonist, TM30089. Inhibition of CRTH2-mediated activation of ILC2 reduced dengue infection (Figure 24). TM30089 significantly reduced dengue virus infection in PGD_2 and IL-33 treated ILC2. The latter implicated the inhibition of action

of endogenously produced PGD_2 from ILC2 (Figure 24a), and a functional relevance of endogenously produced PGD_2 in ILC2 in the setting of dengue viral infection. With unstimulated ILC2 the reduction of infection through CRTH2 antagonism is not significant, likely due to the fact that unstimulated ILC2 do not produce adequate levels of PGD_2. Given that the lipid mediator LTE_4 has been reported to be increased in individuals with severe dengue(123), and as above, associates with elevated ILC2 dengue infection, it was investigated whether inhibition of LTE_4-mediated ILC2 activation had a similar effect. Montelukast, a leukotriene receptor antagonist, which demonstrates selectivity to the cysteinyl leukotriene receptor type-1, inhibited exogenous LTE_4-mediated dengue virus infection of ILC2. However, it did not appear that ILC2 produced LTE_4 endogenously to promote ILC2 activation and dengue viral infection (Figure 24b). Inhibition of the autocrine action of type 2 cytokines IL-4 and IL-13 through blockade of shared receptor IL4Rα, did not affect the level of dengue viral infection in ILC2 (Figure 21c). These data further suggest that inhibition of exogenous PGD_2 or LTE_4 has a significant effect in reducing dengue infection in ILC2 and endogenously produced PGD_2 plays a role in dengue infection of activated ILC2.

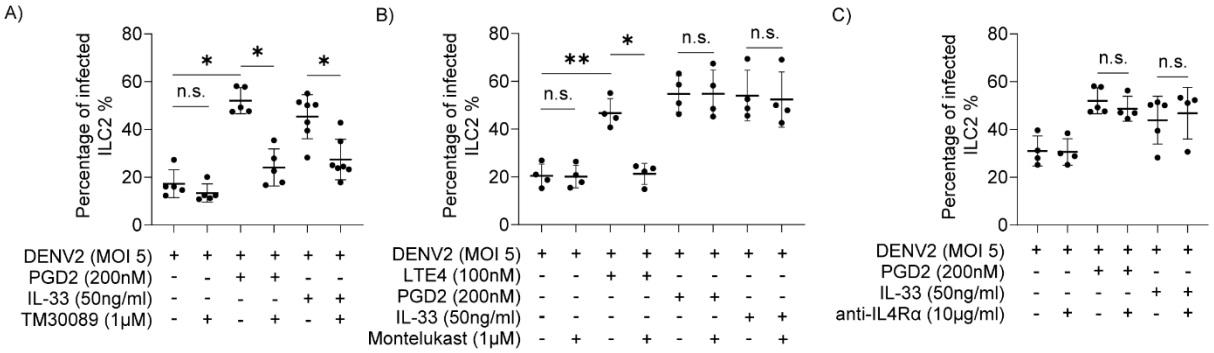


Figure 24: CRTH2 inhibition reduces dengue infection in ILC2. (**A**) ILC2 were treated with CRTH2 antagonist (TM30089 – 1µM) for 1 hour before treatment with PGD_2 (200nM) or IL-33 (50ng/ml) for 24 hours. ILC2 were infected with DENV2 infection

(MOI 5), and infection was assessed. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments (n=5). *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**B**) ILC2 were treated with Montelukast (1µM) for 1 hour before treatment with PGD_2 (200nM), IL-33 (50ng/ml), LTE_4 (100nM) for 24 hours. ILC2 were infected with DENV2 infection (MOI 5), and infection was assessed. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments (n=5). *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**C**) ILC2 were treated with IL4Rα antagonist (10µg/ml) for 1 hour before treatment with PGD_2 (200nM) or IL-33 (50ng/ml) for 24 hours. ILC2 were infected with DENV2 infection (MOI 5), and infection was assessed. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test. (n=5, data representative of 2 independent experiments). *P <0.05, **<0.01, *** <0.001, n.s. not significant.

4.3.6 Sorting and expanding Th2 and Tc2 cells

For a comparison of infection of type 2 innate cells in dengue infection with the counterparts of the acquired immune system, we expanded Th2 and Tc2 cells. CRTH2 is a reliable marker to identify Th2 and Tc2 cells(126, 313). Flow cytometric cell sorting and expansion over 4-6 weeks was used to enrich Th2 and Tc2 populations from different donors; Th2 cells were enriched as CD4+ CRTH2+ cells and the Tc2 cells were enriched as CD8+ CRTH2+ cells (Figure 26a). Before use, the expanded Th2/Tc2 were checked for purity (Figure 26b). The lines which were >95% pure were used for experiments. For cell culture, expansion and *in vitro* experiments ILC2 media supplemented with IL-2 was used.

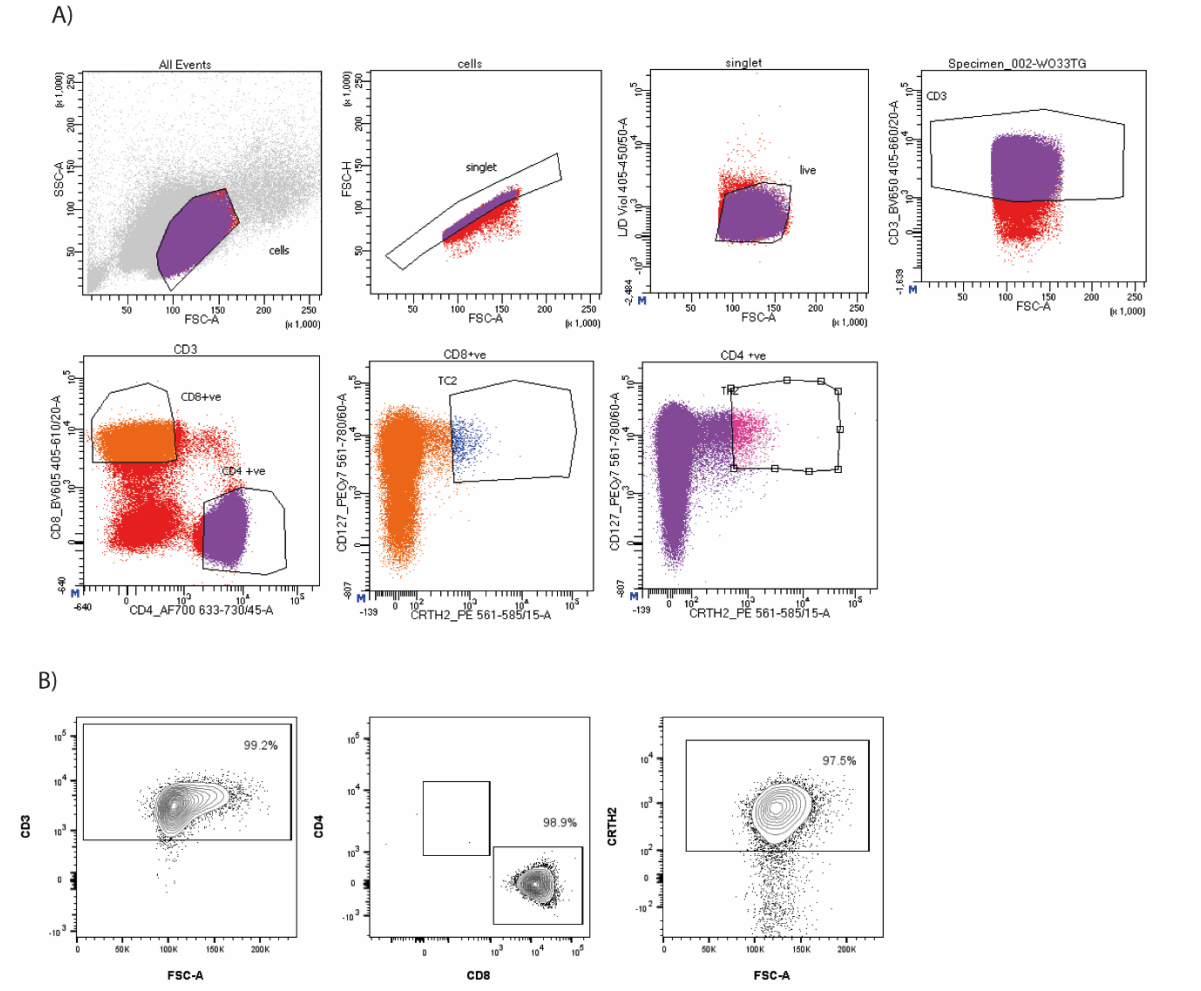


Figure 26: Th2 and Tc2 cell sorting and checking for purity. (**A**) CD3+ CD4+ CRTH2+ Th2 and CD3+ CD8+ CRTH2+ Tc2 cells were sorted from healthy peripheral blood mononuclear cells. (**B**) The purity of Tc2 cells (CD3+ CD8+ CRTH2+) was confirmed by flow cytometry following *in vitro* expansion for 4-6 weeks.

4.3.7 Dengue viral infection in Th2 and Tc2 and the effect of CRTH2 antagonism

Further, it was aimed to investigate whether type 2 immune cells of the acquired immune system respond to PGD_2 and the impact on dengue viral infection. Previous *in vitro* studies have demonstrated the inhibitory effect of fevipiprant on CRTH2 mediated reactivity in Th2 and ILC2 cells(131, 314). PGD_2 activated Tc2 cells to produce type 2 cytokines and PGD_2 in an autocrine manner and showed additional effects such as cell migration and suppression of apoptosis(126).

Infection of polyclonal T cells (pan-T cells), Th2 and Tc2 cells was examined under unstimulated and activating conditions. Pan-T cells were minimally permissive for dengue infection *in vitro,* and the infection rate was <2% (Figure 27a). Activated T cells (with CD3/28 beads 1:1 ratio) had nearly 5% dengue viral infection and remained low (Figure 27a). Comparatively, Th2 and Tc2 cells had significantly higher infection rates at an unstimulated state, and the infection significantly increased upon activation with PGD_2 (Figure 27b-c). In PGD_2 activated Th2 and Tc2, inhibition of CRTH2 receptor significantly reduced dengue infection (Figure 27b-c). Supernatants from activated infected Th2 and Tc2 secreted significantly higher infectious viral particles than unstimulated counterparts (Figure 27d).

Compared to the pan-T cells, Th2, Tc2 and ILC2 had higher levels of dengue infection. ILC2 had significantly higher infection than Th2 and Tc2 (Figure 27e). Additionally, unlike CD3+ pan-T cells, Th2 and Tc2 underwent productive infection and secreted significantly higher infectious viral particles with PGD_2 mediated activation (Figure 27d). There was no significant difference of dengue infection and viral titre of the supernatants between Th2 and Tc2, although the trend was higher in Tc2 cells. Importantly, ILC2 showed higher productive infection compared to Th2 or Tc2 or pan-T cells (Figure 27e,f).

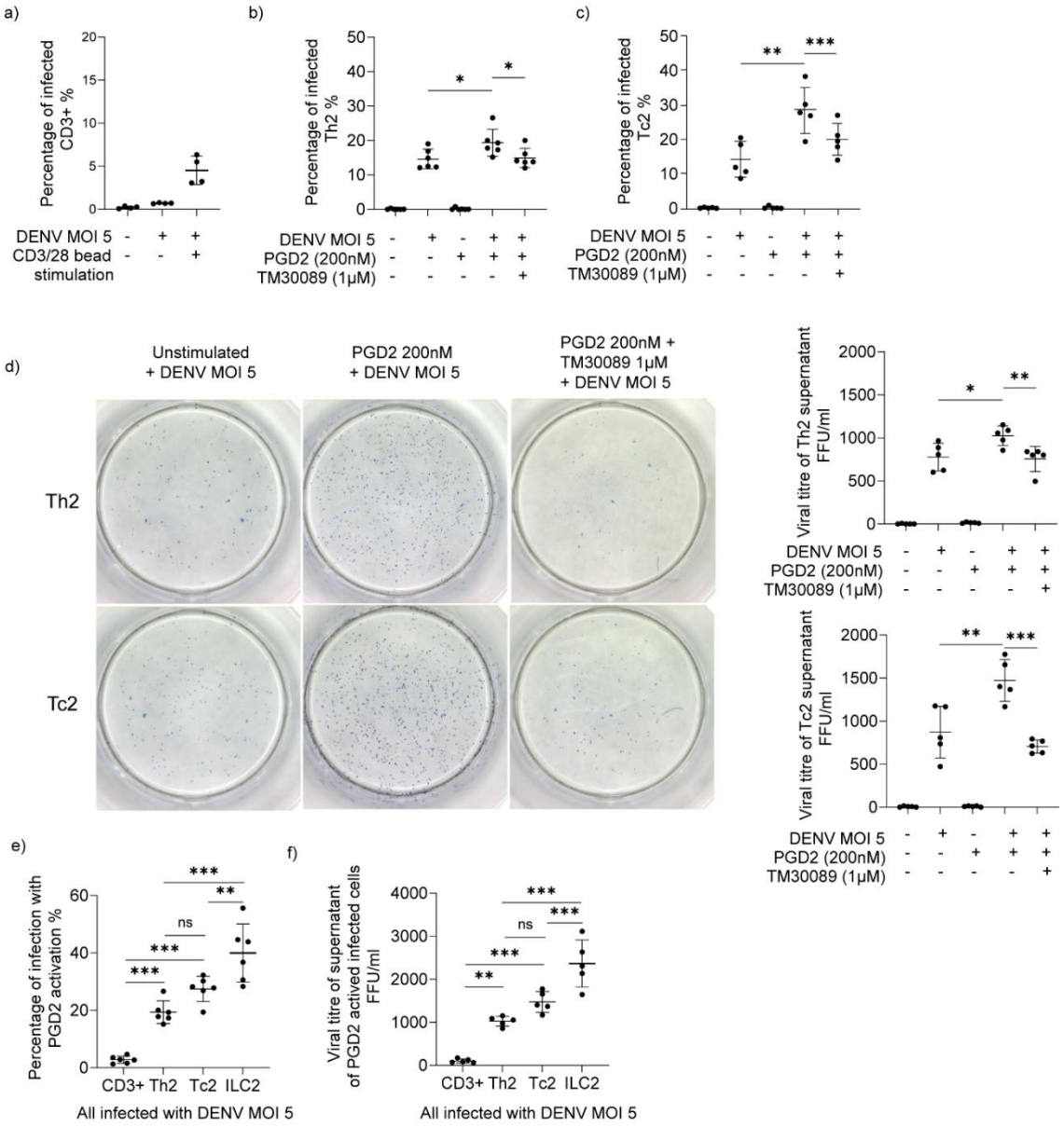


Figure 27: Dengue infection of pan-T cell, Th2 and Tc2. (**A**) CD3+ MACS separated polyclonal T cells (pan-T cells) from healthy individuals were infected with DENV2 infection (MOI 5), and infection was assessed after 48 hours of incubation in T cell media with IL-2. For CD3/28 bead stimulation 1:1 bead to cell ratio was used and activated for 24 hours prior to infection. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments (n=4). (**B**) Th2 cells (CD4+ CRTH2+) from healthy individuals were sorted and expanded over 4 weeks. Unstimulated and PGD2 (200nM) activated (for 24 hours) Th2

were infected with DENV2 infection (MOI 5), and infection was assessed. In the blocking condition, Th2 were treated with CRTH2 antagonist (TM30089 – 1µM) for 1 hour before treatment with PGD2 (200nM). Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments (n=6). P *<0.05. (**C**) Tc2 cells (CD8+ CRTH2+) from healthy individuals were sorted and expanded over 4 weeks. Unstimulated and PGD2 (200nM) activated (for 24 hours) Tc2 were infected with DENV2 infection (MOI 5), and infection was assessed. In the blocking condition, Tc2 were treated with CRTH2 antagonist (TM30089 – 1µM) for 1 hour before treatment with PGD2 (200nM). Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 3 independent experiments (n=5). P ** <0.01, *** <0.001. (**D**) 48 hours after infection, supernatants from mock and infected (PGD2 treated and PGD2 + TM30089 treated) Th2 and Tc2 cells were collected and added on Vero cells and then FFA assay was performed. Each focus is representative of an infected Vero cell. (n=5). P *<0.05, ** <0.01, *** <0.001. (**E**) CD3+ pan-T, Th2 and Tc2 from same healthy individual was incubated with PGD2 (200nM) for 24 hours and were infected with DENV2 infection (MOI 5), and infection was assessed after 48 hours. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 2 independent experiments (n=6). P ** <0.01, *** <0.001. (**F**) 48 hours after infection supernatants from PGD2 treated infected CD3+ pan-T, Th2 and Tc2 (DENV – MOI 5) were collected and added on Vero cells and then FFA assay was performed. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 2 independent experiments (n=5). P ** <0.01, *** <0.001, n.s. not significant. All error bars represent mean+SD.

4.3.8 PGD_2 metabolites in dengue fever

From the above data, PGD_2 plays a role in exacerbating ILC2 infection and dengue viral replication. To understand the PGD_2 and its metabolite levels in humans, concentrations of PGD_2 metabolites in the urine of patients with DF and DHF over the course of illness was measured. Inflammatory lipid mediators such as platelet activating factor (PAF) and secretory phospholipase A_2 levels relevant to the arachidonic acid pathway and urinary leukotriene levels are found to be higher in patients with DHF(109, 123, 146). PGD_2 is a product of arachidonic acid metabolism, dependent on cyclooxygenase and haematopoietic PGD-synthase. Whilst evidence suggests PGD_2 production occurs in platelets, macrophages, T helper cells and DCs, levels are 100–1000 times lower than that synthesised by activated MCs indicating that PGD_2 production largely reflects MC activity(315). PGD_2 is an unstable compound and is rapidly degraded and excreted as more stable urinary metabolites(316). In order to determine the changes in urinary prostaglandin metabolites throughout the course of dengue illness, serial 11β-$PGF_{2\alpha}$ concentrations in the urine were measured in 12 patients with DF and 10 patients with DHF. In patients with DF, urinary PGD_2 metabolite levels (11β-$PGF_{2\alpha}$) gradually declined from day 3 of illness onwards. While in patients with DHF the levels gradually increased up to day 6 (Figure 28a). Additionally, 11β-$PGF_{2\alpha}$ levels were significantly higher in the critical phase of patients with DHF compared to the levels 24 hours before entering critical phase (Figure 28b). While PGD_2 and its metabolites activate ILC2 function, PGE_2 is considered to be inhibitory to ILC2 function(143). Urinary levels of the PGE_2 metabolite PGE-M did not show such a trend in patients with DF and DHF (Figure 28c). These results in part support the hypothesis that PGD_2 plays a role in DHF progression and the pathway constitutes a promising therapeutic target.

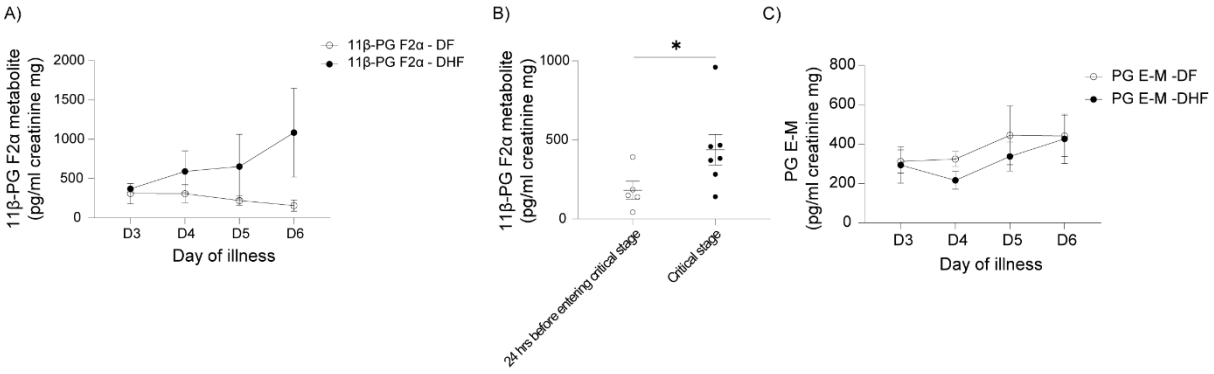


Figure 28: Urinary PGD_2 metabolite 11β-$PGF_{2\alpha}$ levels in patients with acute dengue. Urinary (**A**) 11β-$PGF_{2\alpha}$ levels were measured by quantitative ELISA, in those with DF (n = 12) and those who progressed to develop DHF (n = 10) in serially collected samples on 3rd, 4th, 5th and 6th days of illness. Error bars represent standard error of mean (SEM) and each dot represent the mean. (**B**) 11β-$PGF_{2\alpha}$ levels were measured by quantitative ELISA, in those with DHF (n = 7) 24 hours before entering critical stage and during critical stage. Statistical significance was tested using T test. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. Each dot represents one donor. (**C**) Urinary PGE_2 metabolite PGE-M levels in patients with acute dengue (DF, n=12 and DHF, n=10). Error bars represent standard error of mean (SEM) and each dot represent the mean.

4.3.9 Characterisation of receptors facilitating dengue virus entry in ILC2

The binding of the virus to its specific receptor(s) is a major factor of cellular affinity and is a critical determinant of pathogenesis. In summary, dengue viral host cell entry occurs via clathrin-mediated endocytosis, a process driven by the interaction between viral particles and cellular receptors(91). Virus-receptor complexes then enter the early endosomes, where the acidic environment triggers an irreversible trimerization of the viral E glycoprotein that results in fusion of the viral and host-cell membranes(317) and the release of genomic RNA into the cytosol.

For this process different receptor mediated mechanisms have been identified, mainly through glycosaminoglycans, such as heparan sulfate and C-type lectins, the adhesion molecule of dendritic cells (DC-SIGN), liver endothelial cells (L-SIGN) and mannose receptor (MR) of macrophages. Heparan sulfate (HS) is a ubiquitous receptor among glycosaminoglycans family and is used by many viruses to bind to target cells(318). Furthermore, as elaborated before, FcRγ is another receptor that facilitates dengue virus attachment, and plays a role in secondary dengue infection leading to enhanced virus uptake and replication(296).

Dengue virus can also infect cells that lack these lectins. Other DV entry mediating molecules include lipopolysaccharide (LPS) receptor CD14 and stress-induced proteins such as heat-shock proteins 70 and 90, ER chaperonin GRP78 and 37/67 kDa high-affinity laminin(86). Susceptibility of the host tissues or cells to the virus is intimately dependent on the abundance and distribution of these cell receptors(86). Additionally, there are two distinct families of transmembrane receptors, TIM and TAM that signal to promote the engulfment of apoptotic cells(319). It has been found that TIM proteins bind directly to phosphatidylserine (PtdSer) on the surface of DENV particles, while TAM proteins bind indirectly to viral PtdSer via their natural ligands Gas6 and ProS, which act as bridging molecules. Dengue virus exploits the TIM and TAM receptors and the apoptotic cell clearance pathway to gain entry in to target cells(319).

It is therefore evident that a number of receptors for DENV exist suggesting that DENV does not use a unique, specific receptor for its entry into the cell, but recognises and binds to diverse molecules(320). Firstly, *ex-vivo* RNAseq analysis was explored to see whether ILC2 express recognised receptors to facilitate virus recognition/entry. *HSP70, HSP90, CD14, CD81* and *TIM-3,* known receptors facilitating viral attachment were expressed in ILC2. However, only *TIM-3* was significantly upregulated in DF and DHF (Figure 28a).

Next, ILC2 expression of DC-SIGN, Mannose receptor, HSP70, HSP 90, TIM-3 was examined by flow cytometry. No expression of these receptors was detected on surface staining ILC2 either at an unstimulated state or an activated state in the presence of PGD_2 (Figure 28b), although infection was evidently increased with activation by PGD_2 (Figure 27e). Although a specific receptor to mediate dengue infection in ILC2 was not detected, it was demonstrated that dengue infection could be reduced by inhibiting heparan sulphate (Figure 28c). Heparin (Stemcell #07980) incubated 1 hour before dengue virus infection at a concentration of 100µg/ml significantly reduced dengue infection in ILC2 (Figure 28c). Lower concentrations of heparin did not have a significant effect of reducing infectivity. Similarly, it was shown that heparin or heparan sulphate significantly reduced dengue viral infection in endothelial cells(150) and in human T cells(272). This suggested that dengue virus infection in human ILC2 could be directed through heparan sulphate-containing proteoglycan receptors.

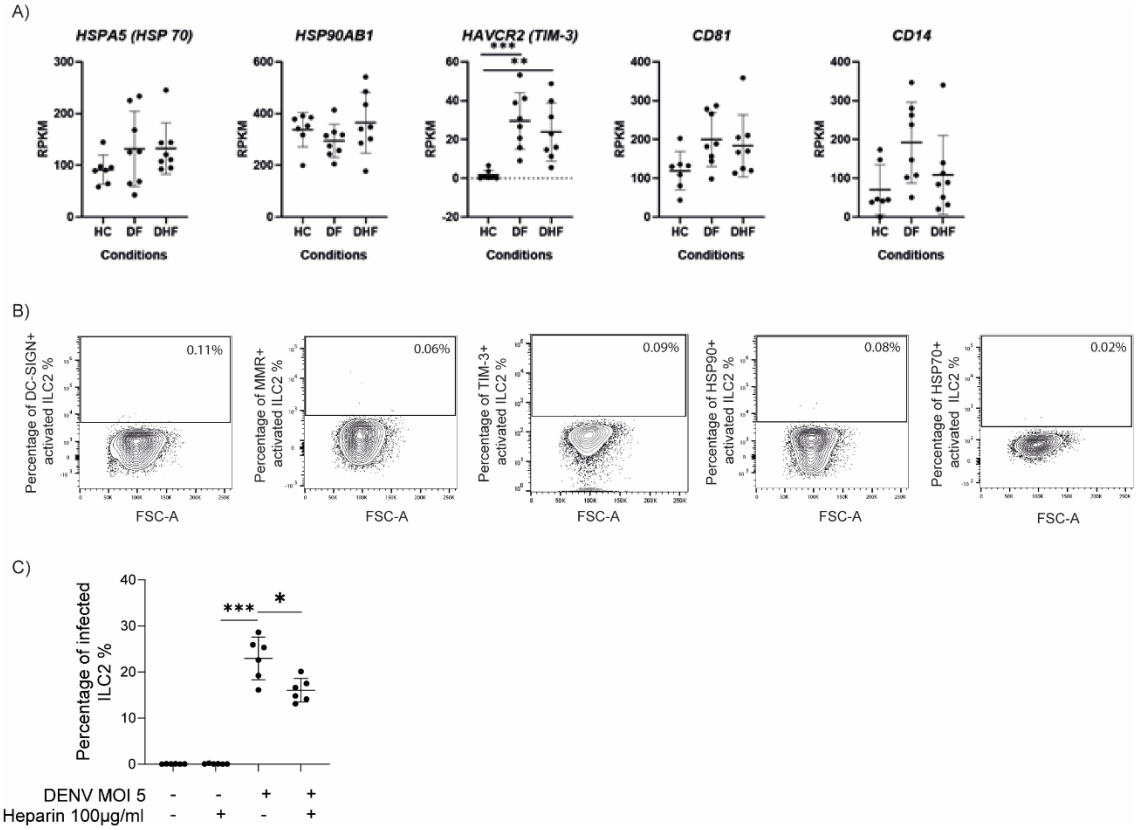


Figure 29: Differential expression of probable receptors that could mediate dengue viral entry in ILC2 and staining of those receptors in ILC2. (**A**) Relative gene expression of *HSPA5, HSP90, TIM-3, CD81, CD14* receptors. *<0.05, **P<0.01, ***P<0.001. One-way ANOVA followed by Tukey's multiple comparisons was performed to determine statistical significance. Mean ± SD. (**B**) Receptors facilitating dengue virus attachment in ILC2. ILC2 were activated with PGD_2 (200nM) for 24 hours and DC-SIGN, MMR, TIM-3, HSP90, HSP70 surface receptors were stained. (**C**) *Ex vivo* sorted and expanded ILC2 were incubated with Heparin (Stemcell #07980) 100µg/ml concentration and incubated for 1 hour before infection with a MOI of 5. Viral infection was assessed after 48 hours by staining for intracellular DENV E protein (n=5, representative figure of 3 independent experiments).

4.4 Discussion

Earlier, we showed that ILC2 are activated in dengue fever and permissive to dengue viral infection *in vivo*. The data reported in the current chapter showed that ILC2 can be infected by DENV *in vitro*. Additionally, ILC2 contibuted to highly productive viral replication, predominantly when activated by PGD_2. Indeed, dengue virus can promote degranulation of mast cells(188, 219). It was showed that the PGD_2 metabolite, 11β-$PGF_{2\alpha}$ is increased in the critical phase of dengue and has a rising trend in severe disease similar to the other mast cell products such as leukotrienes and chymases, which are elevated in dengue infection (123, 321). Serum chymase levels are higher in DHF than DF patients; and chymase levels are higher in patients who had pleural effusions and gallbladder wall thickening(322). In dengue models, leukotriene blockade is associated with resolution of vascular leakage(40, 122). Both PGD_2 and LTE_4 activate ILC2 to produce type 2 cytokine responses(127, 137) and it was evident that both mediators increased viral replication in ILC2, strikingly more with PGD_2 likely due to the fact that it induces autocrine activation of ILC2. Still, the effect of PGD_2 on vascular leakage or endothelial resistance has not been examined. Given the activation of innate type 2 pathways upon dengue viral infection leading to productive viral replication it would be interesting to explore this avenue.

Previously, it has been noted that RSV infection can up-regulate hematopoietic prostaglandin D synthase expression and increased PGD_2 release by cultured human primary airway epithelial cells. Moreover, in a neonatal mouse model of severe viral bronchiolitis, CRTH2/DP2 antagonism decreased viral load, immunopathology, and morbidity(287). Furthermore, it was shown that CRTH2 deficient mice had a reduction in exacerbation of allergic airway inflammation and decline in lung function when challenged with viral ds-DNA(323). It was shown that CRTH2 mediated activation could

be a potential mechanism to suppress protective type I interferon responses and exacerbate viral replication in ILC2 during dengue virus infection. Collectively, this signifies importance in studying allergic inflammatory pathways in viral infections. In a study profiling transciptomic signatures of influenza viral infection, a strong transciptional signal of mast cells was also observed(324). In a specific influenza strain infection, mice who lacked mast cells exhibited a reduction of weight loss and lung pathology compared to wild-type(325).

ILC mostly reside within tissues, particularly in mucosal and skin barriers, where they play a variety of tissue specific inflammatory roles. Despite the similarities noted in activation signals and type 2 cytokine responses, there are key differences in distribution and phenotype of ILC2 in mice and humans(157). In a murine model, ILC2 were seen to be largely absent in human gastrointestinal tract, but enriched on helminth infection and orchestrate a type 2 cytokine mediated helminth worm expulsion(195, 196). Among tissue types, ILC2 have been shown to comprise the main ILC population in the skin(326), but also represent a noteworthy ILC population in lung and abdominal adipose tissue(327). Therefore, it is a possibility that ILC2 could play a role in mosquito bitten skin in dengue disease. Functionally, a significant difference of dengue infection rate of ILC2 isolated from skin and blood was detected. In the absence of tissue cues, the differences of phenotype may have diminished over the expansion period of 6-8 weeks in culture and this may have led to the indifference in DENV infection rates.

In addition, infection of pan-T cells was detected to be low even at an activated state at a MOI of 5 in our experiments. Recently, Silveira *et al* (272) showed that T cells are permissive to infection *in vitro* at higher MOI levels than we used. Authors showed that at MOI of 10, T cells had around 15% of infection and at MOI of 100 showed very high infection rates. From our data we observed that pan-T cells had a significantly lower

infection rate compared to Th2, Tc2 and ILC2 cells at a MOI of 5. Furthermore, at the same MOI, Th2 and Tc2 cells have a significantly higher infection than pan-T cells, although this infection rate remained significantly lower than that the infection rate of ILC2. We used same sorting and expansion techniques for Th2, Tc2 and ILC2, however, Th2 and Tc2 grow much faster than ILC2 and at 4 weeks of expansion the experiments were performed, while ILC2 were sufficiently expanded later around 6 weeks after sorting. ILC2 may be of further relevance as they are resident in human skin and infiltrate rapidly and are capable of secreting large amounts of cytokines upon stimulation. The regulatory elements of effector gene loci such as the type 2 cytokine loci in ILC2, are accessible even in resting ILC and these loci are pre-formed even in ILC precursors. Interestingly, key loci of ILC regulome has shown to be primed even before terminal differentiation in contrast to CD4+ Th cells which undergo dramatic remodeling of chromatin after activation(328). This explains the mechanism for which ILC2 could rapidly mediate rapid type 2 cytokine responses. The enrichment of ILC2 during skin inflammation (164) and the capacity of ILC2 to migrate to inflamed skin are further important characteristics for a rapid immune response in ILC2(329). In addition, it has been proposed that ILC2 modulate naive T cell activation in a cell contact-dependent manner, favoring Th2 differentiation while suppressing Th1 differentiation(330). Activated T cells in acute dengue infection acquire skin homing markers and migrate to skin to involve in immune surveillance (225, 331). This could promote further infection of T cells via differentiation to Th2 and Tc2 counterparts and subsequently assist viral dissemination to peripheral organs.

In summary, we have shown that ILC2 are permissive for dengue infection and undergo productive viral replication. In addition, type I interferons control dengue infection in ILC2 and induce anti-viral genes and proteins with exogenous stimulation. In addition,

viral dissemination would be further enhanced by Th2 and Tc2 infection, as they are more permissive to dengue infection than CD3+ T cells. We also showed that PGD_2 are elevated in critical phase of dengue infection.

5 Chapter 5: Interaction of ILC2 with monocytes

5.1 Introduction

5.1.1 Role of monocytes and dendritic cells in dengue viral infection

Mononuclear phagocytes are important in the control of dengue infection and in the development of protective immune responses against the dengue virus. Mononuclear phagocytes such as monocytes, DCs and macrophages are thought to contribute to the pathogenesis of dengue through ADE(59) and through overproduction of pro-inflammatory cytokines(40, 332) as discussed previously. Upon inoculation into the dermis, dengue virus infects keratinocytes and Langerhans cells. Thereafter, the virus spreads via the blood and infects DCs and tissue macrophages in several organs(213, 333, 334). Tropism of the dengue virus to many cell types and the replication efficacy influences viral load and in turn promotes systemic spread.

Mononuclear phagocytes are Fc receptor bearing cells that facilitate dengue virus attachment and predispose to dengue viral infection(87). Interestingly, it has been reported that dengue viremia is partly due to the massive replication of virus in mononuclear phagocytes in the draining lymph nodes, where after the newly produced viral particles then travel to the bloodstream and infect mostly monocytes(335). Theoretically, viraemia will be of utmost importance in dengue for the transmission of the virus to another host through a mosquito bite. Another study showed that in peripheral blood, frequencies of myeloid DCs remain stable in mild to moderate dengue disease, while the frequencies of monocyte derived DCs and pDCs seem to be reduced in the blood in dengue-infected patients who developed severe disease(336). Chemotaxis of these antigen presenting cells to the tissue was thought to be an explanation for these findings.

The skin is a primary barrier to the environment and provides a first line of defense against invasion of microbial pathogens and importantly is the initial site of infection and viral replication in dengue. Indeed, innate cells of the cutaneous immune system could mediate early immune responses that initiate viral replication. Langerhans cells (LC) are self-renewing and reside in the epidermis, which represent a main target for dengue infection(337). It has been shown that LCs were the main dengue virus-infected haematopoietic cells in the epidermis, with over 90% infection, and the remaining epidermal haematopoietic cells that were mostly γδT cells were not dengue infected in mice(286). Human moDCs generated *in vitro* support dengue infection, with immature DCs being 5-10 times more permissive for dengue infection than mature DCs. This correlated with immature DCs having 3-fold higher MFI for DC-SIGN than mature DCs (88). DCs take up antigen, sense the presence of invading pathogens, and migrate to draining lymph nodes, where they prime naive T cells. Macrophages are tissue-resident cells that are specialized in phagocytosis and local antigen presentation to effector and memory T cells(338). Macrophages are shown to produce TNF-α, IFN-α, IL-1β, IL-8, IL-12, CCL3 and CCL5 after in vitro dengue infection. TNF-α co-localises with macrophages in haemorrhagic tissues in patients with dengue, suggesting that macrophages may contribute to vascular leakage(339). Furthermore, Schmid *et al.* proposed the dermis as the main site of early dengue replication and showed that dengue infection in the skin occurs in two waves: initial infection of resident classical DCs (cDCs) and macrophages, followed by infection of monocytes and moDCs that are recruited to the dermis(84, 340). In this study, Ly6C– CD11b+ cDC, CD103+ cDCs and macrophages were the main infected cells up to 24 hours post-inoculation. However, the total frequency of these cells remained low and was around 3-4% of all CD45+ cells present in the dermis. Later, at 48–72 hours post inoculation, monocytes and moDCs

became the main targets of viral replication in the dermis. Dengue virus-infected monocytes and moDCs made up 79% of all infected cells by 72 hours and were the major targets for virus replication constituting a second wave of infection(340). In another study, IL-13, which is a key cytokine produced by ILC2 and Th2 cells upon activation, was shown to promote migration of activated DCs to draining lymph nodes for Th2 priming(194). Further, in *Ifnar*$^{-/-}$ mice, dengue injected mice had significantly higher dermal Ly6C^{high} monocytes compared to control mice who had PBS injection and was further increased in mice with dengue infection and ADE. CD11C+ DCs were additionally found to be significantly increased in dengue virus infected mice in the same study. Therefore, it was hypothesised that Ly6C^{high} monocytes entered inflamed tissues and gave rise to moDCs(340). This suggested that monocytes and their differentiation to moDCs could promote dengue viral dissemination.

5.1.2 Changes in the phenotype of dendritic cells with PGD_2/DP1 agonists

Dendritic cells have a major relevance in the establishment of interferon mediated innate and adaptive responses through activation of lymphocytes. MoDCs and Langerhans cells are partly characterised by high surface expression of CD1a. Comparatively, plasmacytoid DCs are largely considered to be CD1a(-) and exhibit broad antiviral activities and are only mildly permissive to viral infections compared to dermal DCs (341). However, recently CD1a+ BDCA-2+CD123^{int} DCs that have overlapping features with pDCs were described to infiltrate skin in inflammatory states(342).

Earlier, it was proposed that CRTH2/DP2 receptor antagonists may play a role in restricting viral replication in ILC2. The CRTH2 receptor is predominantly present in type 2 cytokine producing cells(131). The DP1 receptor is another receptor present in a range of innate cells including mononuclear phagocytes that could play a role in anti-viral responses. DP1 agonists and antagonists have been used to investigate DP1 receptor

dependent responses; the DP1 agonist BW245C relaxes vascular smooth muscle, and the selective DP1 antagonist BWA868C blocks the vasodilator effect of PGD_2(343). Moreover, administration of BW245C reduces the proliferation of antigen specific T cells and T cell cytokine production. PGD_2 may suppress DC migration to prevent further immune stimulation, thus contributing to the resolution of inflammation(344). In addition, *in vitro* PGD_2 stimulation downregulates CD1a expression and also increases expression of HLA-DR(345). Consequently, these functional changes would bring about changes in T cell responses to lipid and peptide antigens.

Additionally, CD1a+ DCs have been shown to be important in harboring HIV infection where CD1a+ enriched DCs infected with HIV could transmit infectious virus to T cells without cell-cell contact and the CD1a+ DCs had productive infection over a protracted period of time(346). Whether prolongation of infection is related to differentiation of DCs or related to CD1a is incompletely understood. Cluster of differentiation 1 (CD1) molecules are structurally related to MHC class I heavy chain molecules and also associate with β2-microglobulin(347, 348). In humans, CD1 molecules are divided into CD1a, CD1b, CD1c and CD1d by the sequence homology. CD1a, b and c are present in antigen presenting cells while CD1d is present in a broader range of hematopoietic and non- hematopoietic cells. CD1 molecules can capture self and foreign lipids and present these lipids to T cells and initiate T cell responses via T cell receptors (TCRs) (347). These T cells can secrete large amounts of cytokines such as IFN-γ and TNF-α and can express cytolytic properties(349). These cytokines have a profound antiviral effect and have been shown to play a significant role in persistent viral infections(350). In addition, CD1a restricted T cells produce Th2 and Th17 cytokines and contribute to allergic and inflammatory skin diseases(351). In bacterial infections, DCs that express CD1 molecules can stimulate CD1 restricted T cells and present increased amounts of

antigenic self-lipids upon activation by bacterial PAMPs through TLRs. Several bacterial infections induced CD1a-restricted and CD1b-restricted T cells responses and secreted IFN-γ in the absence of exogenously added lipid antigens. Either bacterial infection or LPS treatment induced increased glycosphingolipid (GSL) synthesis in both THP-1 and DCs. Similar to bacteria-infected DCs, DCs treated with bacterial products containing cell wall components also activated glycosphingolipid (GSL) specific T cells. This activation followed a dose-dependent response(352). Such effects on CD1 expression and CD1 functional responses in viral infection are incompletely understood.

5.1.3 ILC2 transcription factors and viral infections

MoDCs show activation with dengue viral infection and phenotypic changes affecting antigen presentation and T cell responses. From our initial RNA sequencing data, it was observed that ILC2 phenotype could alter with dengue infection. ILC2 are GATA3 highly expressing cells that have potent type 2 cytokine producing function. T-bet is one of the vital transcription factors for the development of cytotoxic CD8 cells and Th1 cells in response to virus infection. T-bet is expressed in ILC1 and cNK cells that produce IFN-γ and TNF-α (157, 353) and have a role in antiviral immunity. Additionally, T-bet is co-expressed with ROR-γt and NKp46 in NCR+ ILC3. T-bet expression is required for $CCR6^{-/low}$ ILC3 differentiation into NCR+ ILC3 and subsequent IFN-γ production(354). T-bet can also be induced in human and murine ILC2, via stimulation with IL-12 or IL-18 resulting in the production of IFN-γ(355, 356). T-bet controls the stability of ILC1 and represses ILC2 activity, which may affect type 2 cytokine production. Deletion of T-bet triggers ILC2 expansion and activity both *in vivo* and *in vitro*(357). In helminth infection, it has been shown that T-bet deficiency enhances mucosal immune responses(357). It was observed from RNA sequencing data that the *T-bet* gene had a

higher expression in dengue samples, hence it was sought to investigate the mechanisms of T-bet control in ILC2.

The transcription factor EGR2 (early growth response 2) suppresses excessive immune activation. The EGR family of genes consists of four members (EGR 1-4) that are rapidly expressed following T cell stimulation(358). EGR2 has been widely investigated with respect to T cell proliferation. EGR2-deficient T cells in mice had hyperproliferative features(359, 360). EGR2 has been shown to control excessive proliferation of T cells. While, EGR2 expression has been detected for up to 10 days in anergic T cells, it was downregulated rapidly within 2 days upon TCR activation with co-stimulation. This suggest that T cell proliferation, which occurs through co-stimulatory TCR activation, may be facilitated partly by the downregulation of EGR2. Additionally, the role of EGR2 was investigated in the context of influenza infection. Histological examination of lungs from infected EGR2 knockout (KO) mice showed extensive tissue destruction and lymphocytic infiltration, and had higher viral loads in their lungs than wild-type (WT) mice. In addition, cytokine and chemokine expression in EGR2 KO mice was dysregulated after influenza infection(361). Th1/Th17 differentiation was found to be lower in EGR2 KO mice compared to WT mice(361). This may suggest that lack of EGR2 expression facilitated a hyperimmune response in viral infection which could be related to excessive immune activation.

Given the evidence of functionally relevant cross-talk between moDC and ILC2, the relevance to dengue viral infection was next investigated, with a focus on the role of PGD_2 pathways.

5.2 Hypothesis and aims

On the basis of these preliminary findings, the work of the chapter investigated the hypothesis that an ILC2: monocyte dialogue occurs during dengue virus infection. Specifically the work aimed:

- To determine whether ILC2 affect dengue infection in monocytes/DCs
- To ascertain whether infected DCs affect ILC2 infectivity/proliferation

5.3 Results

5.3.1 Dendritic cells are activated with dengue viral infection

As shown in previous literature, MoDCs are permissive to dengue infection. With differentiation of monocytes to moDCs, CD1a expression is known to be increased and down regulation of CD14 expression is observed. First, differentiation of moDCs over 4-5 days with GM-CSF and IL-4 was performed. Resulting moDCs were infected with dengue virus (MOI 2.5). Infection was tested after 36-48 hours with staining for intracellular envelope protein by flow cytometry. Infection was detected to be 10-20% (Figure 30a) in moDCs. Additionally, it was observed that infected moDC were activated with increased expression of CD86 (Figure 30b) and HLA-DR (Figure 30c). However, moDCs which were at terminal stage of in *vitro* differentiation, did not show a significant difference in CD1a expression with dengue viral infection (Figure 30d) but secreted a significantly higher amount of IFN-α than mock-infected moDCs (Figure 30e). However, when the monocytes were infected at an earlier time point of differentiation with GM-CSF and IL-4, approximately 48 hours after initiation of *in vitro* differentiation, the cells showed a significant down regulation of CD1a expression upon infection with the dengue virus (Figure 30f). These differentiating monocytes demonstrated a higher trend of infection rates reaching nearly twice the rate of moDC infection (Figure 30g), although this needs further exploration on the mechanisms leading to higher infection.

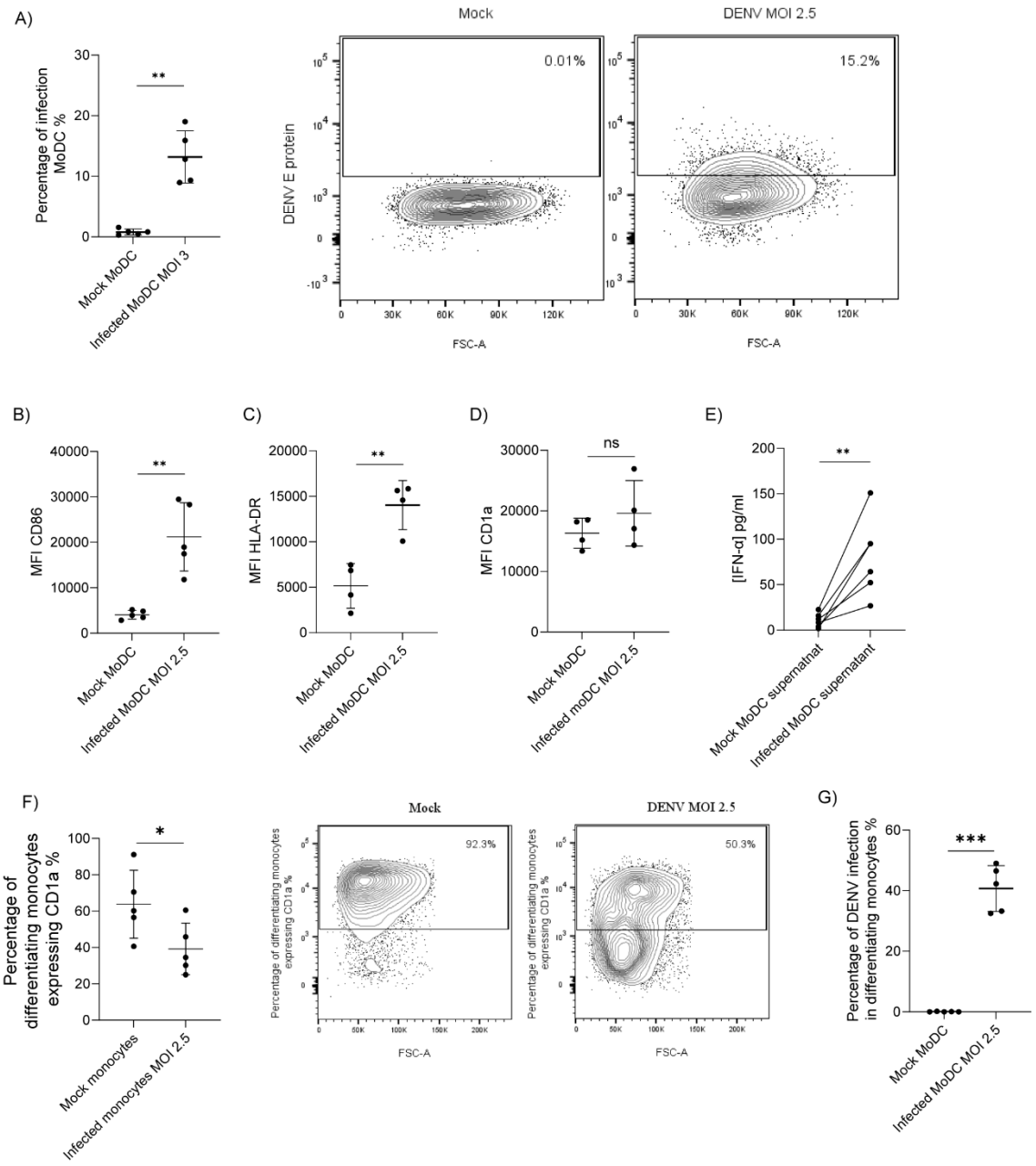


Figure 30: Monocyte derived dendritic cells (moDC) are activated with dengue viral infection. CD14+ separated monocytes were differentiated to moDCs with GM-CSF (100ng/ml) and IL-4 (10ng/ml) over 4-5 days. The cells were harvested and infected with dengue virus with a MOI of 2.5 (n=5, data from 2 independent experiments). (**A**) Dengue viral infection was assessed by staining for intracellular dengue viral envelope protein staining 36-48 hours after infection. Cells were harvested and surface stain for (**B**) CD86, (**C**) HLA-DR and (**D**) CD1a (n=4, data from 2 independent experiments).. (**E**) IFN-α was

performed on the supernatant of mock and infected moDCs. Similarly, CD14+ monocytes were incubated with GM-CSF (100ng/ml) and IL-4 (10ng/ml) over a shorter duration of 48 hours and infected with dengue virus with a MOI of 2.5 (n=4, data from 2 independent experiments). Cells were harvested and surface stained for (**F**) CD1a and (**G**) dengue virus envelope protein. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, data representative of 2 independent experiments (n=4). *P <0.05, ** <0.01, *** <0.001, n.s. not significant.

5.3.2 Activated ILC2 increase DC-SIGN and MMR expression in differentiating monocytes and increase dengue infection

Having found that ILC2 can undergo productive infection, whether ILC2 could influence infectivity of other innate cells such as monocytes was investigated. DC-SIGN and macrophage mannose receptor (MMR) are expressed on immature DCs and macrophages and are known to facilitate viral attachment(362). It has been shown that type 2 cytokines such as IL-4 and IL-13 can increase infection of monocytes and MoDCs by upregulating DC-SIGN and MMR (87, 363). ILC2 supernatants increased CD1a, DC-SIGN and MMR expression in monocytes (Figure 31a). DC-SIGN and MMR expression in monocytes reached 70-80% positivity at 48 hours after treatment with ILC2 supernatants (Figure 31a-c). Supernatants from activated ILC2 significantly upregulated DC-SIGN and MMR expression in monocytes compared to supernatants from unstimulated ILC2 (Figure 31b-e). Expression of DC-SIGN and MMR receptors was dependent on GM-CSF secreted by ILC2, as blocking GM-CSF reduced DC-SIGN and MMR expression and dengue infection (Figure 31b-f). Inhibiting IL-4Rα, which is the shared receptor for IL-13 and IL-4, did not significantly change expression of DC-SIGN, MMR expression or dengue infection (Figure 31b-e). Additionally, it was evident that supernatants from activated

ILC2 enhanced dengue infection in monocytes compared to supernatants from unstimulated ILC2 (Figure 31f).

Furthermore, blocking of the mannose receptor with Mannan (Mannan from *Saccharomyces cerevisiae*), which is known to bind to MMR and reduces attachment of dengue virus(87), significantly reduced dengue infection in differentiating monocytes treated with ILC2 supernatants (Figure 32a). In addition to the reduction of percentage infection, mannan significantly reduced cell death with dengue viral infection in differentiating monocytes (Figure 32b). Blocking DC-SIGN did not affect the percentage infection of differentiating monocytes (Figure 32c).

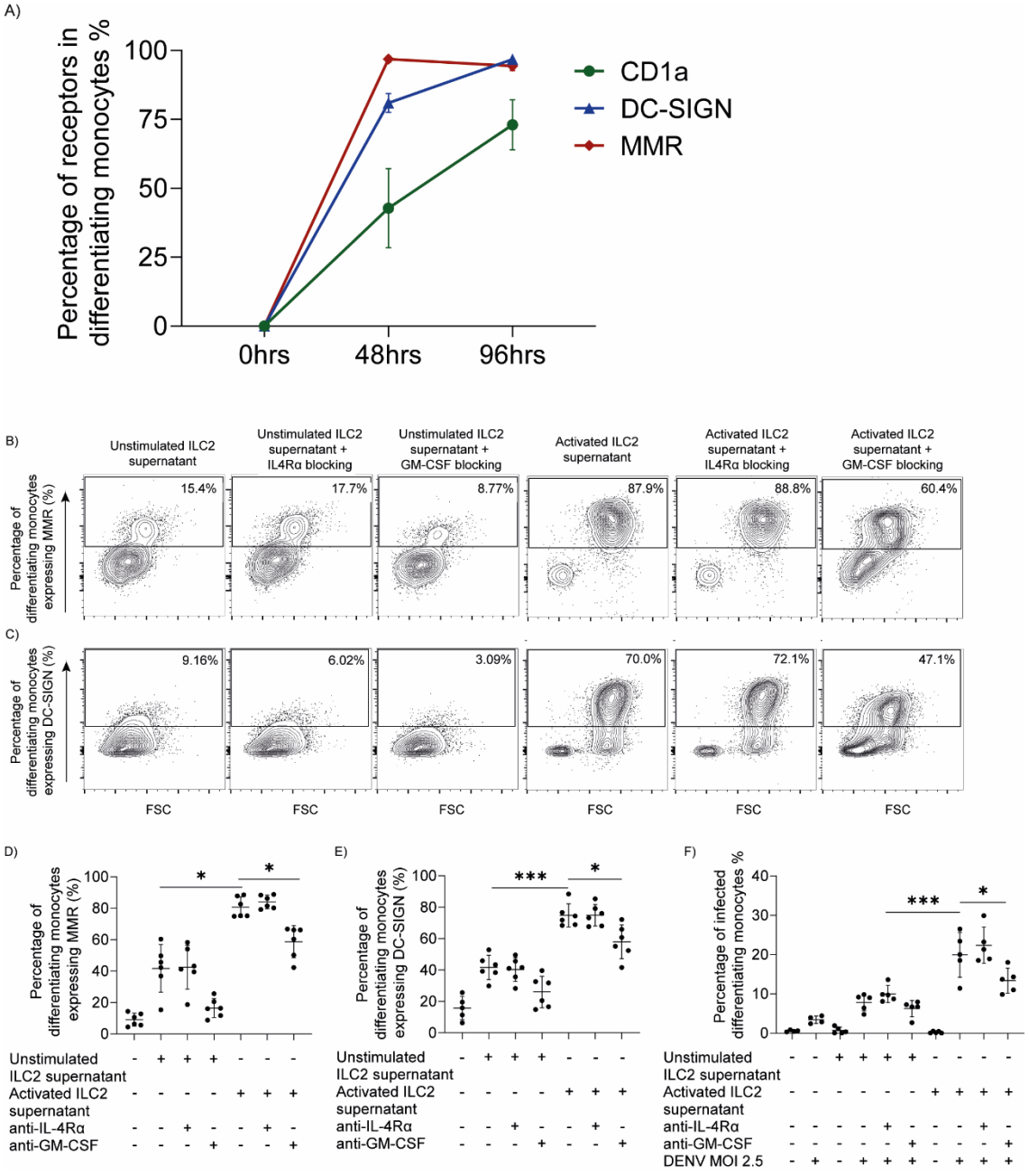


Figure 31: ILC2 supernatant affect receptor expression of monocytes. (**A**) ILC2 supernatants were added on CD14+ monocytes and incubated for 4-5 days. Surface receptor expression of DC-SIGN, MMR and CD1a at 0, 48 and 96 hr time points (n=5, data from 2 independent experiments). Unstimulated and activated ILC2 supernatants were added to CD14 MACS-separated monocytes and (**B**) DC-SIGN and (**C**) MMR expression was measured after 48 hours by flow cytometry (n=5, data from 2 independent experiments). (**D**, **E**). In the blocking conditions anti-GM-CSF and anti-IL-4Rα was

added for 1 hour before adding supernatants of ILC2. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, (n=5) data representative of 3 independent experiments. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**F**) Unstimulated and activated ILC2 supernatants were added to monocytes and cells were infected with DENV2 at a MOI of 2.5 and intracellular dengue envelope protein was stained after 48 hours by flow cytometry. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, (n=5) data representative of 3 independent experiments. *P <0.05, ** <0.01, *** <0.001, n.s. not significant.

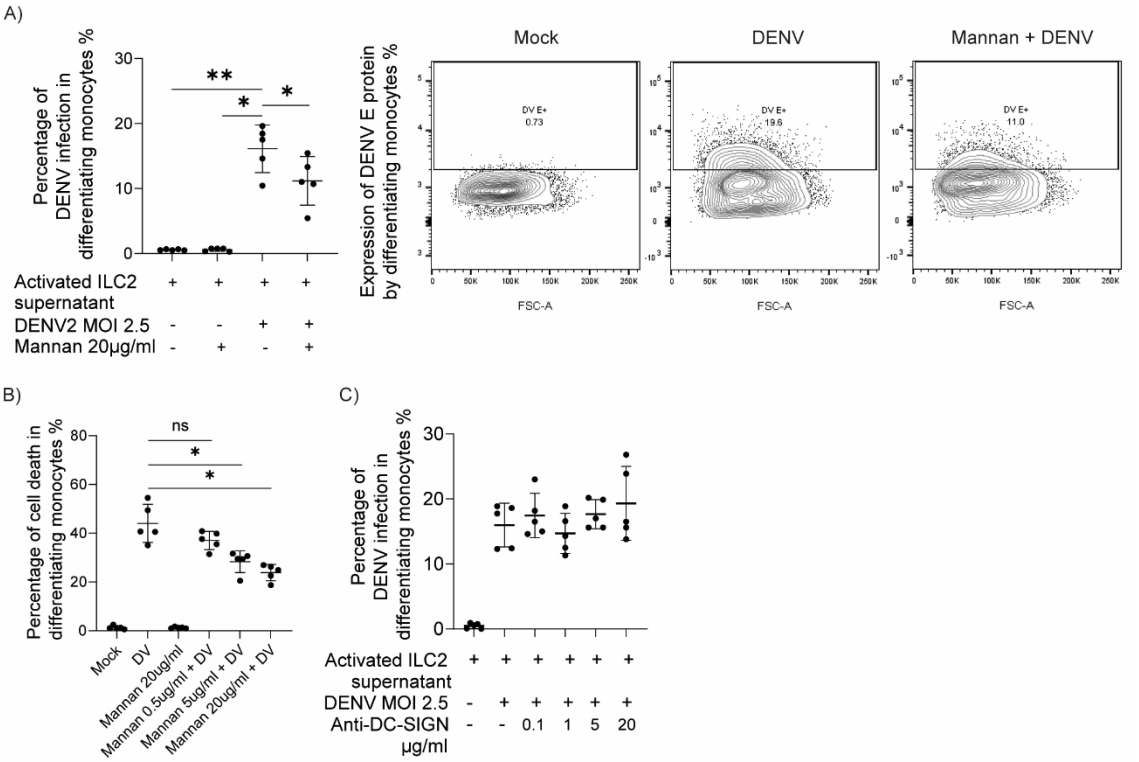


Figure 32: MMR facilitates dengue viral infection in differentiating monocytes. Monocytes were treated with activated ILC2 supernatants for 48 hours and infected with DENV2 (MOI 2.5). In the blocking conditions, monocytes were incubated with Mannan 20µg/ml (Sigma, M7504) for 1 hour prior to infection. (**A**) Infection rate was measured by staining for intracellular dengue envelope protein after 48 hours by flow cytometry (n=5, data from 2 independent experiments). (**B**) Live/dead stain was analysed to

represent death rate of differentiating monocytes. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, (n=5) data representative of 2 independent experiments. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**C**) Monocytes were incubated with anti-DC-SIGN (10µg/ml - MAB161 - clone 120507) for 1 hour prior to infection. Infection rate was measured after 48 hours by flow cytometry. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, (n=5) data representative of 3 independent experiments. *P <0.05, ** <0.01, *** <0.001, n.s. not significant.

5.3.3 Infected DCs reduce dengue infection in ILC2 through type I interferon

As the data showed that ILC2 can increase dengue viral infection in differentiating monocytes, it was explored whether infected DCs influence dengue infection in ILC2 to explore functionally relevant two-way effects. Supernatants of mock and infected DCs were collected and added to ILC2, which were subsequently infected with dengue virus with a MOI of 5. Infected DC supernatants significantly reduced dengue viral infection in ILC2 compared to mock DC supernatants (Figure 33). Inhibiting IFNAR1 increased infection of ILC2 (Figure 33), revealing a type I interferon mediated protection from viral infection.

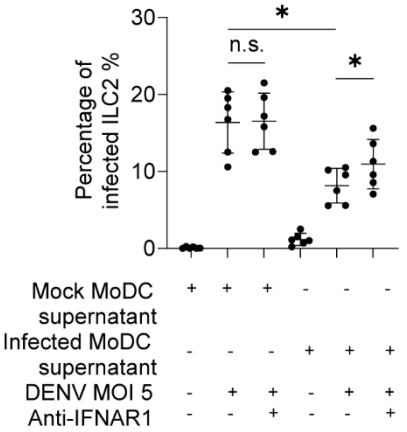


Figure 33: Infected dendritic cells reduce infection in ILC2 in a type I interferon dependent manner. Mock and infected moDC supernatants were collected 48 hours after infection. Mock and infected supernatants were added to ILC2 and were infected with dengue virus with a MOI of 5. Intracellular dengue virus envelope protein was assessed after 48 hours after incubation in ILC2 media. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, (n=6) data representative of 2 independent experiments. *P <0.05, n.s. not significant.

5.3.4 Prostaglandin D2 affects differentiation of monocytes

The local environment plays an essential role in DC differentiation, and cytokines such as GM-CSF and IL-4 are used in *in vitro* systems for monocyte differentiation to DCs. Additionally, circulating monocytes have the potential to differentiate into various other cell types such as macrophages, liver Kupffer cells or even microglia in the central nervous system(364). Hence, monocytes mediate a broad range of effector functions in the immune system such as producing inflammatory cytokines and reactive oxygen species, stimulating effector T-cell proliferation, and mediating tissue repair(365). From our initial experiments, it was evident that GM-CSF secreted from ILC2 mediated phenotypic changes in monocytes. Therefore, it will be vital to study other mediators or cytokines that could affect monocyte differentiation and function.

As above, PGD_2 and its metabolites acts through variety of cells through DP1 and DP2 receptors. While DP1 receptors have been found to be present on monocytes, immature DCs and mature DCs, CRTH2 was detected at very low levels in monocytes(366). BW245C is a DP1 receptor-selective agonist, that has been shown to induce hypotension primarily through vasodilation of arterioles(367). Additionally, it has been reported to have weak EP2, as well as an EP4 receptor agonist activity facilitating vasodilation for vascular smooth muscles(367). It was observed that DCs differentiated in the presence of

PGD_2 acquire an unusual phenotype and show an impairment in the stimulation of naive T cells. PGD_2 induces functional changes in antigen processing in antigen presenting cells(366) by affecting maturation of monocyte derived dendritic cells. To elaborate, PGD_2 treated DCs displayed increased endocytic and antigen processing activities, they had reduced capacities to activate antigen specific naive T cells(345). DP1 receptor agonist treated WT chimeric mice had significantly reduced expression of the maturation markers CD86, CD40, and MHC II on lung DC(343, 344).

It has been shown that migration of monocytes is induced via CRTH2 activation, whereas DP dependent activation inhibits chemokine-driven migration of DCs(366). Inhalation of a selective DP1 agonist showed to suppress the features of asthma by targeting the function of lung DCs (343). Furthermore, DP1 agonist (BW245C) reduced activation, proliferation and cytokine production by draining lymph node T cells (IL-4, IL-10, IFN-γ) (343).

Addition of DP1 agonists to differentiating monocytes (differentiated with GM-CSF and IL-4) altered the morphology of the resulting DCs(Figure 34a,b). After 48 hours of treatment these cells had lesser clustering and cellular extensions than monocytes treated without DP1 agonists (Figure 34a,b). Next, monocytes were incubated with GM-CSF and IL-4 for 48 hours and treated with or without DP1 agonist (BW245C) and surface receptor expressions of relevant receptors were assessed. Differentiating monocytes treated with DP1 agonists had significantly less CD1a expression than control monocytes (Figure 34c,d). Additionally, treatment with PGD_2 significantly down-regulated CD1a expression in a dose dependent manner (Figure 34e,f), although DP1 agonists had a more pronounced effect than PGD_2 (Figure 34c-f). With inhibition of PGD_2 action using BW868C (DP1 antagonist) a trend of reduction of down-regulation of CD1a expression in differentiating monocytes was observed at highest concentrations of PGD_2 (Figure

34g). It will be important to assess these changes at different concentrations of DP1 inhibitor. Moreover, DP1 agonists significantly upregulated mean fluorescence intensity (MFI) of HLA-DR, HLA-ABC, CD1d expression (Figure 34h-j).

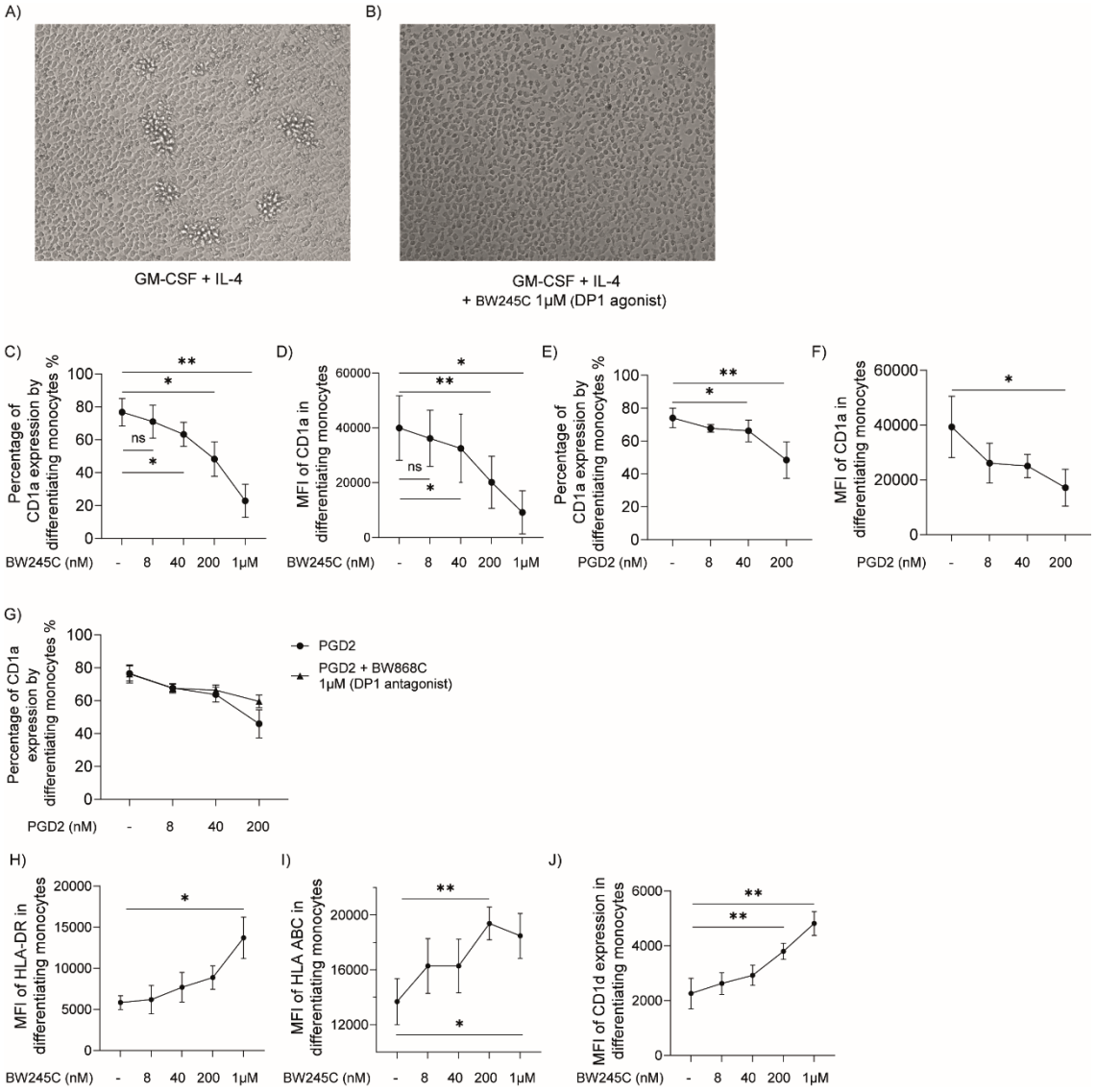


Figure 34: Surface receptor expression of differentiating monocytes with PGD_2 stimulation. CD14+ monocytes were incubated in media containing GM-CSF (100ng/ml) and IL-4 (10ng/ml) for 48 hours with or without DP1 agonists. Morphology was assessed by light microscopy in monocytes treated with (**A**) GM-CSF and IL-4 and (**B**) GM-CSF and IL-4 with BW245C (DP1 agonist) 1μM concentration. Next, CD14+ monocytes were incubated in media containing GM-CSF (100ng/ml) and IL-4 (10ng/ml)

for 48 hours with BW245C (DP1 agonist) 1µM or PGD_2 (200nM). Surface stain for CD1a (**C-F**), HLA-DR (**H**), HLA-ABC (**I**), CD1d (**J**) was performed. Percentage and/or mean fluorescence intensity (MFI) was assessed. One-way ANOVA with Tukey's comparison was used to analyse the statistical significance. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. Additionally, (**G**) monocytes were incubated with PGD_2 (200nM) and inhibition with BW868C 1µM (DP1 antagonist) 1 hour prior to incubation with PGD_2. CD1a percentage in differentiating monocytes were assessed. (n=5, all above from 3 independent experiments).

5.3.5 DP1 agonists lower dengue virus infection in differentiating monocytes

Next, infectivity of monocytes treated with DP1 agonists was investigated. Monocytes were incubated with DP1 agonists for 24 hours before infection with dengue virus, and subsequently the excess virus was washed away and the cells incubated in GM-CSF and IL-4 for 36 hours. Infection was assessed by staining for intracellular dengue envelope protein. BW245C (DP1 agonist) significantly reduced infectivity of differentiating monocytes (Figure 35a). Supernatants of these monocytes were collected and IFN-α levels assessed. The level of IFN- α produced by infected monocytes and DP1 agonist treated monocytes did not show any significant difference (Figure 35b).

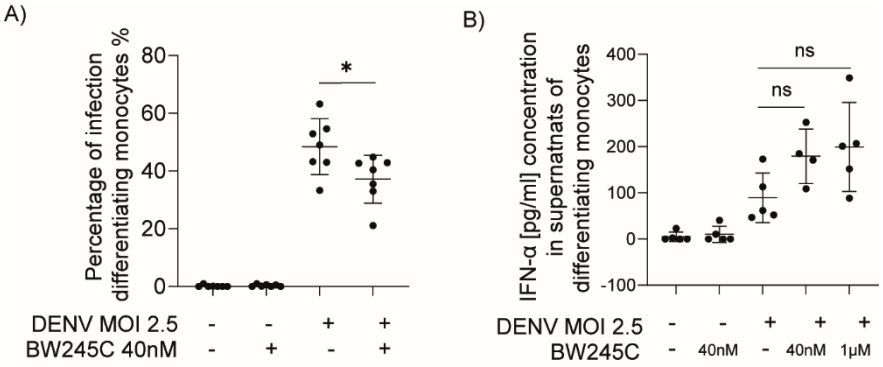


Figure 35: DP1 agonists reduce dengue viral infection in monocytes. (**A**) Monocytes were incubated in media containing GM-CSF (100ng/ml) and IL-4 (10ng/ml) for 24

hours with or without BW245C (DP1 agonist) 40nM. Cells were harvested and infected with dengue virus for 2 hours with a MOI of 2.5. Cells were washed with media and plated in media containing GM-CSF and IL-4 for 36 hours. Infection rate was assessed by staining for intracellular dengue envelope protein (n=6, experiment repeat twice). (**B**) Supernatant was collected and IFN-α ELISA was performed. Single outlier was removed. Experiment was performed twice (n=5), but require further optimisation.

5.3.6 ILC2 phenotype in dengue viral infection

ILC2 are characterized by high expression levels of the Th2 master regulator *GATA-3*, which endows ILC2 with the ability to produce the Th2 effector cytokines, IL-5 and IL-13 following stimulation by upstream cytokines including IL-33, IL-25, and IL-2. *T-bet* is the master regulator for ILC1 cells. *T-bet* is absolutely required for the generation of ILC1 but not for cNK cells. Besides its critical function during ILC1 development, *T-bet* is required for the maintenance of ILC1 cells and production of ILC1 effector cytokines(169). From the RNA sequencing analysis, it was evident that ILC2 have a higher *T-bet* expression in patients with dengue compared to HC (Figure36a), albeit at a substantially lower level than GATA3. In addition, *NFIL3* is a critical transcription factor for all ILC(368), and shows significant change in expression during dengue infection (Figure 36a). ILC2 cells have been shown to exhibit phenotypic plasticity in response to infectious or noxious agents, characterised by a lower expression of the transcription factor GATA-3 and a concomitant switch to being ILC1-like cells that produced interferon-γ (IFN-γ). IL-12 and IL-18 have seen to regulate this conversion. The mechanisms for type I interferon mediated effects on Th2 cells have been studied previously. It has been reported that production of type 2 cytokines by Th2 cells could be suppressed by overexpression of *T-bet*. Exposure of Th2 cells to interferons and IL-12 *in*

vivo seems to affect their plasticity and lead to acquisition of hybrid Th1-Th2 functions(184, 369, 370).

During viral infection, ILC2 have been observed to cluster within inflamed areas and acquired an ILC1-like phenotype. Mechanistically, these ILC1-like cells augmented virus-induced inflammation in a manner dependent on the transcription factor T-bet. Notably, IL-12 converted human ILC2 cells into ILC1-like cells(371). Rapid production of IL-12 by tissue-resident conventional DCs promoted ILC1 production of IFN-γ in a STAT4-dependent manner to limit early viral burden(208). IL-12 is the major cytokine that can activate STAT4, and is synthesised predominantly by DCs and macrophages. IL-12 is composed of two subunits, the light chain p35 (IL-12A) and the heavy chain p40 (IL-12B). Numerous studies have suggested that IL-12 acts as a pro-inflammatory cytokine, which induces liver injury by activating NK and NKT cells to produce IFN-γ(372). IL-12-mediated an increase in IFN-γ production, cellular proliferation, enhanced NK cell cytotoxicity and Th1 cell differentiation, which were all impaired in STAT4 knockout mice. Also, STAT4 difficient lymphocytes had a capacity to differentiate towards Th2 cells (372, 373). In another murine model, IL-12 with IL-18 induced cytolytic function and production of IFN-γ by T, NKT, and NK cells, thereby promoting liver inflammation (374, 375).

As above, ILC2 receive activation signals from alarmins including IL-33 and from lipid mediators such as PGD_2, which promote the type 2 phenotype. To investigate the expression of T-bet in ILC2, *in vitro* expanded ILC2 from healthy individuals were stimulated with IL-33, PGD_2, IL-12 and IFN-β for 16 hours and intracellular transcription factors, T-bet and GATA3 were stained. Both PGD_2 and IL-12 increased T-bet expression in ILC2 over unstimulated ILC2 although the level of T-bet expression remained considerably low compared to GATA3 expression (Figure 36b,c). Supernatants were

collected from activated ILC2 from above mentioned stimulatory conditions after 24 hours. IFN-γ was detected in ILC2 treated with IL-12 but not with ILC2 treated with PGD_2 (Figure 36c). To further explore the mechanisms of ILC2 cytokine production, the regulation EGR signaling was investigated.

EGR2 and 3, are transcription factors that are essential to suppress Th1 differentiation in Th2 and Th17 conditions *in vitro* and also to control IFN-γ-production(376). It was observed that *EGR2* was significantly expressed upon PGD_2 stimulation of ILC2. IL-12, IL-33 and IFN-beta did not induce expression of *EGR2* in comparison to unstimulated ILC2 (Figure 36e). *EGR2* is expressed in ILC2 early at 30 minutes, after activation with PGD_2 (Figure 36f), but not with IL-33, IL-12 or IFN-beta. This may translate to a functional significance of the EGR2 protein in ILC2 in the context of PGD_2 activation. EGR2 binds to T-box region of *T-bet* and alters functionality in T cells and suppresses T-bet-induced epigenetic alterations. EGR2 function was defined earlier with TCR mediated activation of T cells and here it was observed that it may have a functional significance in cytokine mediated activation in ILC2, to suppress function of T-bet.

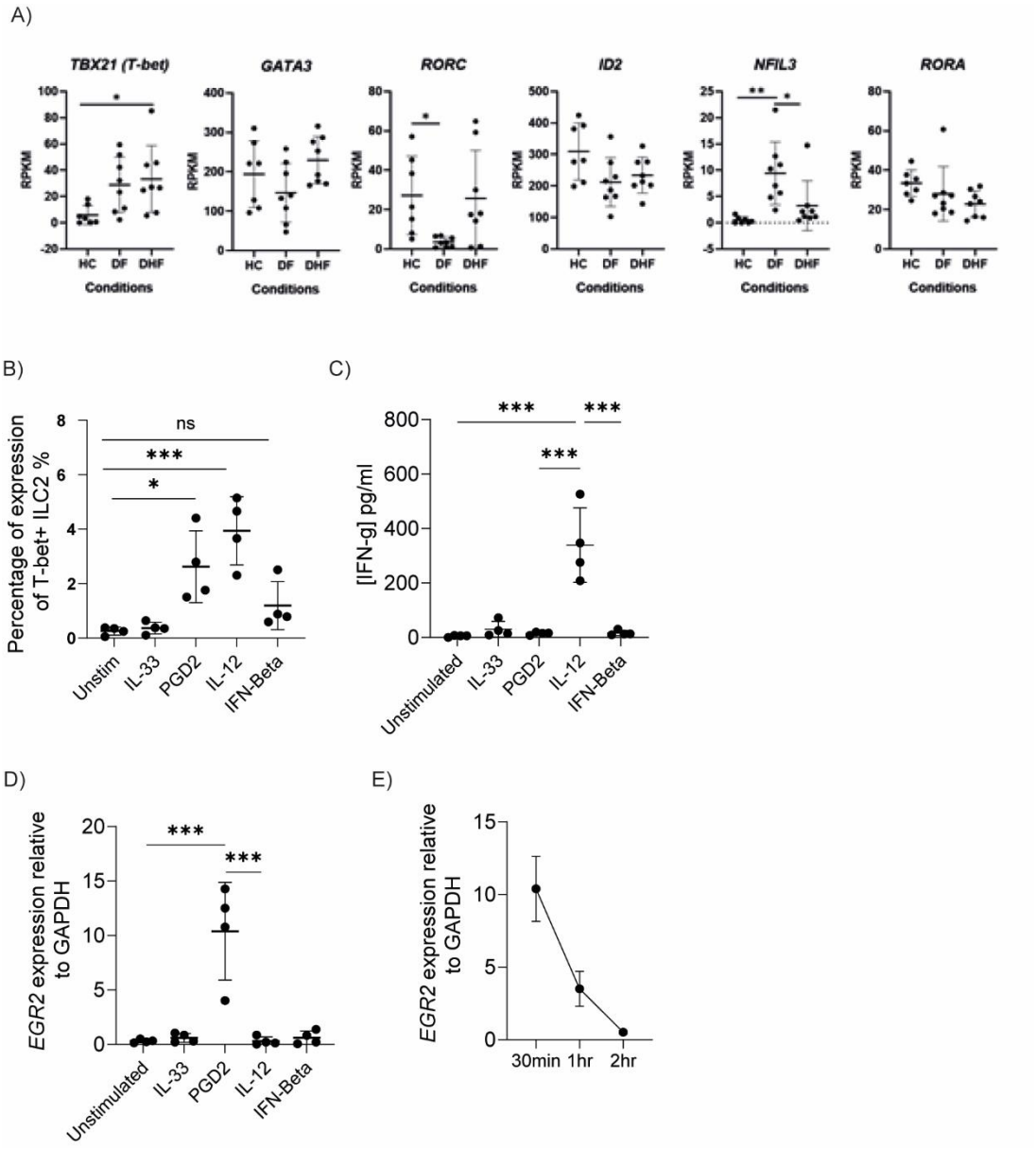


Figure 36: T-bet and EGR2 expression in ILC2. (**A**) Gene expression of transcription factors *TBX21, GATA3, RORC, ID2, NFIL3, RORA* of human blood derived ILC2 of HC, DF and DHF, determined by RNA Sequencing and represented in RPKM values. Statistical analysis performed using adjusted p-value from the DEG result. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**B**) ILC2 were incubated with IL-33 (50ng/ml), PGD_2 (200nM), IL-12 (50ng/ml), IFN-β (250IU/ml) for 16 hours and transcription factors (T-bet and GATA3) were stained and assessed by flow cytometry. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test,

(n=4) data representative of 3 independent experiments. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**C**) ILC2 were incubated with IL-33 (50ng/ml), PGD_2 (200nM), IL-12 (50ng/ml), IFN-β (250IU/ml) for 24 hours and interferon-γ ELISA was performed on the supernatant. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, (n=4) data representative of 3 independent experiments. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**D**) ILC2 were incubated with IL-33 (50ng/ml), PGD_2 (200nM), IL-12 (50ng/ml), IFN-β (250U/ml) for 30 minutes and qRT-PCR was performed. *EGR2* expression was normalised to GAPDH gene expression. Statistical significance was tested using one-way ANOVA with Tukey's multiple comparison test, (n=4) data representative of 3 independent experiments. *P <0.05, ** <0.01, *** <0.001, n.s. not significant. (**E**) ILC2 were incubated with PGD_2 (200nM) in a time course of 30 minutes, 1 hour, 2 hours and qRT-PCR were performed. (n=4) Data representative of 3 independent experiments. *EGR2* expression was normalised to GAPDH gene expression.

5.4 Discussion

Mononuclear phagocytes are important in the control of infection and in the development of protective immune responses against viral infection. In dengue infection, these cells facilitate attachment of dengue virus to mononuclear phagocytes and promote infection and favour dengue viral replication(87). Infected antigen presenting cells migrate to lymph nodes to present viral peptides to T cell and activate antigen specific immunity. However, viruses can take advantage of innate immune cells like ILC2 as a trojan horse for transporting the virus from tissues to secondary lymphoid tissues for dissemination of infection to peripheral tissues. Interestingly, viremia in dengue is the result of the massive replication of virus in mononuclear phagocytes in skin and draining lymph nodes, and newly produced viral particles then travel to the bloodstream where they predominantly infect monocytes(377). It was shown that activated ILC2 increase viral infection of monocytes through a GM-CSF-dependent rather than an IL-4/13 dependent mechanism, and may contribute to systemic viral dissemination. In contrast, Miller *et al* showed that in monocytes and macrophages, direct IL-13/4 stimulation increased MMR and DC-SIGN expression(87). Comparatively, unstimulated ILC2 did not significantly affect DC-SIGN and MMR expression, nor did they increase dengue infection in monocytes. Therefore, ILC2 activation, which may be induced by PGD_2 metabolites that were high in the urine of patients with dengue hemorrhagic fever, could contribute to GM-CSF secretion and assist viral dissemination indirectly via monocyte infection.

Relatively less is known about the regulation of CD1a molecules in antigen presenting cells in viral infections. The peroxisome proliferator-activated receptor γ (PPARγ) pathway, acting through the production of all-trans-retinoic acid (ATRA) which binds the retinoic acid receptor (RARα) has been shown to downregulate CD1a expression(378). Moreover, a study noted that activation of the activating transcription

factor/cAMP Response Element Binding Protein (ATF/CREB) family decreased *CD1A* transcription, through binding of the *CD1A* promoter in human monocytes(379). Interestingly, BW245C-treated DCs of mice showed a rise in nuclear-associated CREB-1(343). This may suggest a mechanism of down regulation of CD1a with DP1 agonists, which could be via CREB. Also, from our data, dengue infection at an earlier stage of differentiation in monocytes reduced CD1a expression. This has been observed in another study where Herpes Simplex virus-1 (HSV-1) infection downregulated levels of CD1a, CD80, CD40, and CD54 markedly when compared to those of mock-infected controls(380). The mechanism behind this downregulation and its contribution for pathogenesis is unexplored.

PGD_2 acts in both pro-inflammatory and anti-inflammatory immune responses depending on the receptor (DP1 or DP2) and the tissue of action. In contrast to the action of PGD_2 through CRTH2/DP2 receptor, which increased productive infection in ILC2, PGD_2 acts on monocytes through the DP1 receptor and mediated changes in CD1a, CD1d and HLA-DR expression. This change in surface protein expression theoretically, could affect peptide and lipid capture and antigen presentation to T cells and could subsequently affect T cell responses. Further experiments need to be performed to investigate the changes of subsequent T cell responses with PGD_2. Additionally, DP1 agonists reduced dengue infection in monocytes suggesting therapeutic relevance. Additionally, the supernatants from infected differentiating monocytes treated with DP1 agonists had a trend of higher IFN-α levels than the condition without DP1 agonists although it is not statistically significant. Similarly, in RSV infected airway epithelial cells DP1 agonism had anti-viral function by up-regulating IFN-λ production, which in turn, increased IFN-stimulated gene expression, accelerating viral clearance(287). Therefore, we suggest that DP1

agonists may have a protective role in dengue viral infection and would be an interesting area to pursue exploration.

Interestingly, the role of EGR2 was previously defined in the context of TCR stimulation and was shown to be associated with hyperproliferation of T cells leading to an autoimmune phenomenon. There is an interaction between T-bet and EGR2 where the latter could suppress Th1 phenotype and IFN-γ production(361). Through binding to the T-box domain of T-bet, EGR2 alter DNA binding which results in suppression of T-bet mediated differentiation of effector CD8 and Th1 cells. (376), In the context of a viral infection, Th1 and CD8 effector function would be important for viral clearance. First, it was observed a differential higher expression of T-bet in ILC2 in dengue infection in comparison to healthy samples. Unexpectedly, a low-level increase of T-bet expression was observed with PGD_2 activation, which was similar to the T-bet expression with IL-12 stimulation. IL-12 induced changes in ILC2 effector function, biasing the response towards ILC1-like cells producing IFN-γ(355). Although PGD_2 activation in ILC2 led to an increase T-bet expression it did not necessarily increase IFN-γ secretion, instead induced type 2 cytokine production. Therefore, it was suggested that EGR2 expression in ILC2 with PGD_2 activation could mediate suppression of T-bet function and assist ILC2 to maintain type 2 cytokine production. This suggests that in addition to TCR stimulation, EGR2 expression could be induced by PGD_2. Theoretically, EGR2 may assist ILC2 to maintain type 2 phenotype and thereby undergo activation and enhancement of dengue viral infection and thus could contribute to the pathogenesis of severe dengue infection. Further investigations need to be performed to show functionality of EGR2 in dengue infection.

6 Discussion and future perspectives

The RNA sequencing data presented here showed that ILC2 were highly activated in dengue infection compared to healthy samples. This result gave an early indication that ILC2 are involved in dengue infection. In addition to this, ILC2 frequency was found to be higher in severe disease. Usually in dengue infection a significant reduction in lymphocyte counts have been observed and unconventional T cells such as MAIT cells were decreased in parallel with the total lymphocyte count during the acute phase(223). Invariant NKT cell frequency remained static in severe disease and during the course of the illness(237). In addition to ILC2, follicular helper T cells (T_{FH}) which is a subset of CD4+ T cells providing B cell help was found to have higher frequency in severe disease. Frequency of T_{FH} peaked when reaching the critical phase of DHF(381). Given that ILC2 are resident in barrier tissues, these novel data raised the possibility that early effects of ILC2 at the time of the initial phase of infection, may play a role in the progression. Moreover, ILC2 activation markers were validated *in vitro* which showed that CD25 expression was dependent on PGD_2 mediated activation, and CD38 expression was dependent on IFN-β stimulation.

Additionally, it was shown that PGD_2 metabolites were found to be higher in patients with severe dengue compared to milder disease, and the PGD_2 metabolite levels were significantly increased during the 'critical phase' of dengue infection. Interestingly, PGD_2 could suppress protective type I interferon responses. This provides a novel perspective on lipid mediators which plays a role in ILC2 activation in dengue infection. Furthermore, detection of higher PGD_2 metabolites in dengue may translate to increased chemotaxis of ILC2 and Th2 cells and additionally could facilitate proliferation of ILC2. In asthma and allergy ILC2 numbers are higher and PGD_2 metabolites could significally increase early with allergen challenge(315). Therefore, we propose that individuals with

a background of atopy or allergy are predisposed for early PGD_2 metabolite responses, therefore, could have a propensity to have ILC2 activation and impaired type I IFN responses.

As discussed above, Malavige et al, have shown that lipid mediators such as LTE_4 are increased in severe dengue(123). It was shown that PGD_2 metabolites are increased in the critical stage of dengue in DHF. Severe dengue, vascular leakage and bleeding manifestation are usually assocated with secondary dengue infection through antibody dependent enhancement. Dengue virus induce significantly higher mast cell degranulation through an antibody dependent mechanism compared to direct infection alone(120). This signifies that high mast cell degranulation can occur in secondary dengue infection through a mecahnism involving ADE. We propose that higher mast cell degranulation in secondary dengue will lead to abundance of mast cell mediators such as PGD_2 and LTE_4 which mediate pronouced activation of ILC2. We showed activated ILC2 can mediate pronounced productive dengue viral replication. Therefore, we propose that mast cell degranulation in secondary dengue infection could lead to higher ILC2 activation than primary dengue infection leading to increased viral replication and potentially contribute to severe disease.

ILC2 are predominantly tissue resident innate cells that could play a role in responses to dengue virus after a mosquito bite on skin and contribute to productive viral infection. ILC2 could release higher infectious particles to infect bystander cells and additionally assist viral dissemination. Unexpectedly, the dengue viral genome was detected *in vivo* in peripheral blood ILC2 and we showed that *ex vivo* ILC2 from dengue patients had significant infection rates. Furthermore, *ex vivo* sorted and expanded ILC2 are permissive to infection *in vitro*, and could potentially assist viral dissemination. In fact, both blood and skin ILC2 were permissive to dengue infection, which was increased when ILC2

were activated. Other than PGD_2, IL-33 and LTE_4 activation increased dengue virus replication in ILC2, but to lesser degree than with PGD2. The effect of PGD_2 on infection and secretion of viral particles is striking and is further increased by co-activation with other ILC2 activators. Comparatively, tissue resident ILC1 in mice have been shown to promote early antiviral responses at the initial sites of infection via rapid production of IFN-γ before other known tissue-resident innate and adaptive lymphocytes. Furthermore, genetic ablation of ILC1 led to increased MCMV viral load in the liver and increased mortality in the presence of intact NK and T cell responses(208, 382). Therefore, activity of ILC2 promoting a type 2 cytokine environment as opposed to type 1 cytokine responses could be linked with severe dengue infection, as shown by previous authors(73).

The site of the mosquito bitten skin is characterised by an edema that retains virus at the inoculation site. This could lead to a strong inflammatory influx of innate cells at the site, inadvertently facilitating further virus infection by encouraging the entry and infection of virus-permissive myeloid cells(383). PGD_2 is a chemoattractant recruiting ILC2, Th2 and Tc2 cells to the site of infection. In addition to ILC2, we have shown that Th2 and Tc2 cells are permissve to dengue infection and undergo productive infection with PGD_2 activation. Essentially, ILC2 had higher productive infection than the Th2 and Tc2 cells. Comparatively, pan- T cells had very low infection rates compared to enriched Th2, Tc2 and ILC2 cells. This signifies that type 2 cytokine producing innate and T cell conunterparts assist dengue viral infection and likely disseminate infection. Interestingly, mosquito saliva also affects serum cytokine levels, with the most notable trend being an increase in type 2 cytokines compared to unbitten, control mice(384). This type 2 cytokine environment persisted until 7 days post-bite and was relevant to the context of allergic reactions(384). Although type 2 cytokines such as IL-13 and IL-4 were important

in mediating infection in DCs and macrophages, we showed that autocrine PGD_2 production, but not IL-13/4 production increased infection in ILC2.

Moreover, GM-CSF produced by ILC2 increased infection in diffferentiating monocytes. These expriments showed that other mediators supporting a type 2 cytokine environment such as PGD_2 and GM-CSF can assist dengue viral replication in innate cells. This indicate that the initial type 2 cytokine niche in the tissue would be important for the subsequent systemic response in dengue infection. We have shown that dengue infection correlated with greater receptor expression of DC-SIGN and mannose receptor in differentiating monocytes. Mannose receptor mediates attachment of dengue virus to monocytes. Blocking DC-SIGN has previously been shown to modulate dengue virus infection of monocytes (291).

6.1 Translational potential of CRTH2 blockade in dengue infection.

We propose that inhibiting action of CRTH2 activation would have favorable responses in reducing productive infection in ILC2, Th2 and Tc2 cells. Moreover, due to the fact that PGD_2 suppresses expression of anti-viral genes and proteins, inhibiting PGD_2 responses may have a favourable outcome for anti-viral defense. PGD_2 is a metabolite in the arachidonic pathway and previous studies have shown that this pathway is activated in dengue (109) and downstream lipid mediators such as leukotrienes are shown to contribute to pathogenesis of dengue and vascular leakage(40, 123). Although inhibition of the CRTH2 pathway could lead to reduction of viraemia of dengue infection, it has been clinically observed that use of NSAIDs, which block COX 1 and 2 enzymes at an early step of the arachidonic acid pathway, is associated with higher mortality and bleeding manifestations and derangement of liver enzymes(385, 386). Since, arachidonic acid pathway leads to many downstream effects through a range of mediators, this outcome could occur either due to inhibition of protective mediators of dengue infection

or due to additional side effects of these medication outside arachidonic acid pathway. One of the downstream mediators TXA_2, which mediates vasoconstriction and platelet aggregation, is suggested to play a protective role in dengue infection(387). Additionally, PGD_2 shows clues of a protective role through DP1 activation, which is discussed later. Furthermore, PGE_2 has shown to increase viral replication(115), but suppresses ILC2 activation(181, 388).

Therefore, we propose that inhibition of the CRTH2 receptor could have therapeutic potential in dengue viral infection. CRTH2 inhibitors are investigated in allergic diseases such as asthma and allergic rhinitis and considered to be well tolerated given orally(314, 389, 390). Such medications are more economically affordable comparatively, especially for tropical countries. Interestingly, dengue viral infection shares certain features of allergic inflammation such as mast cell activation and release of mast cell mediators such as leukotrienes and histamines(123). Furthermore, vascular leakage, which is a clinical hallmark of severe dengue infection, was shown to occur due to increased capillary permeability. Anaphylaxis and urticaria been have shown to have a similar pathogenesis (144) with altered capillary permeability, although the plasma leakage is much more pronounced in dengue infection. In addition, a randomised clinical trial using oral CRTH2 blockade in asthma, observed reduced infections, including influenza in asthmatics in the CRTH2 blockade group (391). However, Fevipiprant, the CRTH2 blocking medication, did not show a statistically significant reduction in asthma exacerbations, but had a modest reduction in exacerbation rates at a higher dose(389). These data suggest a novel mechanism of inhibition of ILC2 to modulate dengue viral replication. It is still unclear that these beneficial effects can be extended to other viral infections. Comparatively, activation through PGD_2 led to higher infection than other individual activators and endogenous PGD_2 production from ILC2 amplified the effects

on viral replication. This is of significant functional relavance to the autocrine activation of ILC2 that may amplify dengue viral replication.

6.2 Mast cell based therapies in dengue infection

Furthermore, it has been shown that mechanical skin injury, similar to scratching, can lead to mast cell (MC) expansion in a ILC2 dependent manner. In fact, keratinocytes released IL-33 that was detected systemically after skin injury, activating ILC2 resulting in IL-4 and IL-13 mediated MC expansion and propagation of allergic inflammation(312). In addition, in patients with systemic mastocytosis with D819V somatic mutation, which have a high mast cell burden, had significantly higher ILC2 in peripheral blood than healthy controls and the Th2 cell frequency was increased although CD4+ T cell frequency was not different. This is likley to be due to excessive mast cell activation and release of mediators such as PGD_2(392). These studies signify the cross-talk between ILC2 and MCs in a setting of allergic inflammation. Mosquito bites usually give rise to urticaria, which suggests MC activation in the mosquito bitten skin. In addition, mediators of allergic inflammation such as histamine, leukotrienes (123) were found to be elevated in severe dengue and MCs were found to be infected and activated in dengue infection(40). These effects are much more pronouced in secondary dengue(122), which is associated with higher morbidity and mortality. In addition, *in vitro* studies showed that sensitisation of MCs with 4G2 significantly increased the magnitude of MC degranulation in response to DENV2 infection, compared to virus exposure alone. Further increase in concentration of 4G2 antibody resulted in increasing MC degranulation in a dose-dependent fashion(119), suggesting increased degranulation of MCs in the presence of pre-formed antibodies, as seen in individuals with previous dengue disease. From what is known MC degranulation increases endothelial permeability *in vitro*, and also promotes vascular leakage *in vivo*. Therefore, higher

activation of MCs in subsequent dengue infection could enhance the extent of vascular leakage during DENV infection(122).

In addition we found that PGD_2 is elevated in severe dengue, which strengthens the mast cell involvement in dengue infection. Through this mediator, a range of type 2 cells in innate and adaptive immune system are activated and likely contribute to the pathogenesis of dengue. Therefore, it is possible that individuals who are predisposed to atopy or allergy may have a more pronounced type 2 response in the bitten site, which could influence the earliest events of viral replication.

MCs are known to secrete range of molecules, including proteases such as chymase and tryptase, vasoactive amines such as histamine and serotonin, lipid mediators such as LTB_4, LTE_4, PAF, PGD_2, PGE_2 and proteoglycans such as heparin. Most of these mediators have shown to be elevated in severe dengue(109, 146, 153, 322). In addition, serotonin induces platelet activation, aggregation and induces thrombocytopenia in dengue infection(393). Although, vascular leakage has not been linked to serotonin in dengue infection, murine models have shown that activation of 5-HT receptors prompted significant plasma extravasation in the airway and upper gastrointestinal tract and this was reduced by pharmacological inhibition of the 5-HT2A receptor(394). Additionally, vascular endothlial frowth factor (VEGF) and sVEGFR-1 levels are significantly increased in DHF and DSS compared with those having DF and healthy controls(151).

Given the consistent evidence of MC activation in dengue infection, several MC mediator based therapies have been investigated. Although primary reduction of DHF was not observed, in the post-hoc analysis, patients who received rupatadine, which is a PAF and histamine blocker, had lower vascular leakage manifestations and bleeding(395). This suggested early initiation of Rupatadine, less than 3 days from onset of symptoms, may

have beneficial effects. However, these data are from a post-hoc analysis and may be associated with some bias. Furthermore, montelukast, a CysLT1 receptor inhibitor, which blocks the leukotriene pathway, showed a significant reduction of the risk of having DSS in dengue infection(396). In an *in vivo* animal model, a potent tryptase inhibitor, nafamostat mesylate was shown to reduce DENV induced vascular leakage(153). In addition to above, we suggest that CRTH2 blocking medications could be used to reduce productive viral infection and reduce the supression on interferon pathways induced through PGD_2. However, an effect of CRTH2 blockers on vascular leakage would an interesting avenue to be explored. Due to the broad link of MC mediators and dengue infection, MC stabilisers have also been examined as options for treatment of dengue. MC-stabilizing drug ketotifen and cromolyn have shown to reduce vascular leakage in *in vitro* models(397). Ketotifen has been shown to reverse transcriptome changes induced by dengue virus in an immunocompetent mouse model(398). In addition to dengue virus, MC mediators are shown to increase lung injury in influenza animal models. Therefore, ketotifen administered in influenza models showed a reduction in epithelial cell apoptosis. Moreover, combination of ketotifen and oseltamivir imporved survival than either alone(399). Moreover, vitamin D has been shown to have a mast cell stabilising action(400) and vitamin D levels are found to be reduced in patients with dengue infection(401). High dose vitamin D therapy has been shown to associate with decreased pro-inflammatory cytokine production and increased resistance to dengue virus in macrophages differentiated from monocytes obtained from healthy donors(402). Interestingly, vitamin D deficiency was strikingly high in Sri Lankan children with obesity, approximating 75%(403). It is still unexplored whether vitamin D supplimentation will have any morbidity benefit in dengue infection, through mast cell stabilisation mechanism. Moreover, in addititon to the above, CRTH2 inhibitors could be

explored as a medication to treat dengue viral infection with additional benefits on viral replication and IFN signaling.

6.3 Diverse actions of PGD_2 through DP1 receptor

PGD_2 acts through both DP1 and DP2/CRTH2 receptors. In murine models, action of PGD_2 through DP1 receptor, mediates the attenuation of anaphylaxis. Also, systemic DP1 deficiency, but not CRTH2 receptor deficiency ($CRTH2^{-/-}$) significantly increased dye extravasation compared with that seen in WT mice. Interestingly, *in vivo* DP1 activation strongly enhances endothelial barrier formation(111). The mechanism of this is thought to be through nitric oxide (NO) production. PGD_2 inhibits nitric oxide production through activation of DP1 receptors in endothelial cells. In addition, deletion of the DP1 receptor enhanced croton oil-induced vascular hyper-permeability *in vivo* (404). This signaling is also involved in the endothelial barrier enhancement mediated by DP1 receptors in anaphylaxis.

As such, the effect of PGD_2 and its metabolites in vascular leakage would be worth investigating. Nakamura *et al* have demonstrated that mast cell-derived PGD_2 inhibits murine anaphylaxis. In murine models, DENV-injected tissues have been shown to contain many activated MC located near vessels with evidence of MC degranulation. Additionally, authors have observed evidence of fluid pooling or oedema in infected tissues. This signifies that MCs could be involved in dengue vascular leakage and possibly with severe disease manifestations(122).

From our data we have shown that DP1 agonists alter the phenotype of differentiating monocytes and reduced dengue viral infection significantly. In a previous study on RSV viral infection, DP1 agonists induced IFN-λ expression and induced downstream antiviral responses(287). PGD_2 significantly suppressed IFN-γ production from iNKT cells

through a DP1 receptor mediated pathway(405). However, although DP1 agonist BW245c decreased the severity of bronchiolitis in virus infected mice with increased levels of IFN-λ production and decreased viral loads, and this unexpectedly increased mortality in mice. This unexpected finding was associated with viral spread to the parenchyma, and an increased cellular infiltrate of TNF-α-producing cells. Thereafter, the authors observed that this increase in mortality could be significantly attenuated following the administration of anti-TNF-α to DP1 agonist treated mice(406). Therefore, it is still unclear how DP1 agonists could influence dengue viral replication in a broader perspective. Additionally, why PGD_2 is pro-inflammatory in one setting while it has anti-inflammatory actions in others needs further investigation. Moreover, PGD_2 has been found to suppress cytotoxicity of NK cells(407) and reduced type I cytokine responses. This action of PGD_2 was shown to be mediated through DP1 receptor and NK cells had a low expression of CRTH2/DP2 receptor. CD16 cross linking induced calcium mobilisation in NK cells is an essential step for cytolytic function, and DP1 agonists have shown to blunt the release of calcium to carry out these cellular functions(408). This suppressing effect of PGD_2 on NK cell cytotoxicity and IFN-γ production coupled with stimulatory effects on type 2 immune cells could lead to an overall bias toward a type 2 immune response. Additionally, PGD_2 has been shown to induce phenotypic changes in moDCs leading to impaired antigen presentation(345) and modulation of T cell responses. The initial data from the current project on DP1 receptor actions of PGD_2, opens another relevant aspect for dengue pathogenesis which is worth exploring.

7 Conclusions

Here we present data that implicate ILC2 in the immunopathology of dengue virus infection. PGD_2 mediates activation and significantly influences productive viral infection in type 2 cytokine producing cells. Moreover, PGD_2 downregulates type I IFN induced anti-viral responses in ILC2, which can be rescued by exogenous type I IFN. By co-opting a type 2 pathway, dengue virus escapes early type I IFN control and aids viral dissemination directly through enhanced ILC2 infectivity and indirectly through enhanced monocyte infection. With these insights we propose that inhibition of the PGD_2 pathway through CRTH2 as a therapeutic avenue to modulate viral replication.

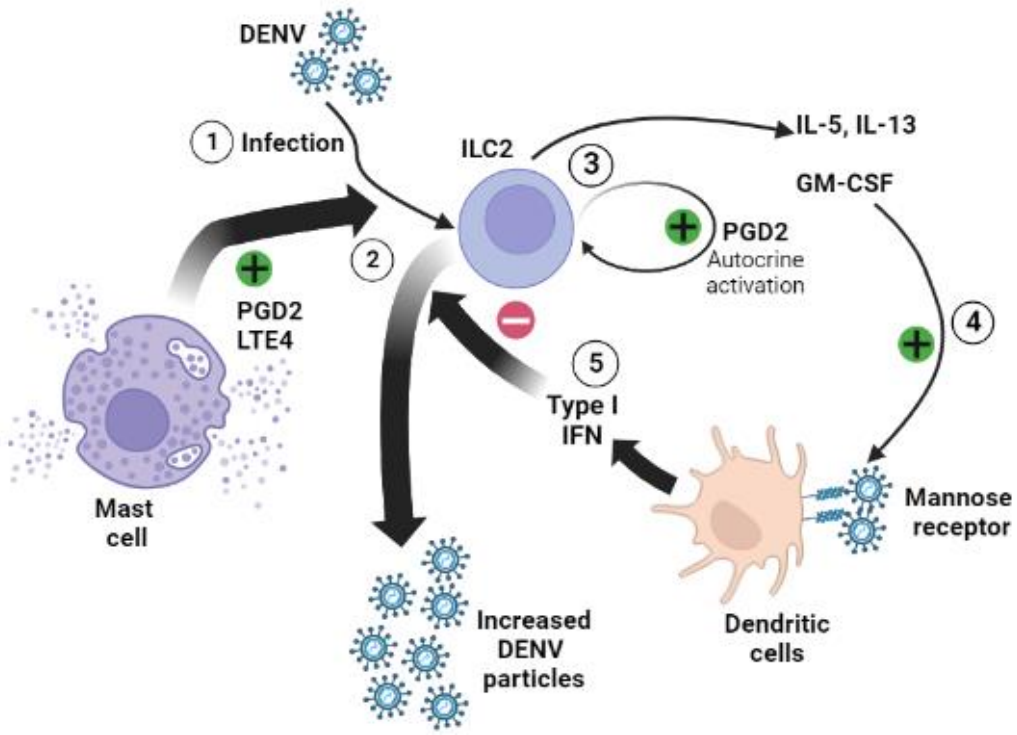


Figure 37: Graphical diagram: 1) ILC2 are permissive for dengue virus infection. 2) Mast cell produced PGD_2 & LTE_4 increase DENV infection of ILC2 through activation and lead to productive viral replication. 3) ILC2 activation mediate production of type 2 cytokines including GM-CSF and PGD_2. Latter mediate autocrine activation and

contribute to productive infection. 4) GM-CSF increase expression of mannose receptor and facilitate DENV infection in differentiating monocytes/dendritic cells. 5) Type I interferons produced by infected dendritic cells inhibit ILC2 infection.

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