STUDIES ON IMMUNE IMPAIRMENT IN CHRONIC HEPATITIS C VIRUS INFECTION

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ABSTRACT

Hepatitis C virus (HCV) is a blood-borne pathogen that infects hepatocytes and causes widespread destruction to the host immune system. Estimates suggest that HCV has infected ~200 million people worldwide, and ~350,000-500,000 people die every year from HCV-associated hepatic and extra-hepatic complications. HCV has developed numerous mechanisms to evade host immune responses to establish persistence. Due to the lack of a preventive vaccine, the mainstay of current treatment is a combination therapy with interferon-α and ribavirin. The molecular mechanisms underlying the establishment of persistent HCV disease remain poorly understood. A better understanding of these mechanisms would aid design newer therapeutic targets and improve the quality of life of HCV-infected patients

Here, we aimed to investigate the role of spontaneous apoptosis of immune cells, expansion of senescent and exhausted virus-specific T-cells, as well as potential depletion of circulating mucosal-associated invariant T (MAIT) cells, with immune impairment in chronic HCV (CHC) disease. We recruited 62 chronically-infected HCV patients and 62 healthy controls (HCs) to conduct a cross-sectional investigation. Peripheral blood mononuclear cells (PBMCs) were isolated, and co-cultured with HCV antigens and phytohaemagglutinin (PHA) individually prior to the investigations (except for apoptosis). Flow cytometry, quantitative real-time PCR (qRT-PCR), ELISA and QuantiGene Plex 2.0 analyses were employed to investigate apoptosis in immune cells. Multiparametric flow cytometry approaches were utilized to examine the phenotypes of immunosenescent T-cells and expression of co-inhibitory molecules on HCV-specific T-cells, together with frequencies of circulating MAIT cells. Expression of molecules associated with T-cell inhibition was confirmed by qRT-PCR.

The ability of HCV to induce apoptosis in immune cells correlated with the increase of apoptotic cells (Annexin V+PI+) and cellular reactive oxygen species (ROS). QuantiGene Plex 2.0 analysis showed differential regulation of apoptotic pathways involved in mitochondrial or activation of death receptors. Besides, the onset of immunosenescence was clearly evident from the up-regulation of HLA-DR, CD38, CD57 and CD127 on HCV-specific CD4+ and CD8+ T-cells of chronic HCV-infected patients. Furthermore, chronic HCV-infected patients displayed relatively significant increase of late-differentiated T-cells. Chronic HCV infection also resulted in significantly increased expressions of PD-1, CTLA-4, CD160 and TRAIL on HCV-specific CD4+ and CD8+ T-cells suggestive of immune exhaustion. Increase in the levels of pro-inflammatory cytokines was also observed in PBMC cultures of chronic HCV-infected patients. MAIT cells of HCs expressed elevated levels of CCR5 and CCR6. Conversely, all these receptors were down-regulated on the circulating MAIT cells of chronic HCV-infected patients. Expression of PD-1 was also higher on MAIT cells of chronic HCV-infected patients relative to controls.

In conclusion, our observation suggests the spontaneous onset of apoptosis signaling in chronic HCV disease, and increased frequency of late-senescent T-cells that lack the potential to survive, possibly contributing to viral persistence. These phenotypically defective HCV-specific T-cells may likely contribute to inadequate virus-specific T-cell responses. Decreased frequency of MAIT cells with elevated levels of PD-1 may result in diminished mucosal defense attributes, and could potentially contribute to HCV disease progression.

ABSTRAK

Virus hepatitis C (HCV) adalah satu patogen darah yang menjangkit sel-sel hati dan sistem imun. Dianggarkan sekitar ~ 200 juta orang diseluruh dunia dijangkiti dengan hepatitis C, dan sebanyak ~ 350000-500000 orang mati setiap tahun daripada manifestasi klinikal yang berkaitan dengan hepatitis C. HCV telah membangunkan pelbagai mekanisme untuk menangkas tindakbalas perlindungan sistem imun untuk mengakibatkan jangkitan kronik yang berpanjangan, dan mekanisme molekul yang mendasari penubuhan jangkitan kronik yang berpanjangan tidak difahami. Dengan memahami mekanisme ini akan membantu menghasilkan sasaran terapeutik yang lebih berfaedah kepada manusia. Malangnya, masih belum ada vaksin untuk mencegah hepatitis c dan pilihan rawatan hanya dengan interferon-alfa dan ribavirin.

Di sini, kami bertujuan untuk meneroka induksi apoptosis secara spontan pada sel-sel sistem imun, pengembangan penuaan pra-matang dan kelesuan sel-sel T yang khusus untuk virus serta kekurangan sel-sel "mucosal-associated invariant T" (MAIT) dalam darah yang berkaitan dengan kemerosotan sistem imun dalam penyakit hepatitis C kronik seperti. Untuk tujuan perbandingan, enam puluh dua pesakit hepatitis C kronik dan enam puluh dua donor darah telah menyertai kajian hirisan lintang ini. Sel mononuklear dari subjeck kajian telah diasingkan and ditumbuhkan dengan antigen HCV dan phytohemagglutinin (PHA) secara individu sebelum assays (kecuali kajian apoptosis). Sitometri aliran, kuantitatif real-time PCR (qRT-PCR), ELISA dan QuantiGene plex 2.0 analisis telah digunakan untuk menyiasat induksi apoptosis pada sel sistem imun. Pendekatan seperti multiparametric sitometri aliran telah digunakan untuk memeriksa proses penuaan pra-matang dan ungkapan penghalang reseptor pada sel-sel T yang khusus untuk HCV berserta dengan frekuensi sel-sel MAIT dalam darah. Ungkapan penghalang reseptor terhadap sel T juga disahkan dengan qRT-PCR.

Keupayaan HCV untuk menginduksi apoptosis pada sel sistem imun korelasi dengan peningkatan “apoptotic bodies” (Annexin V+PI+) dan spesies oksigen reaktif. QuantiGene Plex 2.0 analisis menunjukkan induksi laluan apoptotic berkenaan dengan mitokondria atau pengaktifan reseptor kematian. Selain itu, pra-matang penuaan sel sistem imun disaksikan oleh ungkapan HLA-DR, CD38, CD57 dan CD127 pada sel CD4 dan CD8 yang dari pesakit HCV kronik. Selain itu, pesakit HCV kronik juga memaparkan peningkatan penuaan pra-matang sel-sel T. Jangkitan HCV turut menyebabkan peningkatan kelesuan sel CD4 dan CD8 yang khusus untuk HCV mengungkap reseptor PD-1, CTLA-4, CD160 dan TRAIL. Peningkatan cytokines radang juga diperhatikan pada PBMCs yang diperolehi dari pesakit HCV kronik. Sel-sel MAIT donor darah menyatakan tahap CCR5 dan CCR6 yang tinggi. Sebaliknya bagi pesakit HCV, ungkapan reseptor tersebut telah dikawal ketat. Manakala, ungkapan PD-1 adalah lebih tinggi pada sel-sel MAIT daripada pesakit berbanding dengan donor darah.

Kesimpulannya, pemerhatian kami menunjukkan pembentukan apoptosis secara spontan dalam penyakit HCV kronik, dan peningkatan kekerapan penuaan pra-matang sel T yang tidak mempunyai kemampuan regenerasi, boleh menyumbang kepada pengekalan virus di hadapan jangkitan berterusan. Ketidaksempurnaan fungsi sel-sel T yang khusus untuk HCV boleh menyumbang kepada pembentukan tindakbalas sel T virus tertentu yang tidak mencukupi. Kekurangan frekuensi sel-sel MAIT dalam darah berserta dengan ungkapan PD-1 yang tinggi boleh menyumbang kepada kekurangan perlindungan dan perkembangan penyakit HCV yang ketara.

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Barathan Muttiah

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-mer	the length of an oligonucleotide
°C	degree celcius
%	percentage
µg	microgram
µl	microliter
AICD	Activation-induced cell death
AIDS	acquired immune deficiency syndrome
ALT	alanine transaminases
APAF1	apoptotic-protease-activating factor
APC	antigen presenting cell
APC	allophycocyanin
AST	aspartate
B cell	Bone-marrow-derived lymphocyte
BAX	Bcl-2-associated X protein
Bcl2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BID	BH3-interacting –domain death agonist
Blimp-1	B lymphocyte-induced maturation protein-1
BTLA	B- and T-lymphocyte attenuator
CCR5	chemokine receptor 5
CCR6	chemokine receptor 6
CD	cluster of differentiation
CD4+	helper T cell
CD8+	cytotoxic T cell
CHC	chronic hepatitis C
CIA	chronic immune activation
CMV	cytomegalovirus
COX-2	cyclooxygenase-2
cROS	cellular reactive oxygen species
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CVI	chronic viral infection
Cy	cyanine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTX-1	deltex 1
E	envelope
EBV	epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FADD	Fas-associating protein with death domain
FasL	Fas ligand
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FOXP3	fork-head box transcription factor P3

gp	glycoproetins
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
HC	healthy control
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IDO	indoleamine 2, 3-dioxygenase
IFN-α	interferon alpha
IL	interleukin
iNKT	invariant natural killer T-cells
IU	international unit
K2EDTA	dipotassium ethylenediaminetetraacetic acid
kDa	kilodalton
KLRG1	killer-cell lectin like receptor G1
LAG-3	lymphocyte activation gene 3 virus
LCMV	lymphocytic choriomeningitis
MAIT	mucosal-associated invariant T
MHC	major histocompatibility complex
ml	millilitre
mM	millimolar
MOMP	mitochondrial outer membrane permeabilization
MR1	MHC-I-like related protein
n.d.	no date
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NS	non-structural
ORF	open reading frame
PARP	Poly ADP-ribose polymerase
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD-1	programmed death -1
PE	phycoerythrin
PEG	polyethylene glycol
PerCP	peridinin chlorophyll protein
PGHS	prostaglandin G/H synthase
PHA	phytohaemagglutinin
PI	propidium iodide
qRT-PCR	quantitative real-time PCR
RBV	ribavirin
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SEM	standard error mean
ssRNA	single-stranded ribonucleic acid
TB	tuberculosis
tBID	truncated BID
T-cell	T lymphocyte
TCR	T cell receptor

TGF-β	transforming growth factor beta
Th	T helper
TIM-3	T-cell immunoglobulin mucin domain-3
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
TNFR	TNF receptor
TRADD	TNFR1-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
UMMC	University Malaya Medical Centre

CHAPTER ONE

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Background

Hepatitis C virus (HCV) is a blood-borne pathogen that predominantly infects the epithelial cells of the liver (Chen & Morgan, 2006). Estimates suggest that HCV has infected ~200 million people worldwide, and at least 350000 people die annually from HCV-associated hepatic and extra-hepatic complications (Zignego *et al.*, 2012). Similar to other chronic viral infections (CVIs), HCV appears to harness certain immune evasion strategies to facilitate progression to chronic disease in infected individuals (Xing & Hogquist, 2012). Hence, the current research aims to address certain important immunological concepts that underlie the pathogenesis of chronic HCV infection.

The immune impairment attributes in chronic HCV disease largely remain ambiguous. Accumulating lines of evidence suggest that mechanisms underlying immune impairment are multi-factorial in CVIs. Suggested mechanisms include spontaneous T-cell apoptosis (Zhang *et al.*, 2005), immunosenescence) (Fülöp *et al.*, 2013), exhaustion of virus-specific T cells (Wherry *et al.*, 2007) as well as depletion of circulating mucosal-associated invariant T (MAIT) cells in chronically-infected individuals (Leeansyah *et al.*, 2013). These mechanisms result in impaired responses to foreign antigens and impart sub-optimal regulatory functions in bystander T-cells, which concertedly allows the virus to adapt to intracellular antiviral pressure (Yi *et al.*, 2010).

In a cross-sectional cohort that consisted of chronic HCV-infected patients and healthy controls (HCV negative), we investigated certain immune impairment attributes using various molecular methods. Our investigations will serve as a prelude to understand the role of certain immune impairment mechanisms operational in chronic HCV disease, and will largely be applicable for intervention strategies to improve the quality of life of HCV-infected individuals.

1.2 Objectives

1) To investigate the role of apoptosis in *ex vivo* cultured PBMCs of chronic HCV-infected patients.
2) To study the *in vitro* expression of senescence and activation markers on HCV-specific T-cells of chronic HCV-infected subjects.
3) To investigate the *in vitro* expression of co-inhibitory molecules on virus-specific T-cells and secretion of Th1/Th2/Th17 cytokines in chronic HCV infection.
4) To determine the frequency and phenotypes of circulating MAIT cells in chronic HCV infection.

It has been hypothesized that HCV induces apoptosis in immune cells via the mitochondrial pathway and/or activation of death receptors. Current literature also speculates the likely role of immunosenescence and T-cell exhaustion leading to sub-optimal anti-HCV responses. Further, the role of MAIT cell exhaustion has been seldom investigated in chronic HCV infection. The findings of this research could provide impetus to understand various hitherto unexplained mechanisms potentially operational in chronic HCV infection. Further, this will also aid in the designing of better therapeutic targets, especially the different immune checkpoints investigated aimed at improving host defense attributes in chronic HCV infection.

CHAPTER TWO

REVIEW OF LITERATURE

CHAPTER 2: REVIEW OF LITERATURE

2.1 Hepatitis C Virus

Hepatitis C virus (HCV) is an RNA virus belonging to genus *Hepacivirus* within the *Flaviviridae* family (Pfaender *et al*., 2014). HCV reportedly displays a significant genetic diversity across the global population, and is classified into 7 genotypes and as many as 90 subtypes accounting for their varied levels of persistence potentials in the host, and diverse susceptibility attributes to antiviral drugs (Messina *et al*., 2015). Such variations result in the generation of HCV quasispecies, which primarily results from the error-prone nature of viral replication and reduced proof-reading ability of viral RNA polymerase (Elena & Sanjuán, 2005).

Hepatitis C is an infectious disease that triggers inflammation of the liver, which ranges in severity from acute illness lasting for a few weeks to life-long chronic disease (Park & Rehermann, 2014). Liver is the essential site of HCV replication. Growing evidence suggests that HCV can also propagate within extra-hepatic sites, including cells of the lymphoid and central nervous systems (Revie & Salahuddin, 2011). Studies suggest that HCV positive and negative strands were detected from the peripheral blood mononuclear cells (PBMCs) and bone marrow of chronically HCV-infected individuals (Castillo *et al*., 2005). The disease is spread via multiple blood transfusions and organ transplants (largely before 1992), injecting drug use, vertical, and sexual contact with infected individuals. Besides, body piercing and tattooing with infected tools have also been identified as other modes of transmission (Strader *et al*., 2004).

2.1.1 Structure

HCV has a spherical shape. It contains an RNA genome enclosed within icosahedral capsid structure, which is further enclosed within a lipid envelope derived from the host cell membrane (Khan *et al*., 2014). Two highly conserved viral glycoproteins, E1 (gp31) and E2 (gp70) are embedded within the lipid envelope (Bartenschlager, 1997).

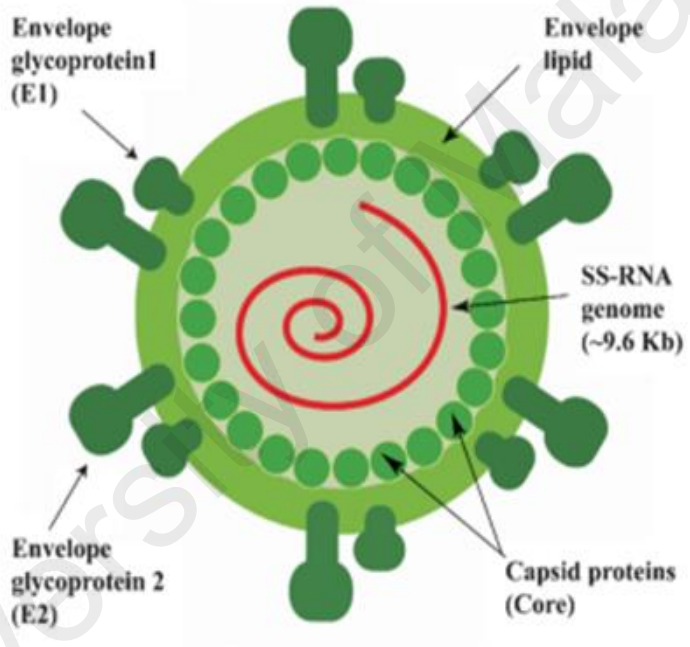

Figure 2.1: Structure of Hepatitis C Virus (Sharma, 2010).

The HCV genome comprises a single-stranded, positive-sense RNA (ssRNA) molecule of 9.6 kb with one long open reading frame (ORF) coding for a large polyprotein of ~3000 amino acids, which endures co- and post-translational proteolytic cleavage by host and viral proteases to produce various structural and non-structural (NS) proteins (incorporated). The structural proteins produced by HCV include core proteins, (p21), E1, E2 and P7; and non-structural proteins comprise NS2 (p21), NS3 (gp70), NS4A (p8), NS4B (p27), NS5A (p58), and NS5B (p68).

The HCV core protein together with other cellular proteins, affect the cellular functions of the host. The E1 protein aids as the fusogenic subunit whereas E2 acts as the receptor-binding subunit of the HCV envelope. P7 protein helps assembly and release of virions in the host. The NS2 protein acts as a protease and is vital for the completion of viral replication cycle. The polyfunctional NS3 protein acts as a serine protease, and inhibits the host's innate cellular defense functions. The RNA helicase plays an essential role in viral RNA replication. NS3 acts as a translocase with unidentified functions. NS4A is a cofactor for NS3 that guides the localization of NS3 and controls it's enzymatic activities.

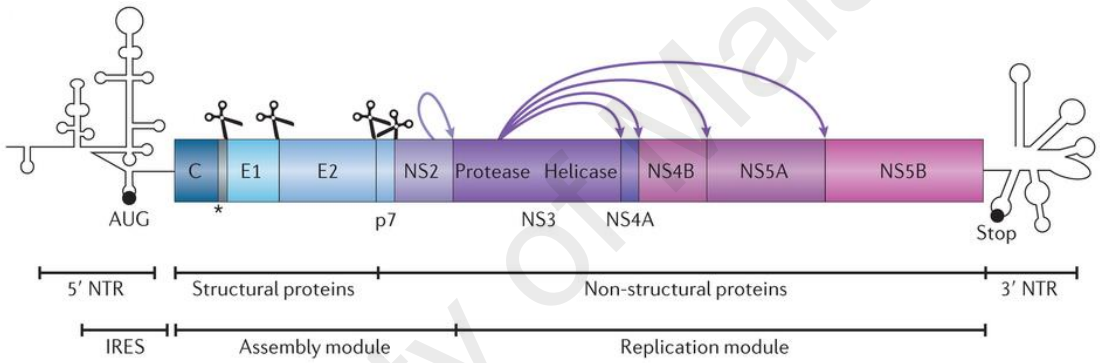

Figure 2.2: Genome Structure of Hepatitis C Virus (Bartenschlager *et al*., 2013).

NS4B is a small hydrophobic 27 kDa protein that plays an important role in the recruitment of other viral proteins. NS5A is a hydrophilic phosphoprotein believed to play a role in viral production, modulation of cell signaling pathways and interferon (IFN) responses. NS5B acts as a RNA-dependent RNA polymerase (RdRp), and plays an important role in the production of new RNA genome (incorporated) (Nielsen *et al*., 2004; Cristofari *et al*., 2004; Krekulová *et al*., 2006; Dubuisson, 2007).

Figure 2.3: Structural and Non-structural Proteins of HCV (Bartenschlager *et al*., 2013).

2.1.2 Epidemiology

The latest global estimates show an increase in HCV seroprevalence over the last 15 years to 3.3%, and as much as 200 million individuals are reportedly infected with the virus (Messina *et al*., 2015). HCV infection is a pandemic owing to its wider degree of geographic variability and distribution. HCV prevalence is reported to be high, ~5-10% in Egypt, Cameroon, Hubei, Mongolia, as well as Pakistan (Khan *et al*., 2010). Several other countries across Latin America, Eastern Europe and the former Soviet Union, Africa, Middle East, and South Asia have reported >2% prevalence, whereas <2% prevalence rate has been reported from the UK, Scandinavia, USA, Western Europe, Australia, and South Africa (Sy & Jamal, 2006; Martins *et al*., 2010). In regards to the absolute number, Australia and Oceania (400,000), USA (14 million), Middle East (16 million), Europe (17.5 million), Africa (28 million) and Asia (83 million) have most of the chronically-infected individuals (Salam & Akimitsu, 2013). Genotype 1 has the largest geographical distribution, as it is reportedly the most common genotype across most of North America, Northern and Western Europe, South America, Asia and Australia. Meanwhile, endemic strains from genotypes 2 and 3 are seen in West Africa, and South Asia, respectively. In Central Africa and Middle East, genotype 4 was

prominent, but data from past decade indicates that HCV genotype 5 is observed among individuals from Southern Africa. Studies from East and Southeast Asia have reported an increase in the prevalence of HCV genotype 6 over the years. Genotype 6 is also predominant in Hong Kong and Southern China. Till date, only one report of infection with genotype 7 has appeared in the literature, from Canada, in migrant from central Africa (incorporated) (Hajarizadeh *et al*., 2013; Messina *et al*., 2015).

The most common mode of HCV transmission is via sharing of contaminated needles or receiving blood or blood products (Averhoff *et al*., 2012). Malaysia has a prevalence of 2.3%, and falls under the intermediate zone of HCV prevalence. HCV is also the leading infection among individuals undergoing multiple blood transfusions (Mohd Hanafiah *et al*., 2013). Recent estimates show that over 400,000 Malaysians are infected with HCV. Nonetheless, analyses suggest that this figure could be an under-estimate as many are probably unaware of their positive infection status. Estimates also suggest that about 80% of HCV-infected patients are aged between 25 and 55 years, and only 4 out of 7 genotypes are found in Malaysia. Genotypes 1 (39%), 2 (4%), 3 (56%) and 4 (1%) have been reported from Malaysians (Mazlam, n.d.).

Figure 2.4: Global Epidemiology and HCV Genotype Distribution (Hussain, 2013).

2.1.3 Natural History of Hepatitis C Disease

HCV causes both acute and chronic liver disease. Acute infection is characterized by the presence of clinical symptoms of hepatitis for at least 6 months, and elevation of liver enzymes following initial infection (Westbrook & Dusheiko, 2014). Acute HCV disease shows the presence of anti-HCV antibody (seroconversion) and HCV RNA. Nonetheless, acute disease that occurs within 5–12 weeks of virus exposure, is only detected in ~15–30% of individuals, and lasts for about 2–12 weeks. Majority of patients remain symptomless during the acute phase, and therefore are seldom considered for diagnosis. It also appears that some acutely-infected individuals achieve spontaneous viral clearance. However, failure of spontaneous virus clearance leads to progression to chronic HCV disease. Overall, ~85% of the acutely-infected individuals progress to develop chronic illness where HCV RNA persists for >6 months of initial exposure. Nearly about half of chronic cases do not progress further, whereas 20% of chronically-infected individuals tend to experience liver scarring (cirrhosis) over a period of 50 years (Tsoulfas *et al*., 2009). Almost 20% of chronic HCV-infected cases develop fibrosis and cirrhosis over a period of 20-30 years (Sulkowski & Thomas, 2005; Kamal, 2014; Lee *et al*., 2014).

Patients progressing to cirrhosis may likely develop hepatic encephalopathy and hepatocellular carcinoma (HCC) requiring liver transplantation.

2.1.4 Treatment

The current treatment for chronic HCV infection is the mixture of pegylated IFN-α and ribavirin (RBV). Conjugation of polyethylene glycol (PEG) side-chain with IFN-α confers an extended bioavailability, warranting only weekly injections rather than three injections per week (Yasin *et al*., 2011). The dosage and length of treatment are based on the HCV genotypes involved (Ghany *et al*., 2009). For HCV genotypes 1, 4, 5 and 6, pegylated IFN-α and RBV treatment is done over a period of 48 weeks. Meanwhile, 12-24 weeks of treatment for genotypes 2 and 3 results in undetectable virus levels at the end of treatment, also known as sustained virologic response (SVR), which is reportedly ~40-50% for genotype 1 and ~80% or more for genotypes 2 and 3 (Zeuzem *et al*., 2009). The newly designed oral regimens such as simeprevir and sofosbuvir work directly by interfering with HCV replication (Elbaz *et al*., 2015) although simeprevir appears to be effective only against genotype 1. Sofosbuvir, a polymerase inhibitor utilized in conjunction with peg-IFN and RBV, is prescribed for patients with genotypes 1, 4, 5 and 6, and results in an SVR of 90% at the end of 12 weeks of treatment (Shiffman, 2014). Currently, there is no vaccine available against HCV, largely owing to the lack of an effective small animal model or cell culture system. Furthermore, the huge genetic heterogeneity of HCV warrants a number of different vaccines to individually protect against each genotype (Scheel & Rice, 2013).

2.2 HCV Persistence

Epidemiological data suggests that only ~15% of the infected individuals spontaneously clear the virus in the first 6 months owing to robust HCV-specific CD4+ (helper) and CD8+ (cytotoxic) T-cell responses that sustain for decades after virus clearance (Barrett *et al.,* 2005; Koziel, 2005; Larrubia *et al.,* 2014). Other factors associated with

spontaneous viral clearance include ethnicity, lack of co-infection (mainly HIV), and rapid decline in HCV RNA levels (Abdel-Hakeem & Shoukry, 2014). Notably, permanent loss of HCV-specific T-cell proliferation and functions during acute HCV infection leads to progression of viral persistence and chronicity (Bowen & Walker, 2005). Chronic HCV infection has relatively stable HCV RNA titers, low levels of liver inflammation, and impaired HCV-specific T-cell functions (Raghuraman *et al*., 2012).

The biology of HCV and the potential mechanisms behind viral persistence are poorly understood, although certain immune mechanisms have been postulated based on findings from other CVI investigations. Firstly, spontaneous induction of apoptosis in virus-infected T-cells has been linked to likely destruction of lymphocytes, and lack of protective CD4+ and CD8+ T-cell responses (Alimonti *et al.*, 2003; Zhang *et al.*, 2005) Secondly, the expansion of virus-specific senescent T-cells in the systemic circulation likely impairs the functional antiviral responses (Molina-Pinelo *et al.*, 2009; Akbar & Henson, 2011; Lang *et al.*, 2013). Thirdly, virus-specific T-cell exhaustion leading to poor proliferation attributes and killing abilities toward infected target cells, and/or production of cytokines, also accounts for sub-optimal T-cell responses. Viral persistence is also linked to expansion of functionally weakened T-cell responses (Wherry *et al.*, 2007; Aubert *et al.*, 2011; Velu *et al.*, 2015). Another newfangled mechanism explains the depletion of circulating MAIT cells in CVIs (Gold & Lewinsohn, 2011; Le Bourhis *et al.*, 2011; Fernandez *et al.*, 2015; Saeidi *et al.*, 2015). The mechanisms associated with functional aberrations, and the nature of immunological interface that occurs between HCV and healthy cells warrant in-depth investigations.

2.2.1 Chronic Virus Infection and Spontaneous Apoptosis of T-Cells

Apoptosis or programmed cell death represents an organized mechanism of cellular suicide key to removal of worn-out cells from the body. It also represents a genetic program important for the development and differentiation of normal cellular functions

(Elmore, 2007). Apoptosis has also been recognised as a host mechanism implicated in the pathogenesis of CVIs and tumorigenesis (Barber, 2001). Conversely, growing evidence also suggests that apoptosis could play a predominant role in microbial disease progression, particularly in the setting of CVIs (Young *et al.,* 1997). Alterations observed in lymphocyte functions during persistent infection likely trigger virus-induced lymphocyte apoptosis where involvement of diverse apoptotic pathways has been suggested to exploit the production of virus progeny and delay virus-specific immune responses by the host (Oldstone, 2006).

The apoptotic pathways employed by viruses during chronic infection are receptor-mediated (extrinsic) and non-receptor-mediated (intrinsic) pathways (Hardwick, 2001). The extrinsic pathway is induced by Fas ligand (FasL) and tumor necrosis factor (TNF), on binding to their cognate cell surface receptors Fas/CD95 and TNFR1, respectively. This ligation triggers a signal to recruit adaptor molecules such as Fas-associating protein with death domain (FADD) and TRADD (TNFR1-associated death domain) that inducts a chain of intracellular events to cause cleavage, and stimulation of procaspase-8 to form caspase-8. Once caspase-8 is active, it can activate executioner caspases such as caspase 2, 8, 9, and 10. Activated caspases catalyze the cleavage of other caspases, which, in turn, activate various cellular proteases and endonucleases. Eventually, this leads to cleavage of structural and regulatory proteins and nuclear DNA of the host cell. The other target of caspase-8 is the BH3-only protein, BH3-interacting-domain death agonist (BID). BID that has been cleaved by caspase-8 (tBID) is capable of directly stimulating pro-apoptotic multi-domain proteins to induce mitochondrial outer-membrane permeabilization (MOMP), to co-engage the intrinsic pathway (Screaton & Xu, 2000; Aggarwal, 2003; Benedict, 2003). Meanwhile, the intrinsic pathway is engaged by the transcriptional or post-transcriptional regulations of Bcl-2 proteins to directly influence the MOMP, pro-apoptotic, and Bcl-2 family members. When MOMP occurs, cytochrome c is released

from the mitochondrial intermembrane space, and induces the oligomerization of apoptotic-protease-activating factor-1 (APAF1) resulting in apoptosome formation. This complex recruits and activates procaspase-9, which cleaves downstream caspases and activates caspase-3. Caspase-3 cleaves various substrates such as the poly ADP-ribose polymerase (PARP), that ultimately causes morphological and biochemical changes characteristic of apoptosis (Jiang & Wang, 2000; Shamas-Din *et al.,* 2013; Renault & Chipuk, 2013). Apoptosis is morphologically and biochemically described by nuclear fragmentation, development of membrane-encased apoptotic bodies containing organelles, plasma membrane permeabilization, cell shrinkage and maturation of executioner caspase 3 that is distinct from necrosis (Saraste & Pulkki, 2000; Zhang & Xu, 2000; Elmore, 2007).

It has been well-documented that T-cells of chronically-infected individuals exhibit increased signs of spontaneous apoptosis via FasL–Fas (Cummins & Badley, 2010), and TNF-α–TNF-R1/2 interactions (Sedger & McDermott, 2014), mitochondrial (Reshi *et al.,* 2014), and Bcl-2 family apoptotic pathways associated with high rates of apoptosis during infection (Petrovas *et al.,* 2005). The onset of immunosuppression attributed to dramatic reduction in Th cell populations is emerging, likely owing to induction of apoptosis in uninfected by-stander lymphocytes in HIV infection, a CVI (Holm *et al.,* 2004). Remarkably, T cells of HIV-infected patients have altered expression of Bcl-2, yet expression of Bax, Bcl-xL, and Bcl-xS does not differ from that of apparently healthy controls (Badley *et al.,* 2000). Epstein-Barr virus (EBV) and HBV infections also have been associated with receptor mediated-apoptosis of helper T-cells and cytotoxic T-cells respectively (Galle *et al.,* 1995).

The apoptotic mechanisms underlying chronicity of HCV largely remain unclear. Recently, researchers have found augmented Fas expression and apoptosis of peripheral T-cells in patients with chronic HCV infection compared to healthy controls (El-Latif *et*

al., 2007). It has also been demonstrated that increased apoptosis of peripheral activated T-cells in chronic HCV infection is due to reduced expression of NF-κB (Bantel & Schulze-Osthoff, 2003) likely contributing to sub-optimal antiviral responses. Non-receptor-mediated apoptosis, where livers of patients with HCV infections showing elevated of reactive oxygen species (ROS) levels indicative of caspase activation, apoptosis and peripheral T-cell deletion, has also been described (Muriel, 2009).

2.2.2 T-Cell Senescence and HCV Infection

During normal human ageing, the immune system becomes less functional as a result of sub-optimal cellular responses to foreign antigens, poor self-tolerance and naïve T-cell turnover in addition to increased frequency of senescent T-cell phenotypes, also called as immunosenescence (Derhovanessian *et al.,* 2008; Chou & Effros, 2013). The functional alternations in immunosenescence appear to be attributed to poor frequency of naïve T-cell repertoire, increased rates of differentiation of naïve T-cells to terminally-differentiated T-cells, telomere shortening, decline in CD4/CD8 ratio and increased number of memory T-cells lacking CD28, leading to increased susceptibility to infections, autoimmune disorders, chronic inflammatory diseases and cancers (Strioga *et al.,* 2011; Tatum & Hill, 2012; Janković *et al.,* 2013).

Accumulating evidence suggests that individuals infected with chronically-infecting viruses experience functional and immunologic impairment via a mechanism called replication senescence (Maue *et al.,* 2009). This confers immune cells susceptible to activation-induced cell death (AICD) as a consequence of persistent exposure to antigen and excessive chronic immune activation (CIA) in infected individuals (Fülöp *et al.,* 2013). Replicative senescent T-cells undergo proliferative arrest leading to premature biological aging, and generally display reduced expression of co-stimulatory receptors

(CD28 and CD27) and T-cell survival molecules (CD127), and increased expression of CD57, a marker indicative of replicative senescence (Chou & Effros, 2013). CVI also facilitates the depletion of virus-specific T-cells via CIA characterized by sub-optimal levels of pro-inflammatory cytokines and over-expression of persistent activation markers (HLA-DR and CD38) (Borkow & Bentwich, 2004). Two co-stimulatory molecules, CD27 and CD28 associated with T-cell proliferation, also act as modulators of T-cell functions (Luciano *et al.*, 2007). Meanwhile, CD127 expression is generally designated in activation, balance, differentiation, and survival of different T-cell subsets (Golden-Mason *et al.,* 2006). CD57 is a marker of replicative senescence (Focosi *et al.*, 2010). CD38 and HLA-DR drive premature immunosenescence by augmenting virus replication in infected cells, which heralds the onset of disease pathogenesis (Claiborne *et al.,* 2015).

The development of senescent T-cells was strongly observed among chronic virus-infected individuals, especially with HIV (Deeks, 2011) and human cytomegalovirus (HCMV) (Solana *et al.,* 2012) infections. Research suggests that the frequency of CD8+CD57+ T-cells was higher among HIV-infected relative to HIV seronegative individuals (Palmer *et al.,* 2005). Recently, others have reported that CD57 is linked to increased resistance to apoptosis and poor proliferation of CD8+ T-cells in HIV infection (Petrovas *et al.,* 2009). Subsequent work on HIV also has shown increased recruitment of intermediate-differentiated CD4+ T-cell subsets (CD28+CD27-) suggestive of immunosenescence (Saeidi *et al.,* 2015). The importance of CD127 expression on T-cells for survival and cytokine responses, especially during chronic HIV infection has also been recommended (Sabbaj *et al.,* 2007). HLA-DR expression on CD8+ T-cells is suggestive of excessive CIA (Deeks *et al.,* 2004; Chou *et al.,* 2013) Meanwhile, in persistent/latent CMV infection, seropositive individuals show expansion of terminally-differentiated T-cells characterized by lack of CD28 as well as accumulation of potentially senescent T-

cells (Fülöp *et al.,* 2013) Increase of CD57 expression has been associated with poor antibody responses to influenza vaccination (Dolfi *et al.,* 2013). An elevated CD4+CD28- T-cells co-expressing CD57 is suggestive of expansion of senescent T-cells in chronic HBV infection (Maly & Schirmer, 2015). A similar phenomenon has also been observed among HCV-infected patients where higher proportions of effector senescent CD8+CD57+ T-cells are seen, which also appears to be markedly higher in liver cirrhosis (Focosi *et al.,* 2010).

2.2.3 Functional Exhaustion of HCV-Specific T-Cells in HCV Infection

Humoral and cellular immune responses persistently interact with each other to provide an effective defense against viral infections (Abdel-Hakeem & Shoukry, 2014). Humoral responses are stimulated following the uptake of viral proteins by antigen presenting cells (APCs) that present viral peptides on MHC II molecules to CD4+ T-cells. Activated Th2 cells will produce specific B cell stimulating cytokines including IL-4, IL-5, IL-6, IL-10, TGF−β that activate naive B cells. This facilitates the differentiation of B cells into memory B cells and plasma cells that produce large amounts of immunoglobulins that inhibit virus binding, entry, or uncoating. These immunoglobulins act primarily to neutralize viral pathogens. On the other hand, cytotoxic CD8+ T-cells recognize viral peptides presented on MHC I molecules. The CD4+ T-cells stimulated by DCs produce IL-2, IFN-γ and TNF-α, and help the cytotoxic T lymphocytes (CTLs) differentiate into memory and effector CD8+ T-cells. CTLs play a role in direct killing of viral-infected cells by producing perforin and granzyme.

Patients acutely infected with HCV tend to achieve spontaneous viral clearance via expansion of short-lived CTLs and establishment of virus-specific memory T-cells (Racanelli *et al.,* 2011; Bengsch *et al.,* 2014). However, a completely contrasting phenomenon termed T-cell exhaustion arises when viruses persist for extended periods of time. T-cell exhaustion is induced by persistent stimulation of T-cells and inflammation

by virus, which eventually would repress protective humoral as well as cell-mediated immune responses (Jelley-Gibbs *et al.,* 2005; Bucks *et al.,* 2009). The hallmark of functional exhaustion of CD8+ and CD4+ T-cells is the up-regulation of co-inhibitory molecules, inability to secret antiviral cytokines, and destruction of virus-infected cells, which negatively impacts T-cell priming, differentiation, effector functions and viral control (Yi *et al.,* 2010). Patients with CVIs typically possess exhausted T-cells expressing multiple co-inhibitory receptors such as programmed death-1 (PD-1), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), T cell immunoglobulin mucin-3 (TIM-3), lymphocyte activation gene 3 protein (LAG-3), killer-cell lectin like receptor G1 (KLRG1), and CD160 (Lee *et al.,* 2010; Larsson *et al.,* 2013). T-cell exhaustion also leads to cytokine imbalance towards production of anti-inflammatory (IL-10 and transforming growth factor-β1 (TGF-β1), rather than pro-inflammatory cytokines (IL-1, IL-2, IL-6, TNF-α, and IFN-γ) during viral infections (Wherry *et al.,* 2007; Garidou *et al.,* 2012). Nevertheless, exhaustion is not only limited to CD8+ T-cell responses as CD4+ T-cells have also been shown to develop functional unresponsiveness (clonal anergy), which is key to optimal B cell development, antibody production, macrophage activation, and/or recruitment of CD8+ effector T-cells at the site of infection (Han *et al.,* 2010; Valdor & Macian, 2010).

Multiple lines of evidence suggest the expression of co-inhibitory receptors and secretion of immunoregulatory cytokines by virus-specific T-cells subsets. Functional impairment of HBV-specific T-cells leads to increased production of IL-10 and TGF-β1, and causes exhaustion of circulating HBV-specific CD8+ T-cells marked by the expression of PD-1 (Peng *et al.,* 2008; Ye *et al.,* 2008). Increased production of TGF-β1 and IL-10 is linked to disease advancement in HIV-infected patients (Larsson *et al.,* 2013). TGF-β1 up-regulates CTLA-4 expression on HIV-specific CD8+ T-cells and suppresses Th1 cytokine production and proliferation (Elrefaei *et al.,* 2010). The

frequencies of TIM-3 expression on HIV-specific CD4+ T-cells and CD8+ T-cells, especially in primary infections and AIDS cases, associate with disease progression (McMahan *et al.*, 2010). Similarly, other investigators reported that HIV-specific T-cells display certain other surface co-inhibitory molecules, for instance, PD-1 (Porichis & Kaufmann, 2012) and LAG-3. Similar observations have also been shown in persistent LCMV infection, where IL-10 and PD-L1 pathways work together to dampen T-cell activation during persistent LCMV infection (Wilson & Brooks, 2010; Yi *et al.*, 2010). Co-expression of multiple inhibitory receptors, such as PD-1, 2B4, CD160 and KLRG1 on HCV-specific CD8+ T-cells is suggestive of impaired antiviral functions (Bengsch *et al.*, 2010). Further, CTLA-4 was reportedly up-regulated on virus-specific CD4+ T-cells of individuals with chronic HCV infection (Raziorrouh *et al.*, 2011). A subset of IL-10-producing HCV-specific CD8+ T-cells present only in individuals who progress to chronic infection, is closely associated Th1 and Th2 cytokine imbalance (Flynn *et al.*, 2011). Cytokine production skewed to Th1 has been suggested to favor spontaneous clearance of HCV infections. In chronic bacterial infections such as TB, higher expression of CD244 on CD4+ T-cells led to significantly reduced levels of IFN-γ (Yang *et al.*, 2013).

2.2.4 Circulating MAIT Cells: Depletion and Exhaustion

A specialized, innate-like T-cells with antimicrobial properties called MAIT cells, represent an evolutionarily conserved αβ T-cell subset in human. Naïve MAIT cells most likely develop in the thymus as well as spleen, and found are in copious levels in the systemic circulation occupying ~1–8% of the total T cell pool. MAIT cells also occur also in the intestinal lamina propria, Peyer's patches of the gut and mesenteric lymph nodes, suggesting their role in the maintenance of mucosal integrity, intestinal immune system and antimicrobial immunity (Gapin, 2009; Martins *et al.*, 2009). They are also found mainly in tissues such as lung and liver (~50% of liver T cells). MAIT cells express an

invariable TCR-α chain (Vα19-Jα33 in mice and Vα7·2-Jα33 in human) in concert with a limited number of TCR-β chains (Vβ2 (TRBV20) and Vβ13 (TRBV6)) in humans and (Vβ6 and Vβ8) chains in mice, capable of secreting a wide range of cytokines, and are readily induced to kill bacteria-infected cells (Le Bourhis *et al.,* 2010; Gold *et al.,* 2011; Ussher *et al.,* 2014).

The TCR of MAIT cells recognizes foreign antigens, usually microbe- (bacteria and yeast) derived vitamin B metabolites that are restricted by mono-morphic MHC-I-like related protein (MR1) along with other co-stimulatory molecules such as CD80 or CD86 required for the activation of MAIT cells (Kjer-Nielsen *et al.,* 2012; Wakao *et al.,* 2013;). Likewise, these T-cell subsets are readily activated by innate cytokines IL-12 and IL-18, either resulting in the expression of pro-inflammatory cytokines IL-4, IL-5, IL-10, IFN-γ, TNF-α, IL-17 and IL-22 or release of perforin and granzymes (Kurioka *et al.,* 2014; Treiner, 2015). The abundance of IFN-γ may then result in the priming and maturation of immature DCs that re-circulate to the lymph node and pledge Th1 immunity. The cells share phenotypic resemblance with iNKT cells, express high levels of IL-12Rβ2 and IL-18Rα besides CD161 (NK-cell marker), together with the semi-invariant Vα7.2 segment and localize to sites of infection via tissue-targeting chemokine receptors, CCR2, CCR5, CCR6, CCR9 and CXCR6 (Dusseaux *et al.,* 2010; Fernandez *et al.,* 2015). MAIT cells merely make up a much minor fraction of this subset, averaging ~15% of the CD161++ CD8+ T-cell population. MAIT cells have been associated with a number of disease settings, including bacterial and viral infections, inflammation-related diseases such as multiple sclerosis and psoriasis, allergy, autoimmune diseases and cancer (Le Bourhis *et al.,* 2011; Simoni *et al.,* 2013).

Murine MAIT cells have been shown to play a paramount role in anti-bacterial immunity, especially against *Escherichia coli*, *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, *Mycobacterium bovis*, *M. tuberculosis*, *Pseudomonas aeruginosa* and

Staphylococcus aureus (Gold *et al.*, 2010; Howson *et al.,* 2015). For instance, during active *M. tuberculosis* infection, circulating MAIT cell frequency is reportedly lowered in the peripheral blood compared to healthy controls, and therefore it is postulated that the presence of mycobacteria in the lungs results in the trafficking of these cells to the site of infection from the peripheral circulation (Napier *et al.,* 2015). The study also report that patients with active TB exhibited elevated expression of PD-1 on MAIT cells, which could be due to depletion of peripheral MAIT cells owing to exhaustion and increased apoptosis during active TB infection (Jiang *et al.,* 2014). On the other hand, no loss of MAIT cells at mucosal sites during pulmonary TB disease has observed (Jiang *et al.,* 2014). Similarly, MAIT cell activation in response to exposure to yeasts such as *Candida albicans*, *C. glabrata,* and *Sacchromyces cerevisiae* envisages the role of these cells in antifungal immunity (Howson *et al.,* 2015). MAIT cells also appear to play an immunopathogenic role against CVIs such HIV, HCV, and HBV. Recent studies have highlighted a decrease in the frequencies of CD161++Vα7.2+ T-cell subsets in the peripheral blood of HIV-infected individuals, which could possibly be due to down-regulation of CD161 and up-regulation of TIM-3 together with PD-1 hinting the role of functional exhaustion (Leeansyah *et al.,* 2013). A latest report suggests the onset of immune aging in MAIT cells due to persistent virus exposure during chronic HIV-1 infection where MAIT cells derived from infected patients expressed high levels of CD57 (Leeansyah *et al.,* 2013). The expression of CD57 on CD8+ T-cells is related to replicative senescence, shortened telomere, and chronic activation (Dock & Effros, 2011). Similarly, during chronic HCV infection the disappearance of peripheral MAIT cells could possibly be due to their recruitment to the site of infection, the liver. Meanwhile, HIV/HCV co-infection has led to a significant decline in the proportion of functional Vα7.2+CD161++ T-cells, with a greater fraction of MAIT cells also co-expressing PD-1 and TIM-3 (MacParland, 2013). Nonetheless, the underlying factors associated with the

disappearance of peripheral MAIT cells during chronic HCV infection largely remain unknown. Further, research also suggests that patients with inflammatory bowel disease (IBD) showed signs of recruitment of MAIT cells far from the blood to sites of inflammation (Treiner, 2015).

CHAPTER THREE

METHODS AND MATERIALS

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Subjects

A total of 62 individuals with chronic hepatitis C disease and an equal number of healthy controls (HCs) were recruited in a cross-sectional study at the Hepatology and Gastroenterology Unit of UMMC, Kuala Lumpur, Malaysia for over a period of 2 years between 2013 and 2015. Chronic hepatitis C disease was defined as HCV infection with persistence of HCV RNA in the blood and with or without abnormal liver functions for at least 6 months (Hoare *et al.,* 2008). Blood samples were collected from participants and were transported to the Diagnostic Medical Microbiology Laboratory of UMMC for routine diagnosis. All chronic HCV-infected patients tested positive for HCV antibodies and HCV RNA, and negative for anti-HIV antibodies and hepatitis B surface antigen (HBsAg). At the time of recruitment, none of the patients were receiving or had received any pegylated IFN-α and RBV in the last 6 months. Additional exclusion criteria were recent illness and/or vaccination within 4 weeks prior to phlebotomy, diabetes mellitus, hypertension/cardiovascular disease, pregnancy and treatment for any form of inflammatory manifestations. Healthy controls were individuals free from HBV, HCV, TB and/or HIV infections. The study was carried out following approval of the protocols by the Medical Ethics Committee (MEC) of UMMC (approved human ethics number: 944.38). Written, informed consent was obtained from all participants before study enrolment. The study was carried out in compliance with good clinical practice, including the International Conference on Harmonization Guidelines and the Declaration of Helsinki.

3.2 Laboratory Investigations

Blood samples of chronic HCV-infected patients were subjected to investigations on liver transaminases including plasma aspartate (AST) and alanine transaminases (ALT), HCV plasma viral load (PVL) and HCV genotyping. Plasma AST levels were valued using a commercial ELISA (IBL America, Minneapoli, Minnesota) according to manufacturer's instructions (Ajakaiye *et al.,* 2011). Plasma ALT was determined using a Hitachi7050 Automatic Analyzer (Hitachi Corp, Tokyo, Japan) by a commercial ALT assay (Wako Pure Chemicals, Osaka, Japan) (Albertoni *et al.,* 2012). The cut-off values were set at 20ng/ml. HCV PVLs of chronic HCV patients were measured using a commercial COBAS AMPLICOR HCV test, version 2.0 (Roche Molecular Systems, Branchburg, NJ). The analytical sensitivity (95% threshold) of the COBAS AMPLICOR HCV 2.0 was 60 and 100 HCV IU/ml with EDTA plasma and serum, respectively (Yu *et al.,* 1999). The total lymphocyte counts (TLCs) for each chronic HCV-infected patients and HCs were determined by flow cytometry.

3.3 Peripheral Blood Mononuclear Cells

Blood samples were obtained in a BD Vacutainer® coated with heparin lithium tubes (BD Biosciences, Franklin Lakes, NJ). Isolation of PBMCs was performed within 8 hours of collection of blood. PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque^{TM} (Amersham Pharmacia, Piscataway, NJ) overlay method according to published literature (Mallone *et al.,* 2011). Briefly, the whole blood was reconstituted in phosphate-buffered saline (PBS) solution in equal volume, and was overlaid on Ficoll-Paque^{TM} gradients and centrifuged for 22 minutes at 2200rpm. PBMCs were collected from buffy coat and washed twice with PBS. The cells were cryopreserved in liquid nitrogen until further use by resuspending in a freezing medium [10% DMSO (dimethyl sulfoxide) in 90% fetal bovine serum (FBS)] (Gibco, Carlsbad, CA). A parallel batch of blood from participants was collected in BD Vacutainer® coated with K2EDTA tubes

(BD Biosciences, Franklin Lakes, NJ). Serum was isolated by spinning the blood at 2500rpm for 10 minutes at room temperature and stored at -80° C until further use.

3.4 Primary Cell Culture

Culture of PBMCs was performed according to published literature (Jeon *et al.,* 2010). Approximately, 1×10^6 cells were added to wells of a 6-well-plate in RPMI1640 medium supplemented with HEPES buffer (25mM), L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100µg/ml), sodium pyruvate (1mM) and 10% of FBS (all reagents procured from Life Technologies, Victoria, Australia). Cells were cultured for 18 hours at 37°C in a 5% CO_2 incubator.

3.5 T-Cell Activation

T-cell stimulation was performed *in vitro* by stimulating PBMCs (3×10^5 cells/ml) in flat-bottomed 6-well plates (BD Falcon, Stockholm, Sweden) with a pool of lyophilized HCV peptides consisting mainly of 15-mer sequences with 11 amino acid overlap, covering the sequences of HCV genotype 1b core protein (Miltenyi Biotec, Bergisch, Gladbach, Germany) and recombinant human IL-2 (rhIL-2). Non-specific activation was done using phytohemagglutinin (PHA) (Thermo Fisher Scientific, Waltham) and exogenous rhIL-2 (Thermo Fisher Scientific, Waltham, MA) in PBMC culture for 48 hours at 37°C in a 5% CO_2 incubator. Negative control was prepared by stimulating PBMCs with 10U/ml of rhIL-2 only (Missale *et al.,* 1996).

3.6 Polychromatic Flow Cytometry

PBMCs were studied for surface expression of target molecules by flow cytometry. For the unstimulated PBMCs, cells were recovered from liquid nitrogen storage, and for the activated PBMCs, cells were recovered by washing once using ice-cold PBS followed by 20% FBS in PBS. The washed PBMCs were resuspended in 100µl staining buffer and were stained with recommended volume of various antibodies tagged with fluorescent dyes against target antigens accordingly. The stained cells were incubated in the dark for

20 minutes on ice. Later, the cells were washed twice and resuspended to a final volume of 300µl with staining buffer. Cells were acquired on a 7-color BD FACSCanto™ II system (BD Immunocytometry Systems, San Jose, CA) using BD FACSDiva™ software. (BD Biosciences), and analysed using FlowJo software (TreeStar, Ashland, OR). Fluorescence minus one (FMO) controls was as used for optimal gating. Isotype controls were also used to set the background for selection of positive cells. Isotype controls used were FITC mouse IgG2a (MAB349051), FITC mouse IgM (MAB555583), PE mouse IgG1 (MAB11711), PE-Cy7 mouse IgG1 (PMG557872), Alexa 647mouse IgM (PMG560806), APC-H7 mouse IgG1 (PMG560167), PerCP-Cy5.5 mouse IgG1 (PMG552834), APC mouse IgG2b (PMG555745), PerCP mouse IgG2a (MAB349054), FITC mouse IgG2b (MAB555742) and FITC goat IgG (GZIC108F). All isotype controls were procured from R & D Systems, Minneapolis, MN).

3.7 Objective 1: Spontaneous Apoptosis in Chronic HCV Infection

3.7.1 Serum Indoleamine 2, 3-Dioxygenase (IDO)

Serum indoleamine 2, 3-dioxygenase (IDO) from chronic HCV-infected patients (n=7) and HCs (n=7) were measured using a commercial sandwich ELISA (Uscn Life Science Inc, Wuhan, Hubei, PRC) according to the manufacturer's instructions, with a detection range of 1.563–100ng/ml. This assay has a sensitivity of 0.61ng/ml for detection of IDO from body fluids. Briefly, standards and sera from both chronic HCV-infected patients and healthy controls were dispensed into appropriate microtiter wells immobilized with a biotin-conjugated anti-IDO. Later, avidin-conjugated to horseradish peroxidase (HRP) was added to each well and incubated for 30 minutes. Later, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added to the wells. The enzyme-substrate reaction was terminated by adding H_2SO_4 and color change was measured using spectrophotometry at 450nm. IDO concentration was estimated by comparing the OD of the samples with the standard curve.

3.7.2 Apoptosis Assay

Detection of apoptotic cells in PBMC culture was performed by flow cytometry using a commercially available BD Pharmingen FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) as described previously. The PBMCs were harvested by centrifuging at 2500rpm for 10 minutes at 4°C and washed with cold phosphate-buffered saline (PBS)/azide solution (PBS plus 0.1% sodium azide). Cells were resuspended in 1× binding buffer and stained with fluorescein isothiocyanate (FITC)-Annexin V® and propidium iodide (PI) according to the manufacturer's instructions. The stained cells were incubated at room temperature for 20 minutes in the dark. Later, the cells were washed with binding buffer and acquired on a BD FACSCanto II (BD Biosciences, San Jose, CA) flow cytometry. Percentages of viable and dead cells were determined using the FACSDiva software (BD Biosciences).

3.7.3 Prostaglandin G/H Synthase (PGHS) /Cyclooxygenase-2 (COX-2) Assay

Cell-based COX-2/PGHS-2 was measured using a commercial sandwich ELISA (R&D Systems, Abingdon, United Kingdom). Standards and PBMC culture samples were added to the corresponding wells pre-coated with anti-COX-2. Unbound COX-2 was washed, and biotin-conjugated anti-COX-2 was added to the wells. After washing, avidin conjugated horseradish peroxidase (HRP) was added followed by washing of unbound avidin-renzyme reagent. Later, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to the wells to facilitate color change in proportion to the amount of bound-COX-2 in the initial step. The enzyme-substrate reaction was terminated by adding H_2SO_4 and the color change was measured using a spectrophotometer at 450nm. The concentration of COX-2 was estimated by comparing the OD of samples with the standard curve.

3.7.4 Cellular Reactive Oxygen Species (cROS) Assay

Cellular reactive oxygen species (cROS) formation in PBMCs culture was measured after 18 hours of incubation using a commercial oxidation-sensitive fluorescent probe, 2', 7'-dichlorofluorescein diacetate (DCFH-DA) ROS detection assay kit (Mitosciences, Cambridge, UK) as previously described after 2 days of culture in RPMI medium according to the manufacturer's instructions. The kit employs a fluorogenic dye DCFH-DA, which measures hydroxyl, peroxyl and any other intracellular ROS. Following diffusion, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound that is subsequently oxidized by intracellular ROS to form a highly fluorescent 2', 7' – dichlorofluorescein (DCF), detectable by fluorescence spectroscopy. Briefly, 5×10^4 cells were seeded in a 96-well plate containing 100µl culture medium per well for 18 hours. Later, the cells were washed with ice-cold PBS and incubated with 50µM of DCFH-DA at 37°C in the dark for 45 minutes. Later, the cells were washed with PBS to remove residual DCFH-DA. Cellular fluorescence was measured at 485 nm (excitation) and 535

nm (emission) using a Varioskan Flash microplate reader (Thermo-Scientific, Waltham, MA).

3.7.5 Measurement of Co-Inhibitory Receptors on T-Cells

T-cells were studied *ex vivo* for surface expression of co-inhibitory receptors associated immune exhaustion using flow cytometry. Antibodies used in the experiments were peridinin chlorophyll protein complex-cyanin-5.5 (PerCP-Cy5.5)-conjugated antibody to CD3 (clone SK7), phycoerythrin-cyanin7 (PE-Cy7)-conjugated antibody to CD4 (clone RPA-T4), allophycocyanin-H7 (APC-H7)-conjugated antibody to CD8 (clone SK-1), PE-conjugated antibody to BTLA (clone J168-540), PE-conjugated antibody to TRAIL (clone RIK-2), fluorescein isothiocyanate (FITC)-conjugated antibody to PD-1 (clone MIH-4), brilliant violet 421 (BV421)-conjugated antibody to CTLA-4 (clone BNI3) (all procured from BD Pharmingen^{TM}, San Diego, CA) and APC-conjugated antibody to TIM-3 (clone 344823) (R & D Systems, Minneapolis, MN).

3.7.6 RNA Extraction and One-Step Quantitative Real-Time PCR

Total RNA was extracted from PBMCs by a spin technology using commercially available RNeasy Mini kit (Qiagen, Solna, Sweden) according to the manufacturer's protocol. Briefly, 1×10^7 PBMCs were homogenized in lysis buffer (RLT buffer, Qiagen, Solna, Sweden). Pure ethanol was added to the culture lysate and mixed well creating conditions to promote selective binding of RNA to the RNeasy membrane. Later, the lysate was applied to the RNeasy Mini spin column to render binding of total RNA to the membrane. RNA was eluted in RNase-free water. RNA concentration and purity were determined using a Nanodrop spectrophotometer. The binding, washing, and elution steps were performed by centrifugation in microcentrifuge tubes. DNase treatment was also carried out to remove any contaminating DNA using an RNase-Free DNase Set (Qiagen, Solna, Sweden). Gene expression of co-inhibitory molecules was determined by a one-step quantitative real-time PCR (qRT-PCR) in a total volume of 20µl that included 12.5µl

of 2× SYBRGreen PCR mix (Applied Biosystems, Stockholm, Sweden), 4.75µl of ultrapure water, 1.25µl of forward and reverse primers (0.2mM each), 0.25µl reverse transcription. Finally, 5µl of RNA sample was added to reach a concentration of 10µg/µl. Reactions were run on an iQ5 Thermal cycler (BioRad, Hercules, CA) using the universal and appropriate thermal cycling parameters. Results were obtained using the sequence detection software iQ5 thermal cycler and analyzed using MS Excel software. Melting curves for the designed primer sets were acquired for quality control purposes. For gene expression quantification, comparative Ct method was used whereby gene expression levels for each sample was normalized against the average Ct values of two endogenous controls (β-actin and tubulin) according to manufacturer's protocol. The primers were designed using the NCBI Primer-BLAST (Basic Local Alignment Search Tool) website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Thornton & Basu, 2011). The primers used against the corresponding genes were synthesized by First Base Labs (First Base, Kuala Lumpur, Malaysia).

Table 3.1: List of Primers used in the Detection of Co-Inhibitory Molecules.

Name	Sequence (5'-3')	Length	GC%	Tm (°C)	Product size (bp)
DTX1 (F)	CCCGGGTGGTGGTGTG	16	75	67.9	134
DTX1 (R)	CATGGTGCCTGTGTCCTGG	19	63.1	68	
BLIMP-1 (F)	CAGCTCGCCCACCTGCAGAA	20	65	74	158
BLIMP-1 (R)	GCCGCAGCGCAGTTCCCTTT	20	65	71	
BTLA (F)	TGCCTGGTTTGTTTTCTTCCAGGC	24	50	70	230
BTLA (R)	TGGGTCATACCGCTGTTCTGCAA	23	52.1	70	
CD160 (F)	GCCTTGTGGCCCTTCAAGCTTTGT	24	54.1	72.8	139
CD160 (R)	TCCCCTGTGCCCTGTTGCAT	20	60	72	
CTLA-4 (F)	GGGCATAGGCAACGGAACCCA	21	61.9	73	174
CTLA-4 (R)	GGGGGCATTTTCACATAGACCCCTG	25	56	72	
FOXP-3 (F)	CAGCACATTCCCAGAGTTCCTC	22	54.5	66	155
FOXP-3 (R)	GCGTGTGAACCAGTGGTAGATC	22	54.5	66	
LAG-3 (F)	CTAGCCCAGGTGCCCAACGC	20	70	73	160
LAG-3 (R)	GCCTGCGGAGGGTGAATCCC	20	70	72	
PD-1 (F)	CTCAGGGTGACAGAGAGAAG	20	55	59	181
PD-1 (R)	GACACCAACCACCAGGGTTT	20	55	59	
TIM-3 (F)	AGGGGACATGGCCCAGCAGA	20	65	73	179
TIM-3 (R)	GCCAGCCCAGCACAGATCCC	20	70	73	
TRAIL (F)	CTTTACCAACGAGCTG	16	50	50	165
TRAIL (R)	GTTATGTGAGCTGCTAC	17	47	51	
β-ACTIN (F)	AGAGGGAAATCGTGCGTGAC	20	55	66	203
β-ACTIN (R)	CAATAGTGATGACCTGGCCGT	21	52.3	66	
TUBULIN (F)	AACACGGGATCGACTTGGC	19	55	65	186
TUBULIN (R)	CTCGGGGCACATATTTCCTAC	21	55	65.6	

3.7.7 QuantiGene Plex 2.0 Assay

Total RNA from PBMCs was extracted using a commercial RNeasy Mini kit (Qiagen, Solna, Sweden), and was later used to measure gene expression of various pro-apoptotic and anti-apoptotic molecules by means of QuantiGene Plex 2.0 assay (Affymetrix, Santa Clara, CA). Briefly, RNA was prepared and a working bead mix was prepared containing lysis mixture, blocking reagent, Luminex® capture beads and probe set. The bead mix was dispensed into the hybridization plate, and 20µl of total RNA was added to each well. For background control wells, 20µl of sterile nuclease-free water was added to the bead mix. The hybridization plate was placed in a shaking incubator at 600rpm/54°C overnight. Later, the wash solution, pre-amplifier, amplifier, label probe, and streptavidin–phycoerythrin (SAPE) solutions were prepared. The hybridized samples were transferred to a magnetic separation plate. Samples were washed and incubated with the pre-made

amplifier solutions for 1 hour (50°C and 600rpm). Subsequently, the SAPE solution was added and the samples were incubated at 25°C for 30 minutes at 600rpm in the dark. The unbound SAPE was washed, and 130µl of SAPE wash buffer was added to each well containing RNA sample. The plate was shaken at 25°C for 3 minutes at 800rpm to resuspend the beads. The plate was directly read using a Bio-Plex 200 system (BioRad, Hercules, CA). Fluorescent readings from blank wells were deducted from fluorescent values for the RNA of interest. The values were normalized against the geometric mean expression of two housekeeping genes, Ppib (peptidylpropyl isomerase B) and Hprt1 (hypoxanthine–guanine phosphoribosyltransferase) for each sample that was run in triplicates.

3.7.8 Statistical Analysis

Statistical analysis was done using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA). The levels of significance for comparisons between two or more independent samples were determined using a two-tailed unpaired Student's t test. Data was presented as mean ± SEM, where n refers to the number of independent experiments. For QuantiGene Plex 2.0 assay, the expression values for each gene were multiplied by a constant, and compared between chronic HCV patients and healthy controls. The values were normalized against the geometric mean expression of two internal control genes *Ppib* and *Hprt1* for each sample, and expressed as fold change (2-fold; highly up-regulated, 1.5- to 2-fold; moderately up-regulated, 1- to 1.5-fold; mildly up-regulated). For expression of immune exhaustion molecules by FACS analysis, the data were transformed and normalized, and a two-tailed non-parametric paired t test was used for revealing significance. The level of significance was considered at $P<0.05$, and measures represented by *$P<0.05$, **$P<0.005$, ***$P<0.001$ and ****$P<0.0001$.

3.8 Objective 2: T-Cell Senescence in Chronic HCV Infection

3.8.1 Immunophenotyping of HCV-Specific T-Cells for Senescence

The HCV-specific and PHA-expanded T-cells from PBMCs of chronic HCV-infected patients (n=20) and HCs (n=20) were subjected to staining for molecules associated with immunosenescence and CIA by flow cytometry. HCV-specific and PHA-expanded T cells were performed using method elaborated in section 3.5- T cell activation. Staining was performed according to protocols set by the commercial manufacturer (BD Biosciences, San Jose, CA). Three antibody panels were used in each duplicate for staining, one that contained PerCp-Cy5·5-conjugated anti-CD3, PE-Cy7-conjugated anti-CD4, APC-H7-conjugated anti-CD8, FITC-conjugated anti-HLA-DR, and PE-conjugated anti-CD38; the second panel that contained PerCp-Cy5·5-conjugated anti-CD3, PE-Cy7-conjugated anti-CD4, APC-H7-conjugated anti-CD8, FITC-conjugated anti-CD57, PE-conjugated anti-CD28, APC-conjugated anti-CD27, and BD Horizon™ (BD Biosciences) BV421-conjugated anti-CD127 (anti-IL-7R); and the third panel that contained PerCp-Cy5·5-conjugated anti-CD3, PE-Cy7-conjugated anti-CD4, APC-H7-conjugated anti-CD8 and FITC-conjugated anti-PD-1.

3.8.2 Statistical Analysis

Mean and SEM were used to describe each variables analysed. Non-parametric unpaired t-test (or Mann–Whitney U-test) was used to compare between the two independent groups, while differences within the groups were analysed using non-parametric one-way Anova (or Kruskal–Wallis test), with Dunn's correction for multiple comparison. Correlations were performed using the Spearman rank correlation co-efficient test. Fisher's exact test was applied for comparison within the categorical results. The measure of significance was represented by *P<0·05, **P<0·005 and ***P<0·001. All analyses were done using GraphPad Prism 6 (GraphPad, La Jolla, CA).

3.9 Objective 3: Functional Exhaustion of HCV-Specific T-Cells in HCV Disease

3.9.1 Measurement of Co-Inhibitory Receptors on HCV-Specific T-Cells

The HCV-specific and non-specific T-cells from PBMCs of chronic HCV-infected patients (n=10) and HCs (n=10) were studied for surface expression of co-inhibitory receptors associated with immune exhaustion by polychromatic flow cytometry. HCV-specific and PHA-expanded T cells were performed using method elaborated in section 3.5- T cell activation. The cells were subjected to two co-inhibitory receptor-staining panels; the first consisted of PerCp-Cy5·5-conjugated anti-CD3, PE-Cy7-conjugated anti-CD4, APC-H7-conjugated anti-CD8, FITC-conjugated anti-PD-1, APC-conjugated anti-TIM-3, BV421-conjugated anti-CTLA-4, and PE-conjugated anti- B and T-lymphocyte attenuator (BTLA); and the second contained PerCp-Cy5·5-conjugated anti-CD3, PE-Cy7-conjugated anti-CD4, APC-H7-conjugated anti-CD8, and PE-conjugated anti-TNF-related apoptosis-inducing ligand (TRAIL). All antibodies were procured from BD Biosciences, Franklin Lakes.

3.9.2 Th1/Th2/Th17 Cytokine Bead Array

A human Th1/Th2/Th17 Cytokines Multi-Analyte ELISArray™ kits (Qiagen, Valencia,CA) was used to estimate cytokine production, evaluating cytokines namely interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (G-CSF) and transforming growth factor beta 1 (TGF-β1) in the supernatants of T-cell cultures. The arrays were performed according to the manufacturer's instructions. Briefly, culture supernatants were collected from the T-cell cultures. The ELISA plate was prepared by incubating collected culture supernatant onto coated with capture antibodies ELISArray plate to bind to their specific targets. Subsequently, the plate was washed to remove unbound proteins, and biotinylated detection antibodies and avidin-horseradish peroxidase conjugate were then added after a few washing steps, respectively. Colorimetric substrate solution was added, that

developed a blue color precipitate in direct proportion to the amount of analytes present in the initial sample. The color development was finally stopped by adding stop solution. The level of proteins was measured on a Varioskan® Flash microplate reader (Thermo-Scientific, Waltham, MA) with an absorbance at 450 nm.

3.9.3 Statistical Analysis

Mean and SEM were used to describe each variables analysed. Data were compared using the Mann–Whitney U-test or Student's T test. The measure of significance was represented by *P<0·05, **P<0·005 and ***P<0·001. All analyses were done using prism 6 for Windows v 6.01 (GraphPad, La Jolla, CA).

3.10 Objective 4: Peripheral Loss and Exhaustion of Mucosal-Associated Invariant T (MAIT) Cells in Chronic HCV Infection

3.10.1 Immunophenotyping of Peripheral MAIT Cells

A total of 50 study participants (chronic HCV-infected patients, n=25 and HCs, n=25) were recruited to investigate into this objective. PBMCs were subjected to immunophenotyping for MAIT cells and chemokine, immune exhaustion and senescence markers on MAIT cells by polychromatic flow cytometry. All antibodies were purchased from BD Biosciences and pre-titrated to determine appropriate working concentrations. Staining with PerCp-Cy5.5-conjugated anti-CD3 (clone UCHT1), APC-H7-conjugated anti-CD8 (clone SK1), APC-conjugated anti-CD161 (clone DX12) and PE-conjugated TCRVα7.2 (clone 3C10) or PE-Vio770-conjugated TCRVα7.2 (Miltenyi Biotec GmbH, Germany) were in the phenotypic characterization of MAIT cells. Surface staining was performed in three different panels of mAbs: BV421-conjugated PD-1 (clone EH12), FITC-conjugated CD57 (clone HNK-1), and PE-Cy7-conjugated TIM-3 (clone SK1); second panel: PE-conjugated CD38 (clone HB7), FITC-conjugated HLA-DR (clone L243), and BV-421-conjugated CTLA-4 (clone UC10-4B9); third panel: FITC-conjugated CD103 (clone Ber-ACT8) and PE-Cy7-conjugated CCR5 (clone 3A9). Doublets were excluded based on FSC-H/FSC-A, lymphocytes were identified on FSC and SSC, and dead cells were excluded using Fixable Viability Stain 510 (BD Biosciences).

3.10.2 Statistical Analysis

Mean and SEM were used to describe each variables analyzed. Due to the sample size and the assumption that the sample population does not follow normal distribution, non-parametric unpaired t test (or Mann-Whitney U test) was selected for comparisons between the two independent groups. Two-sided Mann-Whitney tests for non-parametric data were used to compare the two groups. Differences were considered significant with

*P<0.01, **P<0.001 and ***P<0.0001. Correlations between CD8+CD161++TCRVα7.2+ T cell frequency and HCV plasma viral load, serum AST, serum ALT, and HCV genotype were assessed using Spearman's rank. All analyses were done using Prism 6 software version 6.01 (GraphPad, La Jolla, CA).

CHAPTER FOUR

RESULTS

CHAPTER 4: RESULTS

4.1 Objective 1: Spontaneous Apoptosis in Chronic HCV Infection

4.1.1 Measurement of Serum Indoleamine 2, 3-Dioxygenase (IDO) Levels

IDO is an intracellular enzyme that deprives tryptophan, an aminoacid essential for T-cell survival from the systemic circulation. IDO is also believed to play a key role in facilitating spontaneous apoptosis in immune cells. Hence, we investigated a total of 14 (7 from each group) serum samples to measure the levels of IDO by a commercial ELISA (Uscn Life Science Inc, Wuhan, Hubei, PRC). Our observations showed that serum IDO concentrations were significantly increased in chronic HCV-infected patients as compared to HCs ($P<0.001$). Interestingly, two patients with high plasma viral load showed no significant difference in serum IDO levels relative to HCs. Hence, no significant association could be observed between HCV PVL and serum IDO levels.

4.1.2 Measurement of Signatures of Cellular Apoptosis in Immune Cells

Next, we determined whether there was any evidence of spontaneous apoptosis of immune cells in chronic HCV-infected individuals. Therefore, we determined the frequency of apoptotic cells in bulk PBMCs by flow cytometry using a commercial apoptosis kit (BD Biosciences, San Jose, CA). Our results showed an increase (although not significant) in the frequency of late-apoptotic cells (FITC+/PI+, Q2 panel) in the PBMCs of patients compared to healthy controls. Of the seven patients, only four showed signs of increased spontaneous apoptosis than healthy controls, and the others displayed a similar frequency as healthy controls. Meanwhile, early apoptotic cells (FITC+/PI-, Q4 panel) were also observed in the PBMCs of both the groups where increased frequency of early apoptotic cells was seen in HCV-infected patients as compared to healthy controls.

4.1.3 Measurement of Prostaglandin G/H Synthase (PGHS) /Cyclooxygenase 2 (COX-2)

The higher expression of COX-2 is related to persistent inflammation associated with apoptosis of immune cells (FitzGerald, 2011). Hence, the levels of COX-2 in the supernatants of PBMC cultures after 18 hours of incubation were determined using a commercial sandwich ELISA (R&D Systems, Abingdon, United Kingdom). Our results showed a significant increase in the levels of COX-2 in patients than healthy controls ($P<0.01$). No positive association was seen between HCV PVL and COX-2 levels in our study groups (data not shown).

4.1.4 Determination of Cellular Reactive Oxygen Species (cROS)

Next, we set out to determine the production of cROS in PBMC supernatants by a commercial ELISA (Mitosciences®, Cambridge, UK). The formation of DCF in the PBMC cultures of chronic HCV-infected patients showed significant increase compared to healthy controls ($P<0.001$). The observed increase in cellular ROS indicates increased intracellular oxidative stress that could likely be the mediators of ongoing apoptosis via caspase intermediates, and could also be due to the induction of intrinsic pathway during chronic HCV infection.

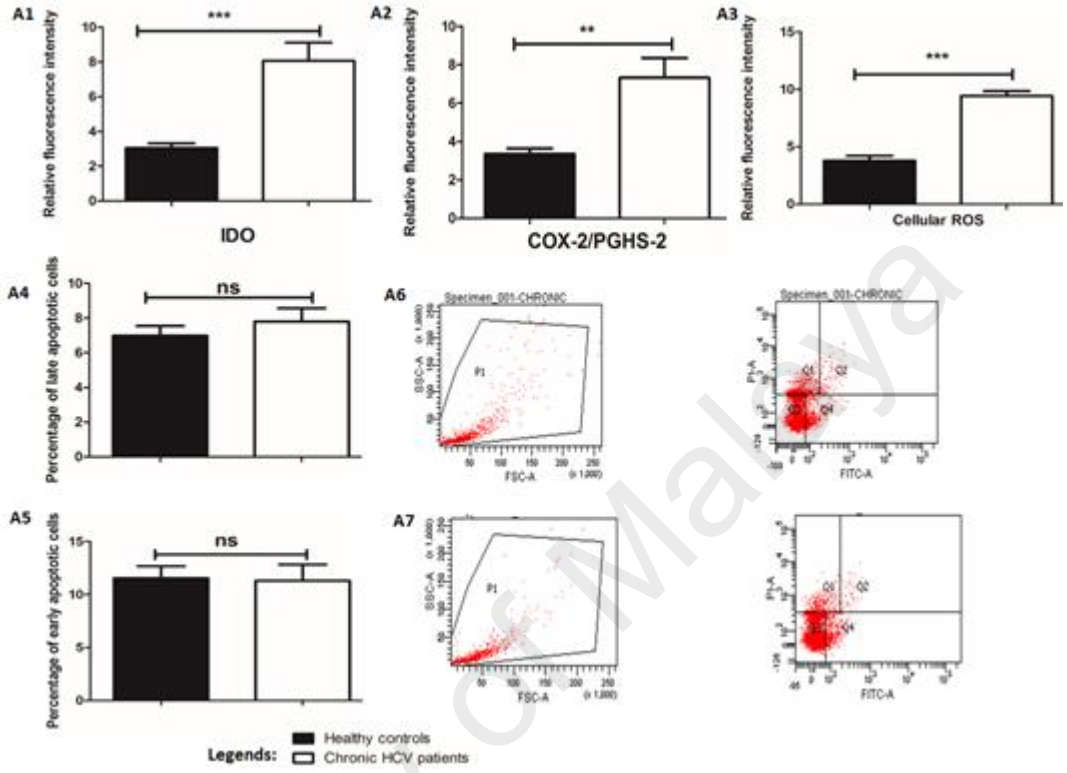

Figure 4.1: Determination of Apoptosis Events and Intracellular Molecules Responsible for Apoptosis in Chronic HCV Patients and Healthy Controls. (A1) serum IDO, (A2) COX-2/PGHS-2 in PBMC culture, (A3) cellular ROS in PBMC culture, (A4) late-apoptotic cells in PBMCs, (A5) early apoptotic cells in PBMCs, (A6) representative FACS image illustrating the levels of early-and late-apoptotic cells in PBMCs of chronic HCV patients and (A7) representative FACS image depicting the levels of early-and late-apoptotic cells in PBMCs of healthy controls. Bulk immune cells gated from forward (FSC) and side scatters (SSC) segregated for early apoptotic, and subsequently for late apoptotic immune cells. Q3; viable cells, Q4; early apoptotic cells, Q1; necrotic cells and Q2; late apoptotic cells. Meanwhile, the levels of significance for comparisons between two or more independent groups were determined using a Mann–Whitney U-test with threshold for significance P=0.025 after Bonferroni correction for 2 comparisons. Data are presented as mean ± SEM, where n refers to the number of independent samples. P<0.05 (two-tailed) was considered as statistically significant. Bars represent mean values. *P<0.05, **P<0.005, ***P<0.001, ns=not significant.

4.1.5 Surface Expression of Immune Checkpoints on T-Cells

CVI contributes to expression of co-inhibitory molecules leading to T-cell exhaustion (Walton *et al.,* 2013). Hence, we measured the surface expression levels of certain co-inhibitory molecules on un-stimulated CD4+ and CD8+ T-cells by flow cytometry. Our findings indicated that the levels of TRAIL on CD4+ T-cells of chronic HCV-infected patients were up-regulated relative to healthy controls ($P<0.05$). Meanwhile, other co-inhibitory molecules such as TIM-3, PD-1 and CTLA-4 on CD4+ T-cells of HCV patients showed an increase although this was not significant as compared to healthy controls. In contrast, CD8+ T-cells from patients showed significant increase in the expression of PD-1 ($P<0.05$) and CTLA-4 ($P<0.05$). Further, down-regulation of TRAIL, TIM-3 and BTLA molecules was observed in CD8+ T-cells of patients as compared to healthy controls. Overall, CD4+ T-cells of patients showed significantly increased expression of TRAIL ($P<0.05$). Hence, we hypothesized that there could be a likely role of spontaneous apoptosis operational in immune cells during chronic HCV infection.

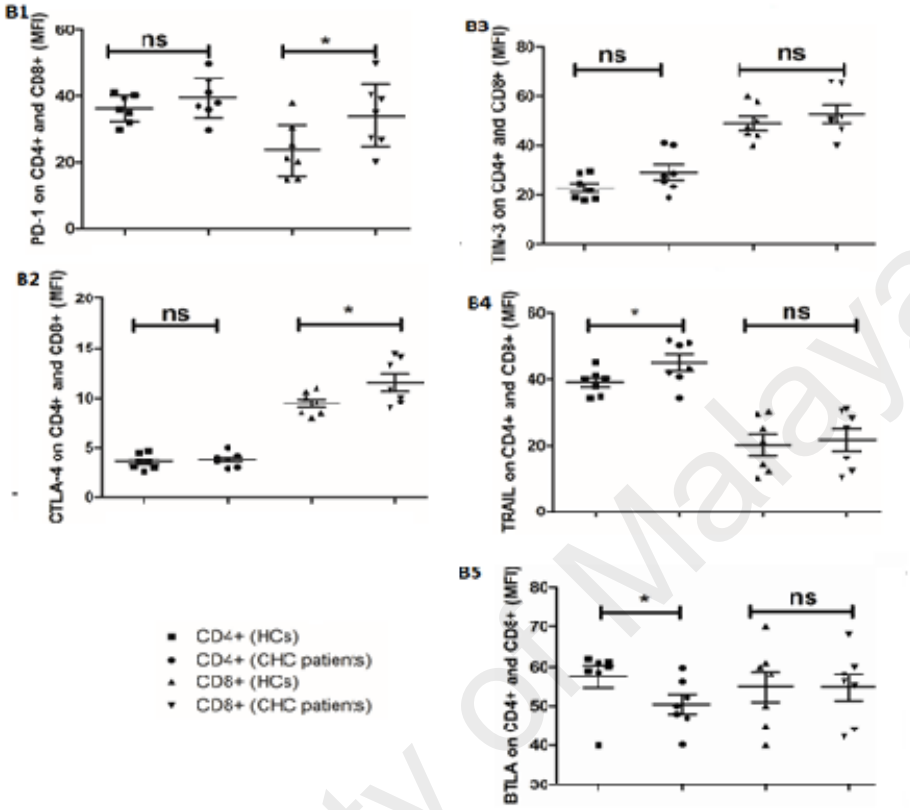

Figure 4.2: Differential Surface Protein Expression of Co-Inhibitory Receptors on CD4+ and CD8+ T-Cells from Chronic HCV-Infected Patients and Healthy Controls. Dot plot shows the expressions of **(B1)** PD-1 **(B2)** CTLA-4 **(B3)** TIM-3 **(B4)** TRAIL and **(B5)** BTLA on CD4+ relative to CD8+ T cells of HCV-infected patients. The levels of significance for comparisons between two or more independent groups determined using a non-parametric Mann–Whitney U-test with threshold for significance P=0.025 after Bonferroni correction for 2 comparisons. Data presented as mean ± SEM, where n refers to the number of independent samples. P<0.05 (two-tailed) was considered significant. *P<0.05, **P<0.005, ***P<0.001, ns=not significant.

4.1.6 Gene Expression of Co-Inhibitory Molecules

We determined the gene expression of co-inhibitory molecules in the PBMCs by qRT-PCR. Our results indicated that the surface expression of BTLA (P<0.001) was highly up-regulated followed by TRAIL (P<0.001) on bulk PBMCs of chronic HCV-infected patients. *DTX1* and *BLIMP-1* were down-regulated (not significant; P=0.5338 and 0.5478 respectively) in CHC patients. *PD-1*, *TIM-3*, and *CD160* were moderately expressed on PBMCs infected with HCV (P<0.01). The expression of *FOXP-3* was significantly higher in CHC patients relative to HCs (P<0.001). *CTLA-4* expression was also significantly up-regulated (P<0.01) among patients compared to HCs. Together, we found that majority of the co-inhibitory molecules examined were up-regulated on immune cells in chronic HCV infection, suggesting that HCV could play a leading role in determining the fate of immune cells.

4.1.7 Expression of Pro- and Anti-Apoptotic Molecules on PBMCs

The increased expression of TRAIL at the gene and surface protein levels in whole PBMCs hints the possible role of spontaneous apoptosis in chronic HCV infection. Furthermore, our results also showed that chronic HCV infection modulates the spontaneous expression of a large array of apoptotic genes in immune cells. Of note, *TNFA*, a potent inducer of apoptosis, was significantly up-regulated in HCV patients than healthy controls. Furthermore, up-regulation of genes involved death receptor-mediated apoptosis such as *TNFRSF1A, FasL, Fas, TRAF, RIPK2, FADD, CASP8* and *CASP10* was also evident. Meanwhile, the apoptosis regulator Bcl-2 members including pro-apoptotic *BID*, *BAX*, *BAK*, *BCL10, BCL2L11* and *BIK* were significantly up-regulated in HCV-infected patients relative to healthy controls. We also found that the death factor genes *CASP 1, CASP 3, CASP 4, CASP 6, CASP 7*, and *CASP 9* were up-regulated together with *NOD1, CARD-6* and *CFLAR* in HCV-infected patients compared to healthy controls. Interestingly, anti-apoptotic factors *BCL2L2*, *BCL2L2* and *MCL-1* were also up-

regulated among immune cells of patients. Together, the preliminary findings demonstrated that the incidence of spontaneous apoptosis in PBMCs of patients was likely to be co-regulated by the extrinsic and intrinsic apoptotic pathways.

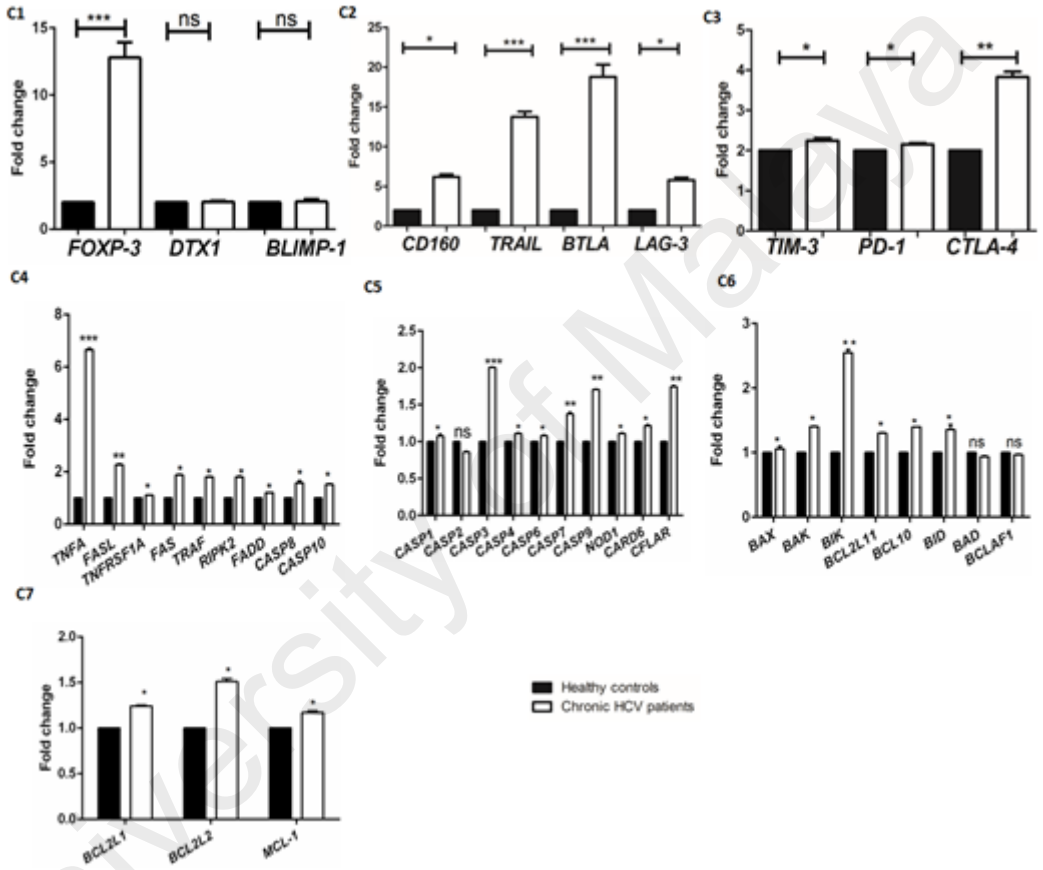

Figure 4.3: Gene Expression of Transcription Factors, Co-Inhibitory Receptors, Pro-Apoptotic Molecules and Anti-Apoptotic Molecules in PBMCs of the Study Population. (C1) transcription factor, (C2) inihibitory receptors, (C3) inhibitory receptors, (C4): molecules involved in death receptors apoptotic pathway, (C5) molecules involved in mitochondrial apoptosis pathway, (C6) pro-apoptotic molecules belong to BCL2 superfamily and (C7) anti-apoptotic molecules. The levels of significance for comparisons between two or more independent groups were determined using a non-parametric Mann–Whitney U-test with threshold for significance P=0.025 after Bonferroni correction for 2 comparisons. Data are presented as mean ± SEM, where n refers to the number of independent samples. P value < 0.05 (two-tailed) was considered as statistically significant. Bars represent mean values. *P<0.05, **P<0.005, ***P<0.001, ns=not significant.

4.2 Objective 2: T-Cell Senescence in Chronic HCV Infection

4.2.1 Surface Expression of Co-Inhibitory Receptors on HCV-Specific T-Cells

PBMCs from 20 chronic HCV-infected patients and 20 HCs were activated to investigate for senescence on HCV-specific and PHA-expanded CD4+ and CD8+ T-cells against CD57, CD127, CD27, and CD28 levels by flow cytometry. The expression of PD-1 was also studied since it could play a crucial role in the ageing of virus-specific T-cells during chronic infection. As expected, we found that virus-specific bulk T-cells of HCV-infected patients displayed a higher percentage of PD-1 with respect to HCs. The HCV-specific CD4+ and CD8+ T-cells showed significantly increased expression of PD-1 relative to healthy controls ($P<0.001$). A similar pattern was also observed among PHA-stimulated CD4+ and CD8+ T-cells of HCV-infected patients that expressed significantly higher levels of PD-1 as compared that of HCs. Meanwhile, correlation between HCV PVL and surface PD-1 expression on HCV-specific CD3+ T-cells was also significant. In general, higher PD-1 expression on HCV-specific T-cells could lead to impaired virus-specific immune response.

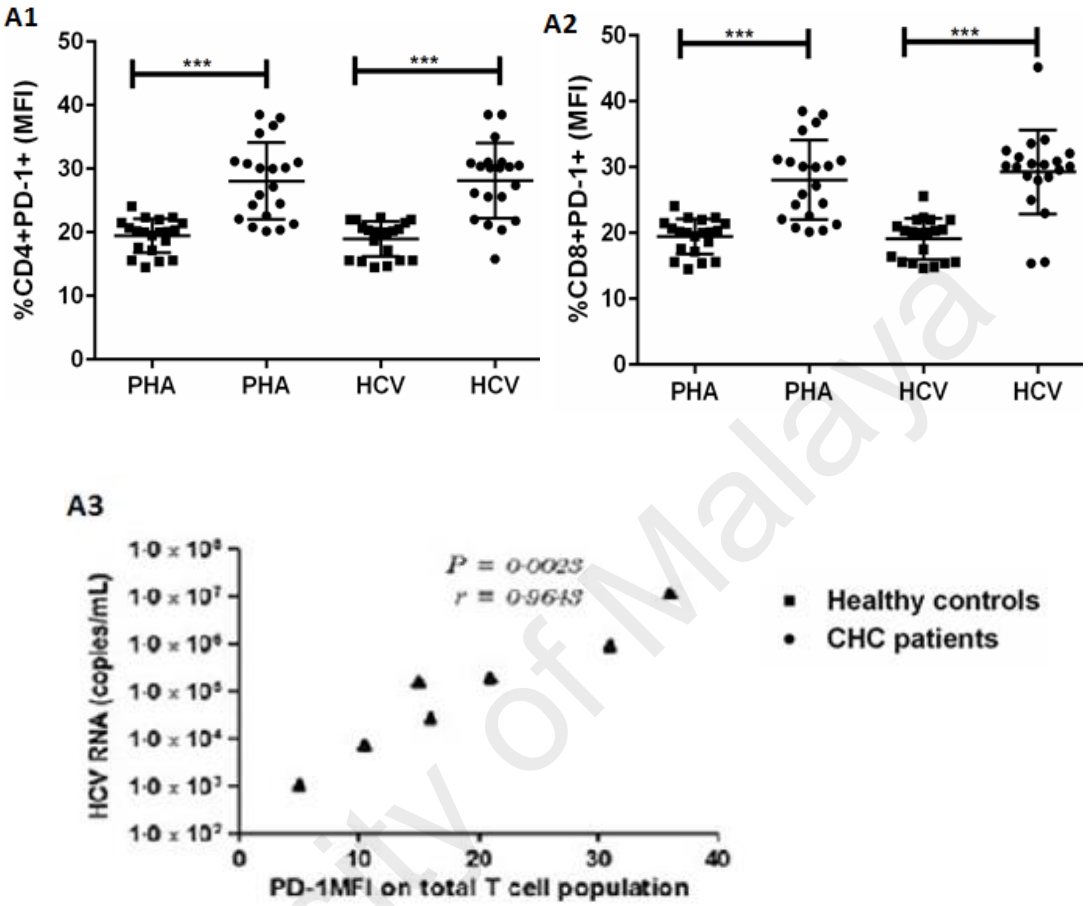

Figure 4.4: Surface Expression of PD-1 on HCV-Specific and PHA-Expanded T-Cells of Chronic HCV Patients and Healthy Controls. (A1) PD-1 on HCV-specific and PHA-expanded CD4+ T-cells of chronic HCV-infected patients, **(A2)** PD-1 on HCV-specific and PHA-expanded CD8+ T-cells of HCV patients compared to healthy controls and **(A3)** Correlation between HCV PVL and PD-1 expression on HCV-specific T-cells. The levels of significance for comparisons between two or more independent groups were determined using a non-parametric Mann–Whitney U-test with threshold for significance P=0.025 after Bonferroni correction for 2 comparisons. Data are presented as mean ± SEM, where n refers to the number of independent samples. P<0.05 (two-tailed) was considered as statistically significant. *P<0.05, **P<0.005, ***P<0.001, ns=not significant. Correlation was performed using non-parametric Spearman rank-order correlation test.

4.2.2 Expression of Chronic Immune Activation Markers on HCV-Specific and PHA-Expanded CD4+ and CD8+ T-Cells

The expression of CIA markers, HLA-DR and CD38 on HCV-specific and PHA-expanded T-cells was studied together with senescence because prolonged immune activation leads to loss of immune cells. As expected, stimulation of T-cell subsets with HCV peptides led to significant increase of CD4+CD38+HLA-DR+ ($P<0.001$) and CD8+CD38+HLA-DR+ ($P<0.05$). In contrast, our findings also revealed that no significant difference in PHA-activated CD4+ and CD8+ T-cells co-expressing CD38+ and HLA-DR was found among HCV patients. The expression of HLA-DR was significantly increased on HCV-specific CD4+ and CD8+ T-cells of patients, whereas PHA-expanded CD4+ T-cells of HCV patients expressed significantly higher levels of HLA-DR. HCV-specific and non-specific CD4+ T-cells of patients evenly expressed significant levels of CD38 ($P<0·001$). Taken together, our data indicates that HCV potentially leads to the onset of CIA in T-cells.

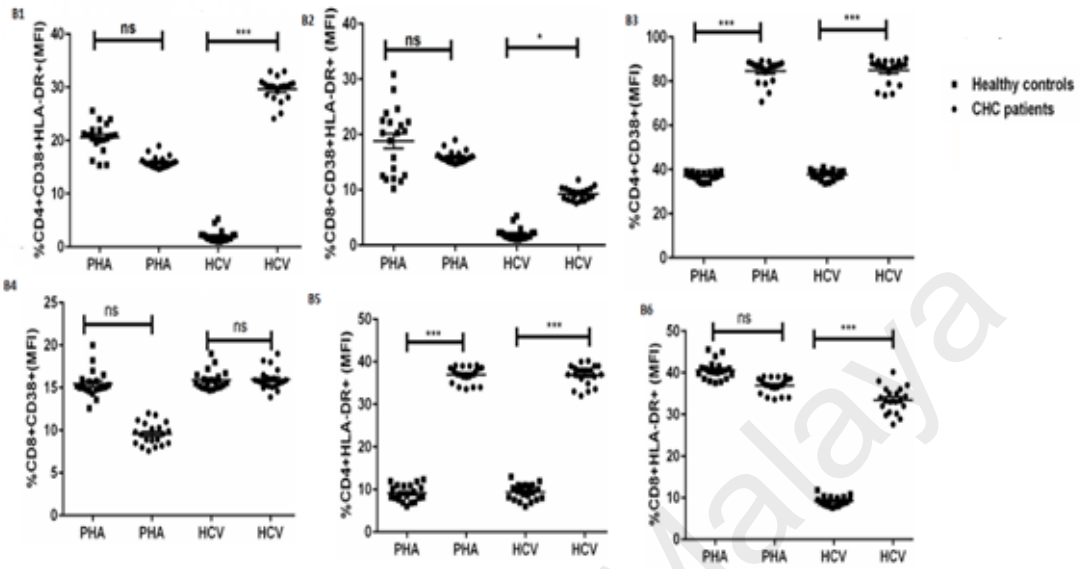

Figure 4.5: Surface Expressions of Immune Activation Markers, CD38 and HLA-DR on HCV-Specific and PHA-Expanded T-Cells of Chronic HCV Patients and Healthy Controls. **(B1)** CD38 and HLA-DR on HCV-specific CD4+ T-cells of HCV patients, **(B2)** HCV-specific CD8+ T-cells from HCV patients co-expressing CD38 and HLA-DR, **(B3)** HCV-specific and PHA-expanded CD4+ T-cells expressing CD38, **(B4)** HCV-specific and PHA-expanded CD8+ T-cells of HCV patients expressing CD38, **(B5)** HLA-DR on PHA-expanded and HCV-specific CD4+ T-cells of HCV patients and **(B6)** HCV-specific CD8+ T-cells derived from chronic HCV patients expressing HLA-DR. The levels of significance for comparisons between two or more independent groups were determined using a non-parametric Mann–Whitney U-test with threshold for significance P=0.025 after Bonferroni correction for 2 comparisons. Data are presented as mean ± SEM, where n refers to the number of independent samples. P<0.05 (two-tailed) was considered as statistically significant. *P<0.05, **P<0.005, ***P<0.001, ns=not significant

4.2.3 Expression of Replicative Senescence on HCV-Specific and PHA-Expanded T-Cells Subsets

The level of expression of CD57 was studied on HCV-specific and PHA-expanded T-cell subsets of chronic HCV-infected patients and HCs. We observed a significant increase in the percentage of HCV-specific and non-specific CD4+ T cells that expressed CD57 in HCV-infected patients relative to HCs (P<0·001). We also observed a similar profile on CD8+ T-cells, which however, was negligible on these T-cell subsets.

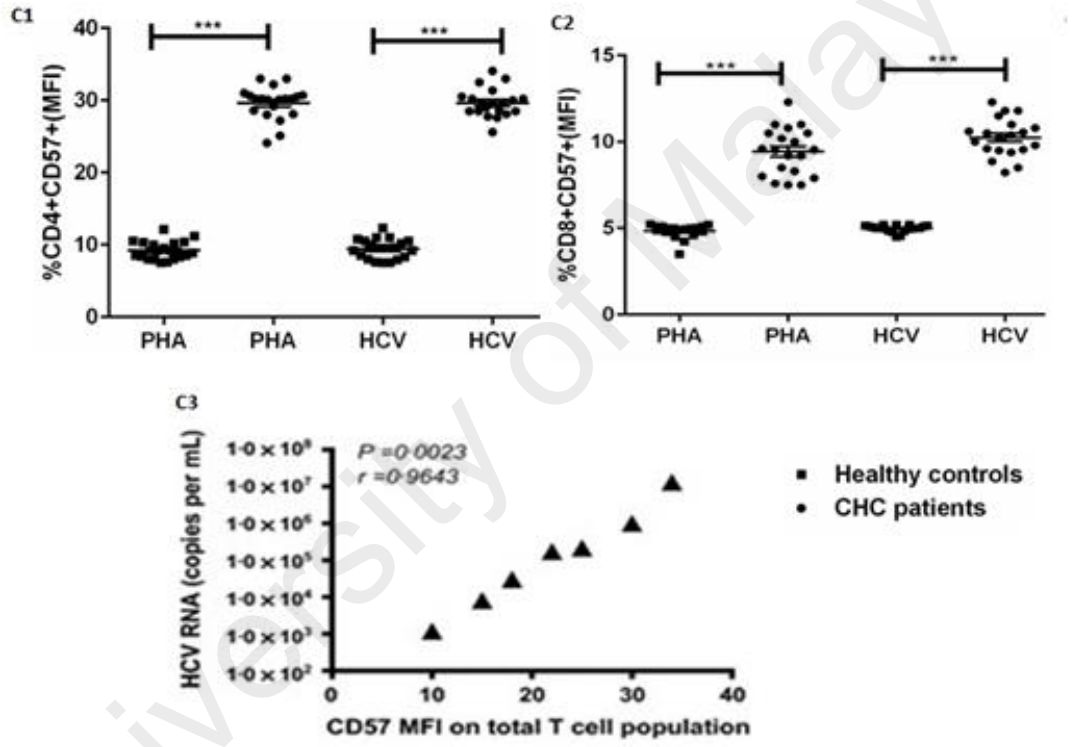

Figure 4.6: Expression of CD57 on T-cells derived from chronic HCV patients and healthy controls. (C1) Expression of CD57 on PHA-expanded and HCV-specific CD4+ T-cells of CHC patients, **(C2)** Levels of CD57 on PHA-expanded and HCV-specific CD8+ T-cells of CHC patients **(C3)** Correlation between HCV-specific total T-cells expressing CD57 and HCV plasma viral load. The levels of significance for comparisons between two or more independent groups were determined using a non-parametric Mann–Whitney U-test with threshold for significance P=0.025 after Bonferroni correction for 2 comparisons. Data are presented as mean ± SEM, where n refers to the number of independent samples. P value < 0.05 (two-tailed) was considered as statistically significant. *P<0.05, **P<0.005, ***P<0.001, ns=not significant. Figure C3: Correlation performed using non-parametric Spearman rank-order correlation test.

4.2.4 Expression of T-Cell Survival Markers on HCV-Specific and Non-Specific T-Cell Subsets

Next, we examined the expression of CD127 on HCV-specific and non-specific CD4+ and CD8+ T-cells using flow cytometry. Our findings showed that HCV patients displayed a significant decrease in CD127 levels on both HCV-specific and non-specific CD4+ T-cells ($P<0.05$) consistent with a previous study. Moreover, non-specific CD4+ T-cells did show reduction in CD127 expression although this was not greater than that observed among HCV-specific CD4+ T-cells. The decrease of CD127 on HCV-specific and PHA-stimulated CD8+ T-cells in HCV-infected patients was significant relative to HCs ($P<0.01$). Meanwhile, a smaller proportion of CD8+ T-cells that expressed reduced levels of CD127 was also observed. Together, our results indicate that HCV infection led to significant increase of virus-specific CD4+ and CD8+ T-cells that have defective CD127 expression.

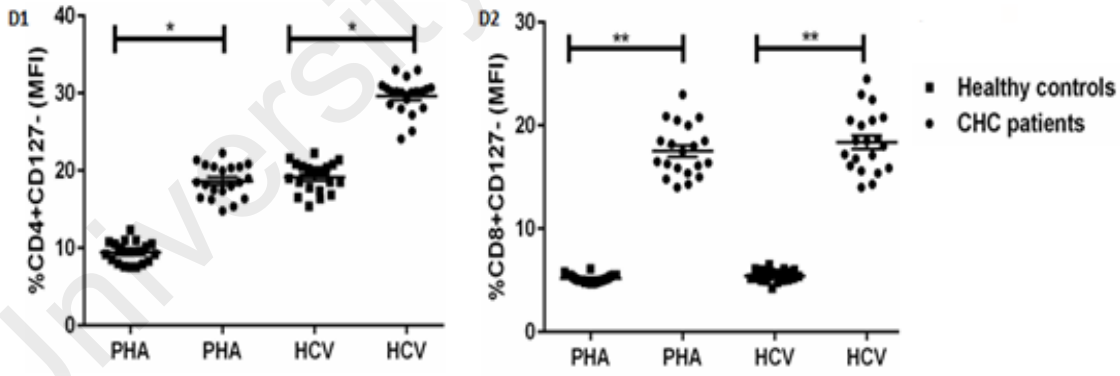

Figure 4.7: Expression of CD127 on stimulated T-cells of chronic HCV patients relative to healthy controls. (D1) CD127 expression of CD4+ T-cells and **(D2)** CD8+ T-cells. The levels of significance for comparisons between two or more independent groups were determined using a non-parametric Mann–Whitney U-test with threshold for significance P=0.025 after Bonferroni correction for 2 comparisons. Data are presented as mean ± SEM, where n refers to the number of independent samples. $P<0.05$ (two-tailed) was considered as statistically significant. *$P<0.05$, **$P<0.005$, ***$P<0.001$, ns=not significant. P values calculated by non-parametric Mann–Whitney U test.

4.2.5 Surface Expression of Differentiation Markers on HCV-Specific and PHA-Expanded CD4+ and CD8+ T-Cells

The differentiation status of HCV-specific and PHA-stimulated CD4+ and CD8+ T-cells was demonstrated by measuring the levels of CD27 and CD28 expression on T-cell subsets. Based on the expression profile of CD28/CD27, CD4+ and CD8+ T-cells were classically distinguished into three subpopulations, such as CD28+ CD27+ (early differentiated T-cell population), CD28−CD27+ (intermediate differentiated T-cell population) and CD28−CD27− (terminally/late differentiated T-cell population). A higher and significant proportion of late-differentiated cell population was observed on both T-cell subsets, CD4+ and CD8+ of patients when stimulated with HCV peptides compared to PHA ($P<0.001$). The formation of late-differentiated CD4+ and CD8+ T-cells was not apparent among healthy controls. There was no significant difference in the proportion of intermediate-differentiated T-cell population in both non-specific and HCV-specific T-cell subsets of patients and HCs. The non-specifically and HCV-specifically stimulated T-cells of HCs showed a significant increase in T-cells belonging to the early differentiation subsets as compared to chronic HCV-infected patients. Together, the data suggests that HCV accelerates the T-cell compartment to differentiate into late-differentiated T-cells resulting in premature ageing. Defective T-cell maturation and differentiation attributes may appear to be one of the key features of HCV disease.

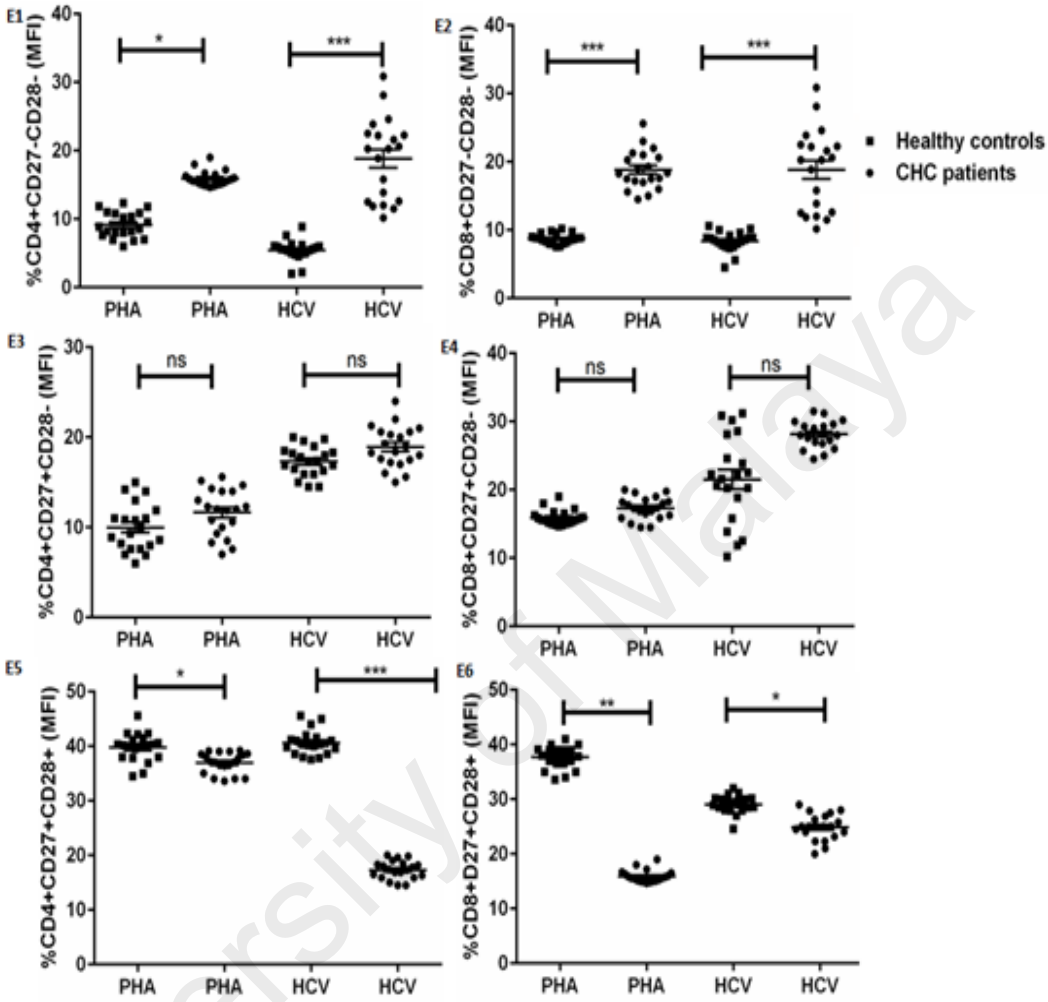

Figure 4.8: HCV-Specific and PHA-Expanded T-Cell Differentiation Subsets. (E1) Late-differentiated (CD27−CD28−) CD4+ T-cells in CHC patients, **(E2)** Late-differentiated (CD27−CD28−) HCV-specific and PHA-expanded CD8+ T-cells, **(E3)** intermediate differentiated (CD27+CD28-) HCV-specific and PHA-expanded CD4+ T-cells, **(E4)** intermediate differentiated (CD27+CD28-) HCV-specific and PHA-expanded CD8+ T-cells, **(E5)** early differentiated (CD27+CD28+) HCV-specific and PHA-expanded CD4+ T-cells and **(E6)** early differentiated (CD27+CD28+) HCV-specific and PHA-expanded CD8+ T-cells. The levels of significance for comparisons between two or more independent groups were determined using a non-parametric Mann–Whitney U-test with threshold for significance P=0.025 after Bonferroni correction for 2 comparisons. Data are presented as mean ± SEM, where n refers to the number of independent samples. P<0.05 (two-tailed) was considered as statistically significant. *P<0.05, **P<0.005, ***P<0.001, ns=not significant.

4.3 Objective 3: Functional Exhaustion of HCV-Specific T-Cells in Chronic HCV Infection

4.3.1 Surface Expression of Co-Inhibitory Receptors on HCV-Specific CD4+ T-Cells

A total of 20 PBMCs from 10 chronic HCV-infected patients and HCs each were used to study the phenotypes and levels of immune exhaustion markers, PD-1, CTLA-4, TIM-3, TRAIL and BTLA in chronic HCV infection. Therefore, the defects in virus specific and non-specific helper T-cells in patients and HCs were investigated by examining the *ex vivo* expression of the co-inhibitory receptors on HCV-specific (HCV peptide stimulated) and non-specific (PHA-stimulated) CD4+ T-cells. The results showed differential expressions of PD-1, CTLA-4, TIM-3, TRAIL and BTLA on the *in vitro* stimulated HCV-specific and non-specific CD4+ T-cells. The chronic HCV-infected patients showed a significantly higher percentage of HCV-specific CD4+ T-cells expressing PD-1 ($P<0.05$), CTLA-4 ($p<0.001$) and TRAIL ($P<0.01$) with respect to HCs. Meanwhile, CD4+ T-cells of patients showed no significance in the levels of TIM-3 and BTLA compared to HCs. Our results also indicated that significant PD-1 expression was seen on HCV-specific CD4+ T-cells rather than PHA-expanded CD4+ T-cells of CHC patients. Interestingly, CD4+ T-cells of CHC patients showed significantly increased levels of CTLA-4 and TRAIL on HCV-specific ($P<0.001$) and non-specific T-cells ($P<0.01$). Together, our data suggest that HCV facilitates the increased expression of co-inhibitory molecules on virus-specific T-cells during persistent infection.

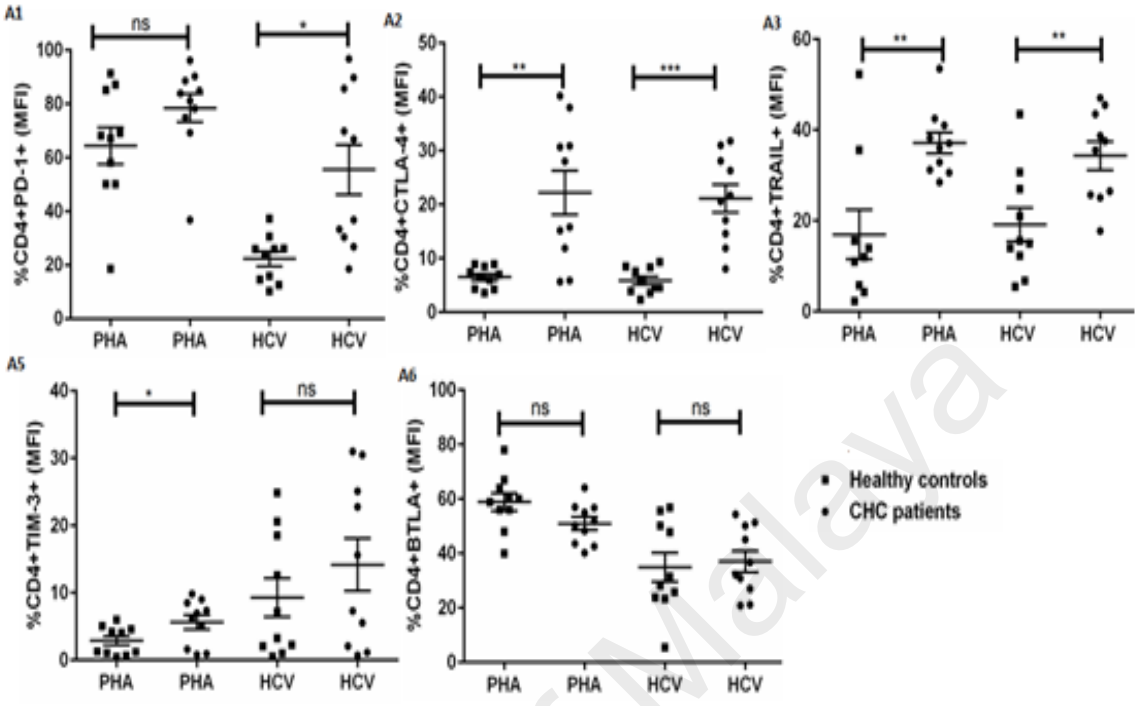

Figure 4.9: Expression of Co-Inhibitory Molecules on HCV-Specific and PHA-Expanded CD4+ T-Cells of Chronic HCV Patients and Healthy Controls. **(A1)** PD-1 expression on HCV-specific CD4+ T-cells, **(A2)** CTLA-4 levels on HCV-specific and PHA expanded CD4+ T-cells, **(A3)** TRAIL expression on HCV-specific and PHA expanded CD4+ T-cells, **(A4)** TIM-3 levels on PHA-expanded CD4+ T-cells and **(A5)** BTLA expression levels. Data are presented as mean ± SEM, where n refers to the number of independent samples. P<0.05 (two-tailed) was considered as statistically significant. *P<0.05, **P<0.005, ***P<0.001, ns=not significant. P values calculated by non-parametric Mann–Whitney U test.

4.3.2 Expression of Co-Inhibitory Molecules on Cytotoxic T-Cells in Chronic HCV Infection

The differential expressions of PD-1, CTLA-4, TIM-3, TRAIL and BTLA were measured on HCV-specific and non-specific CD8+ T-cells of CHC patients and HCs. We found that PD-1 expression was significantly higher on HCV-specific CD8+ T-cell cultures derived from CHC patients ($P<0.01$). However, the frequency of HCV-specific CD8+ T-cells expressing PD-1 was markedly lesser than that of non-specific CD8+ T-cells after 48 hours of stimulation, although this was not significant in PHA-expanded CD8+ T-cells. We also found that the HCV-specific CD8+ T-cells did not express significant levels of TRAIL although this was seen on nonspecific CD8+ T-cells ($P<0.01$). Meanwhile, TIM-3 was significantly increased on HCV-specific CD8+ T-cells in CHC patients ($P<0.05$) although no significance was seen on nonspecific CD8+ T-cells of CHC patients. Similarly, BTLA levels were decreased on CD8+ T-cell responses in CHC patients. Our data suggests that HCV that HCV up-regulates the expression of certain co-inhibitory molecules on virus-specific T-cells potentially leading to immune exhaustion.

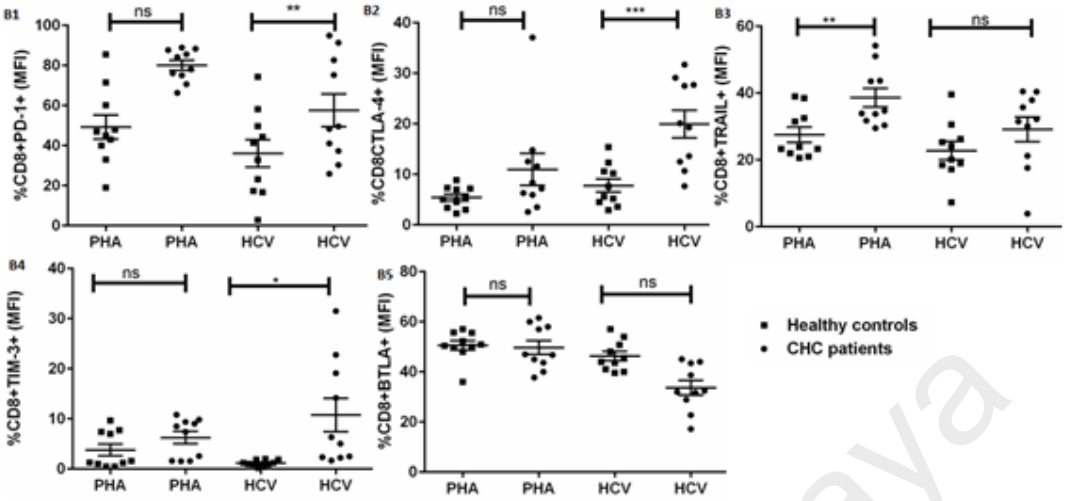

Figure 4.10: Expression of Co-Inhibitory Checkpoints on HCV-Specific and PHA-Expanded CD8+ T-Cells of Chronic HCV Patients and Healthy Controls. (A1) PD-1 on HCV specific CD8+ T-cells, **(B2)** CTLA-4 expression on HCV-specific CD8+ T-cells, **(B3)** TRAIL levels on PHA-expanded CD8+ T-cells, **(B4)** TIM-3 expression on HCV-specific CD8+ T-cells and **(B5)** BTLA expression on both stimulated CD8+ T-cells. Data are presented as mean ± SEM, where n refers to the number of independent samples. $P<0.05$ (two-tailed) was considered as statistically significant. *$P<0.05$, **$P<0.005$, ***$P<0.001$, ns=not significant. P values calculated by non-parametric Mann–Whitney U test.

4.3.3 Secretion of Pro- and Anti-Inflammatory Molecules in HCV-Specific T-Cell Cultures

Deviations in the secretion of pro-inflammatory and anti-inflammatory cytokines in chronic infection may alter virus-specific CVI responses. Hence, the secretion levels of representative cytokines were determined in culture supernatants of HCV-specific and nonspecific T-cells using the Multi-Analyte ELISArray cytokine kit. In the supernatants of HCs, there was a significant increase in most of the Th1 pro-inflammatory cytokines, IL-2, IL-6, IL-17A, TNF-α and G-CSF compared to patients upon stimulation with HCV peptide. At 48 hours of stimulation, no significant levels of IL-12 were detectable. Interestingly, IFN-α was found to be highly expressed in the culture supernatants of HCV-specific T-cells of CHC patients relative to HCs. IL-10 and TGF-β1 levels were increased significantly in HCV-specific T-cell cultures of CHC patients as compared to HCs.

However, the levels of IL-4, IL-5 and IL-13 were not significant in the culture supernatants of CHC patients. Further, the culture supernatants of HCV-specific T-cells of HCs secreted increased levels of IL-13 compared to CHC patients.

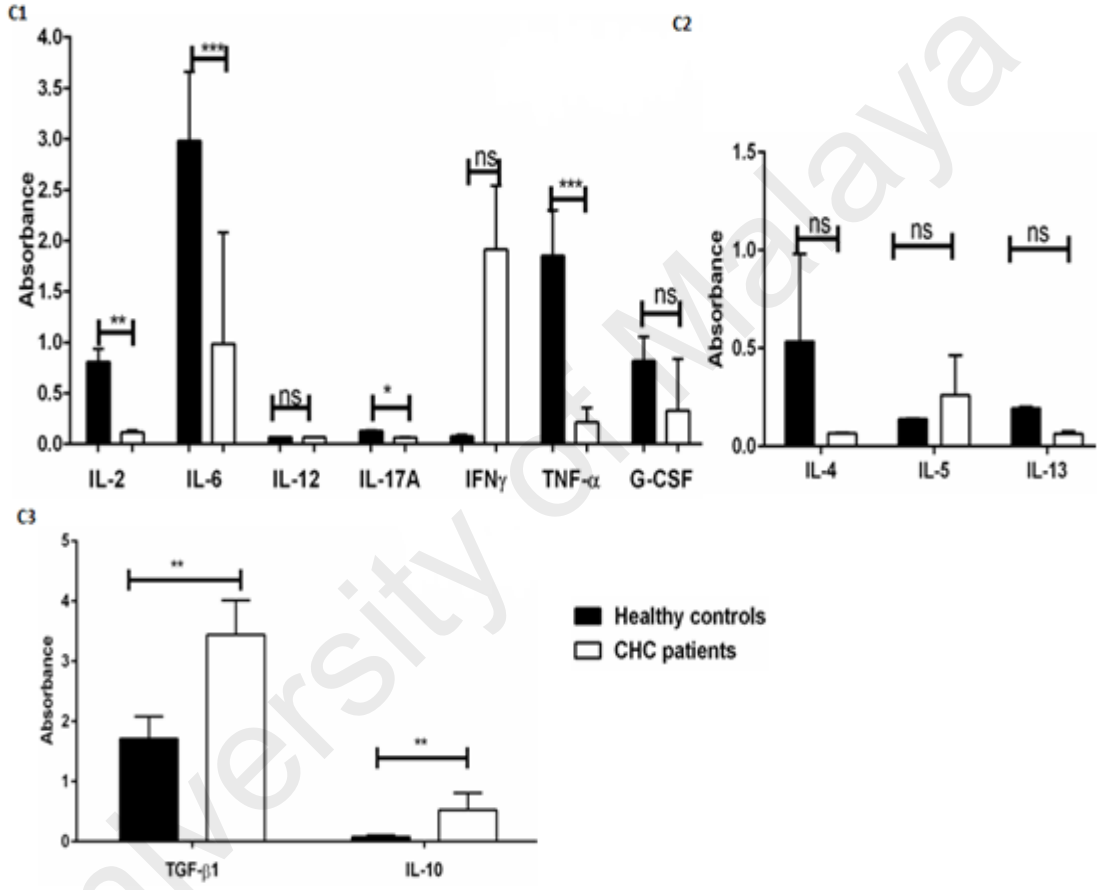

Figure 4.11: Profile of Th1, Th2, and Th17 and Immunoregulatory Cytokine Levels in Cultures of HCV-Specific T-Cells in the Study Population. (C1) Th1 cytokines, **(C2)** Th2 cytokines and **(C3)** immunoregulatory cytokines. The levels of significance for comparisons between two or more independent groups were determined using a Mann–Whitney U-test with threshold for significance P=0.025 after Bonferroni correction for 2 comparisons. Data presented as mean ± SEM, where n refers to the number of independent samples. P<0.05 (two-tailed) was considered as statistically significant. Bars represent mean values. *P<0.05, **P<0.005, ***P<0.001

4.4 Objective 4: Peripheral Loss and Exhaustion of Mucosal-Associated Invariant T (MAIT) Cells in Chronic HCV Infection

4.4.1 Phenotypic Characterisation of MAIT Cells

A non-randomized study consisting of a convenience sample of 50 individuals including 25 chronic HCV-infected patients and 25 healthy controls, was designed to characterize MAIT cells. MAIT cells were defined based on the surface expression of CD161, where functionality was based on the high expression of CD161 (CD161++), and less-functional phenotypes show down-regulation of CD161 (CD161-), together with expression of semi-variant TCR Vα7.2 (marker for antimicrobial properties of MAIT). We found that the frequency of circulating MAIT cells was significantly decreased in CHC patients compared to HCs ($P<0.001$). Our data also showed significantly increased levels of CD8+CD161-TCRVα7.2+ MAIT cells in CHC patients relative to HCs ($P<0.01$). Together, our results indicated that CHC infection down-regulated CD161 levels likely leading to altered phenotypes and function of MAIT cells.

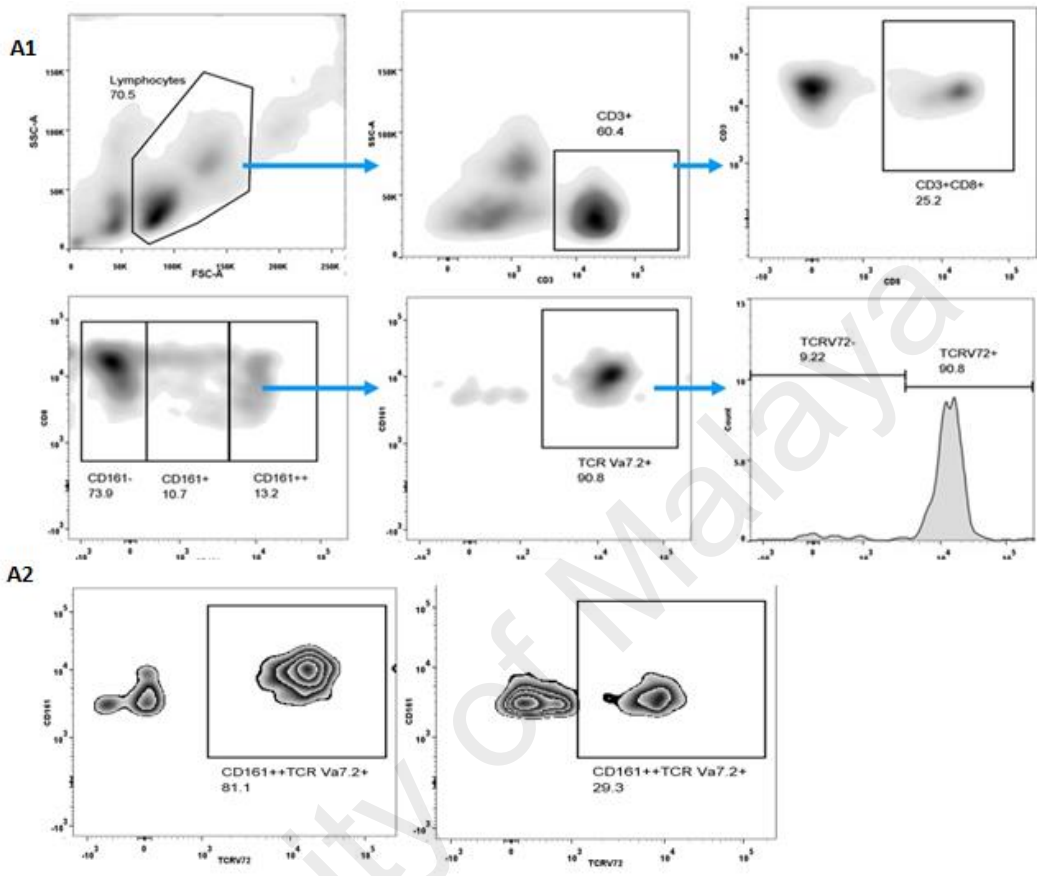

Figure 4.12: Representative FACS Image and Scatter Plot for MAIT Cells in Chronic HCV Patients and Healthy Controls. (A1) Selection strategy for CD8+CD161++TCRVα7.2+ MAIT cells in the PBMCs of the study population. Lymphocytes gated from forward (FSC) and side scatters (SSC) segregated for CD3+ T-cells, and subsequently for CD8+ T cells. CD8+ T-cells with signals for CD161 (i.e. CD161++) were categorized as CD161-, CD161+ (dim) and CD161++ (bright), followed by selection of TCRVα7.2 expressing CD161++ cells. **(A2)** Representative FACS images of MAIT cells for healthy controls (left) and chronic HCV-infected individuals (right)

4.4.2 Characterisation of Cell Homing Properties of Peripheral MAIT Cells

The tissue-homing properties of peripheral MAIT cells were studied for CCR5 and CD103 expressions. Hence, the levels of liver infiltrating chemokine receptor, CCR5 and liver/gut-homing chemokine receptor CD103 were analyzed on functional MAIT (CD8+CD161++TCRVα7.2+) cells of CHC patients along with HCs. The expression of

CCR5 on CD161++TCRVα7.2+ MAIT cells derived from HCs expressed significantly higher levels compared to patients. Nevertheless, no significant difference was found in the expression of CD103 between patients and healthy controls. Together, the results indicate that MAIT cells of patients exhibited decreased in liver infiltrating marker level also expression of CD103 appears to play less likely role in determining the homing characteristics of peripheral MAIT cells.

4.4.3 Signatures of Immune Exhaustion Receptors on Circulating MAIT Cells

The expression of immune exhaustion receptors on MAIT cells could play role in the loss of these antimicrobial cell phenotypes. Our results showed that the CD8+CD161++TCRVα7.2+ MAIT cells of CHC patients expressed significantly increased levels of PD-1 as compared to HCs ($P<0.001$). Meanwhile, we also found that the CHC patients showed significantly increased levels of TIM-3 relative to HCs ($P<0.01$). The CD8+CD161++TCRVα7.2+ T-cells of chronic HCV cases showed significantly higher levels of CTLA-4 as compared to HCs ($P<0.001$). Together, our results concluded that circulating CD8+CD161++TCRVα7.2+ T-cells display an exhausted phenotype and due to increased expression of PD-1, TIM-3 and CTLA-4 in chronic HCV disease.

4.4.4 Premature Immunosenescence and Chronic Immune Activation on Circulating CD8+CD161++TCRVα7.2+ MAIT Cells

The increased frequency of replicative senescent T-cell in CHC disease hints the possibility loss of MAIT cells in the peripheral blood of HCV-infected individuals. Given the high levels of immune exhaustion in chronic HCV infection, the expression levels of senescence marker, CD57 along with CIA markers, HLA-DR and CD38 were investigated on peripheral MAIT cells of CHC patients and HCs. As expected, MAIT cells of CHC patients expressed significantly higher levels of CD57 compared to that of HCs ($P<0.001$). Meanwhile, significant increase of both HLA-DR and CD38 were also

observed on MAIT cells of patients (P<0.001) in comparison with HCs. Overall, the circulating MAIT cells showed increased signs of CIA (increase of CD38 and HLA-DR) and premature immunosenescence (increase of CD57) in chronic HCV infection.

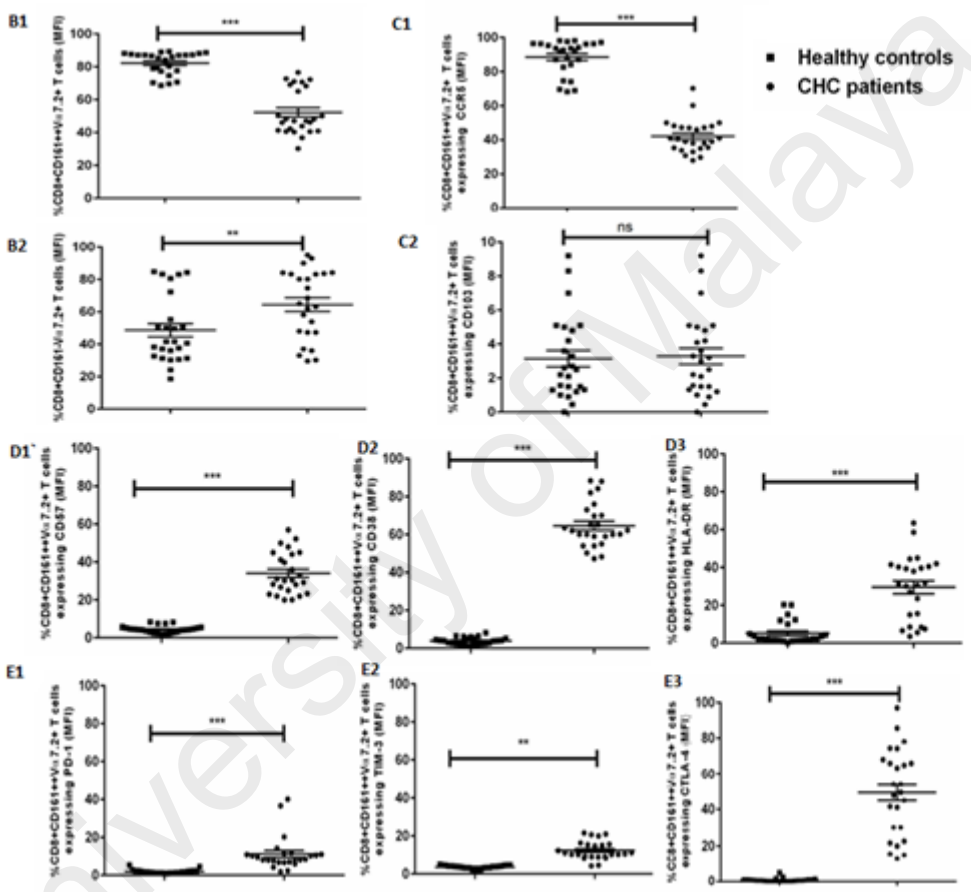

Figure 4.13: Phenotypic and Functional Properties of MAIT Cells. (B1) Frequency of functional (classical) MAIT cells, **(B2)** Frequency of less functional MAIT cells in the study population, expressions of **(C1)** CCR5, **(C2)** CD103, **(D1)** CD57, **(D2)** CD38, **(D3)** HLA-DR, **(E1)** PD-1, **(E2)** TIM-3, and **(E3)** CTLA-4 on classical MAIT cells of patients compared to healthy controls. All graphs show mean and SEM; P values are reported for two-sided Mann-Whitney tests with threshold for significance P=0.025 after Bonferroni correction for 2 comparisons. *P<0.05, **P<0.005, ***P<0.001, ns=not significant

CHAPTER FIVE

DISCUSSION

CHAPTER 5: DISCUSSION

HCV represents a challenging global health concern in ~200 million infected individuals (Hajarizadeh *et al.,* 2013). Clinical data suggests that only ~15% of acutely HCV infected individuals will achieve spontaneous viral clearance despite exuberant virus-specific cellular immune responses (Ward *et al.,* 2002). Failure of virus-specific T-cell responses appears to lead to chronicity in infected individuals. Nearly ~85% of acutely HCV-infected individuals will progress to chronic stage and eventually advance towards life-threatening liver disease, including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) within an interval of 20-30 years (Westbrook & Dusheiko, 2014).

It is so difficult to identify HCV infection very early after exposure, owing to the lack of specific symptoms, it has been quite challenging to study the earliest phase of the T-cell response in humans. Over the last decade several studies have been carried out to explore the mechanisms behind the decline of virus-specific immune responses and viral persistence in chronically infected patients. Many have proposed that HCV adapts several mechanisms to disconcert the host's virus-specific immune responses including viral escape mutations, viral growth at privileged sites, antagonism and T-cell anergy (Cerny & Chisari, 1999; Sewell *et al.,* 2000). It has also been reported that spontaneous apoptosis (Badley *et al.*, 2013), premature aging (Desai & Landay, 2010) and exhaustion of virus-specific T-cells (Yi *et al.,*2010), together with depletion of antimicrobial innate-like MAIT cells (Fernandez *et al.,* 2015) could contribute to viral persistence. These mechanisms are believed to lead to establishment of chronicity in infected individuals. Future therapeutics will require better understanding of these mechanisms whereby viremia is controlled under these circumstances. Here, we characterized the role of certain potential factors/concepts associated with HCV immunopathogenesis and persistence during chronic infection.

5.1 Objective 1: Spontaneous Apoptosis in Chronic HCV Infection

A growing body of literature suggests that HBV-, SIV-, HIV-infected and HCV-HIV-co-infected individuals tend to experience destruction of virus-activated lymphocytes and uninfected immune cells via spontaneous apoptosis either by stimulation of death receptors or mitochondrial damage (Saraste & Pulkki, 2000; Petrovas *et al.*,2005; Reshi *et al.*,2014). Thus, as part of this objective, here we elucidated the occurrence of spontaneous apoptosis in virus-infected immune cells during chronic HCV infection. We found that chronic HCV infection led to induction of apoptosis together with oxidative stress in PBMCs. Interestingly, significant levels of expression of co-inhibitory molecules, transcription factors and immunoregulatory enzymes were clearly evident among CHC patients indicating the possible association between immune exhaustion and apoptosis of immune cells. Our findings also indicated the up-regulation of several pro-apoptotic factors involved in extrinsic and intrinsic apoptotic pathways (Barathan *et al.*, 2015).

5.1.1 Measurement of Serum Indoleamine 2, 3-Dioxygenase (IDO) Levels

Recently, IDO has drawn considerable attention as a mechanism of immune dysfunction associated with immune activation and inflammation (Baban, 2005; Mehraj & Routy, 2015). IDO is induced by proinflammatory cytokines, IFN-γ and TNF-α in patients with bacterial and viral infections (Schmidt & Schultze, 2014). Our findings revealed that HCV-infected individuals expressed significantly increased levels of serum IDO as compared to HCs. High IDO levels in CHC patients indicate the degradation of tryptophan via the kynurenine pathway that essential for proliferation, survival and function of virus-infected T cells (Chen & Guillemin, 2009; Mbongue *et al.,* 2015). Tryptophan deficiency has also been found to be associated with decline in lymphocyte numbers (Hoshi *et al.*, 2012). Up-regulation of IDO may represent a strategy of HCV to escape T-cell immunity and induce immune tolerance. Up-regulation of IDO expression

in liver and plasma has also been reported by others in chronic HCV infection, and has also been suggested to mediate peripheral tolerance (Larrea *et al.,* 2007). A similar observation has also been witnessed in other CVIs, especially in HIV infection where increased IDO activity has been reported in HIV-infected patients, which appears to suppress immune responses to HIV antigens (Mellor & Munn, 2004). However, there was no association between HCV viral load and IDO activity in our study, which is in contrast to a study on HBV viral load and IDO levels (Yong-bing Chen *et al.,* 2009). The cost of IDO ELISA kit was prohibitive and therefore there was only sufficient space for the testing of 7 samples. At the same time in order to overcome the expiry date of the kits as soon as sufficient samples were obtained the assay was performed. This assay was included as a preliminary study to investigate IDO levels in patients with chronic HCV. Therefore due to small subset of samples tested the results should be treated with caution. Further studies using larger sample size should be performed to confirm these data. It must also be noted that a larger number of samples of chronic HCV samples that have high viral loads may produce different IDO levels.

5.1.2 Measurement of Signatures of Cellular Apoptosis in Immune Cells

One mechanism that appears to play a key role in virus-activated T-cell destruction and deletion in HIV infection is activation-induced cell death (AICD) (Young *et al.,*1997). T-cell apoptosis induced by chronically-infecting viruses could lead to immunotolerance and eventually to viral persistence (Nakamoto *et al.,*2002). Hence, here we aimed to determine the role of apoptosis in HCV-infected PBMCs. We found that the PBMCs of CHC patients showed high levels of detectable late apoptotic cells *in vitro* as compared to HCs. We hypothesized that HCV may have prompted apoptosis following acute infection and further led to progression of late apoptosis during chronic infection, which hints to loss the virus-specific T-cells and immune responses (Bantel & Schulze-Osthoff, 2003; Guicciardi & Gores, 2005; Radziewicz *et al.,* 2008). Apoptosis of infected and

bystander cells has been associated with HIV (Silvestri & Feinberg, 2003) and HBV (Schmidt *et al.*, 2013) infections.

5.1.3 Measurement of Prostaglandin G /H Synthase (PGHS) / Cyclooxygenase 2 (COX-2)

COX-2, also known as prostaglandin-endoperoxide synthase 2, is an enzyme induced in response to cellular activation by proinflammatory cytokines and viruses. Furthermore, it plays a key role in the onset of inflammatory responses (FitzGerald, 2011; Steer & Corbett, 2014). COX-2 is responsible for the generation of proinflammatory prostaglandin PGE_2 (Mori *et al.*, 2001), a lipid mediator that has been shown to participate in the regulation of virus replication and suppression of inflammatory responses, preventing the activation of antiviral cellular responses (Sobolewski *et al.*, 2010). Increased activity of COX-2 has been observed in other viral infections, for instance HBV (Yue *et al.*, 2011), cytomegalovirus (CMV) (Zhu *et al.*, 2002), and bovine leukemia virus (BLV) (Pyeon *et al.*, 2000). In contrast, Epstein–Barr virus (EBV) infection has been shown to suppress COX-2 activity in PBMC cultures (Savard *et al.*, 2000). Hence, here we investigated whether chronic HCV infection could regulate the expression of COX-2. As expected, significant elevation of COX-2 in PBMC cultures of CHC patients was witnessed relative to HCs. The up-regulation of COX-2 expression was clearly due to HCV infection potentially leading to chronic inflammation, also regulating virus-activated lymphocyte expansion.

5.1.4 Determination of Cellular Reactive Oxygen Species (cROS)

ROS is defined as oxidized molecules containing superoxide anions ($O^{-}2$), hydrogen peroxide (H_2O_2), singlet oxygen (O_2) and hydroperoxyl radicals (Lang e*t al.*, 2013). At low levels, ROS is usually involved in cell signaling and cellular homeostasis. However, excessive ROS formation could lead to cellular oxidative stress and cell death (Circu & Aw, 2010). Ample evidences have gathered over the past decade suggesting that patients

infected with RNA viruses tend to undergo chronic oxidative stress (Reshi *et al.,* 2014) in addition to bacteria (Spooner & Yilmaz, 2011) and parasitic infections (Percário *et al.,* 2012). Oxidative stress is the key contributor of viral replication, dysfunction immune system, apoptosis and chronic immune activation (Paracha *et al.,* 2013). ROS may regulate both the extrinsic and intrinsic apoptotic pathways of T cells by affecting the expressions of FasL and BCL-2 (Hildeman *et al.,* 1999; Hildeman *et al.,* 2003). Therefore, the formation of cROS in PBMC cultures of chronically HCV-infected individuals, and its association in virus-induced apoptosis were examined. Our findings of increased cROS levels in PBMC culture of CHC patients suggests the onset of oxidative stress resulting in the activation of mitochondrial death pathway. Others have also demonstrated that HCV could promote and manipulate the antioxidant systems of the host leading to chronic disease. Notably, excessive formation of ROS may likely activate *COX-2,* a mediator of chronic inflammation (Hsieh & Yang, 2013).

5.1.5 Surface Expression of Immune Checkpoints on T-Cells

Sustained expression of co-inhibitory signatures namely PD-1, TIM-3, BTLA, CD160, LAG3 and 2B4 on chronic virus-infected T-cells hints the onset of T-cell exhaustion (Yi *et al.,* 2010; Peretz *et al.,* 2012). They have been classically associated with a hierarchical dysfunction of cytotoxic activity, proliferative capacity, and impaired cytokine production by T-cells (Bucks *et al.,* 2009; Wherry, 2011). Deletion of virus-specific T-cells by apoptosis due to co-inhibitory signaling is known to be an important mechanism whereby chronically-infecting viruses persist in the host. Evidence suggests that blockade of the co-inhibitory pathways could salvage the functions of exhausted virus-specific CD8+ T-cells (Larsson *et al.,* 2013; Wherry & Kurachi, 2015; Ye *et al.,* 2015). Meanwhile, impaired virus-specific CD4+ T-cell responses could diminish CD8+ T-cell levels and B-cell activation (Kamphorst & Ahmed, 2013; Misumi & Whitmire, 2014). Our findings of up-regulation of multiple co-inhibitory receptors such as PD-1, CTLA-4,

TRAIL and BTLA expression on CD4+ and CD8+ T-cells in chronically HCV-infected patients suggest the induction of T-cell exhaustion, also suggested by others (Viganò *et al.*, 2012; Marraco*et al.,* 2015; Ye *et al.,* 2015). For instance, up-regulation of PD-1 and CTLA-4 have also been reported on HIV-specific CD4+ T-cells (Kaufmann & Walker, 2009) and HBV-specific CD8+ T-cells (Gu, 2014) correlating with disease progression and defining immune dysfunctions. Furthermore, PD-1, CTLA-4 and TIM-3 have also been found to be up-regulated in chronic viral hepatitis contributing to the loss of T-cell responses against the hepatopathogens (Nebbia *et al.,* 2012; Ye *et al.,* 2015). Expression of TRAIL may also induce apoptosis of activated human T-cells, and hepatocytes (Iken *et al.,* 2006). Based on one study, T-cells of HIV-infected patients are susceptible to TRAIL/APO2-L–mediated apoptosis (Jeremias *et al.*, 1998). Hence high expression of TRAIL on CD4+ of chronic HCV-infected patients in the present study, suggests the induction of apoptosis and deletion of virus-activated CD4+ T-cells also resulting in CD8+ T-cell dysfunction (Wherry, 2011). Although TIM-3 plays a key role in CD8+ T-cell dysfunction (Ferris *et al.*, 2014), in the present study, TIM-3 was not highly expressed on both HCV-activated CD4+ and CD8+ T-cells warranting further studies to unravel the inhibition mechanism of TIM-3 in chronic HCV infection. Overall, the concerted role played by co-inhibitory molecules in immune cell apoptosis during CVIs still remains a gray area of investigation.

5.1.6 Gene Expression of Co-Inhibitory Molecules

Recurrent stimulation of immune responses have been reported to lead to immune exhaustion marked by surface expression of a cumulative range of co-inhibitory molecules, and eventually triggering immune cell apoptosis (Han *et al.,* 2010; Khaitan & Unutmaz, 2011). Interestingly, growing lines of evidence suggests the involvement of transcription factors, BLIMP1, FOXP3 and DTX1 in CD8+ T-cell exhaustion (Welsh, 2009; Larsson *et al.,* 2013), and we also investigated the role of these transcriptional

repressors in CHC infection. We found that the mRNA levels of these transcriptional factors in the immune cells were differentially expressed in the study population. Of note, *FOXP3* was the only gene significantly up-regulated among CHC patients. This assumes importance as *FOXP3* plays a key role as a master regulator in the development and function of regulatory T-cells that would negatively influence T-cell differentiation, and enhances exhaustion (Jenabian *et al.,* 2012). Recent findings also have demonstrated that increase of FOXP3 was associated with the onset of cellular dysfunction in HIV-exposed T-cells (Shankar *et al.,* 2011). Meanwhile, it has also been revealed that chronic HBV-infected patients have elevated frequencies of CD4+ regulatory T-cells in the blood that prevent the proliferation of HBV-specific CD8+ T-cells (Ye *et al.,* 2015). On the other hand, high BLIMP-1 expression has also been associated with increased PD-1, CTLA-4, and CD160 expression in chronic HIV infection (Shin *et al.,*2009). The increased expression of BLIMP-1 in virus-specific exhausted CD8+ T-cells also affects the regulation of terminal differentiation in antibody-producing B cells and cytotoxic T cells (Welsh, 2009). However, our study did not find any significant expressions of *BLIMP-1* and *DTX1* among CHC patients as compared to HCs.

We found that all the inhibitory genes (*TRAIL, LAG-3, BTLA, CTLA-4, PD-1, CD160* and *TIM-3*) were up-regulated in CHC patients, and some of the genes, for instance TRAIL, PD-1, BTLA and CTLA, are known to be involved in immune exhaustion. Similarly, LAG-3 also is known for the negative role in cellular proliferation, activation, and homeostasis of T-cells. It works together with PD-1 to induce T-cell exhaustion (Workman & Vignali, 2003). High expression of LAG-3 occurs on HIV-specific CD8+ T-cells (Juno *et al.,*2015). A strong up-regulation of the LAG-3 has been reported on LCMV-specific CD8+ T-cells in chronically LCMV-infected relative to acutely-infected mice (Jin *et al.,*2010). Further, CD160 has also been reported to be strongly expressed on exhausted virus-specific CD8+ T-cells in chronic LCMV infection (Blackburn *et*

al.,2009). CD160 has also been found to be significantly increased on HIV-specific CD8+ T-cells (Peretz *et al.*,2012). Although the surface expression of TIM-3 was not significant on both the T-cell subsets, it's expression at the mRNA level was significant in whole PBMCs suggesting the association with potential immune exhaustion. Our results are in line with others in stating that co-expression of TIM-3 and PD-1 on HIV-activated T-cells is associated with T-cell exhaustion (Zhang *et al.*,2015). In chronic HBV cases, high number of CD4+ and CD8+ T-cells expressed TIM-3 as compared to healthy individuals (Nebbia *et al.*,2012). To the best of our knowledge, ours is one of the very few studies that has evaluated the expression of various co-inhibitory receptors (*TRAIL, LAG-3, BTLA, CTLA-4, PD-1, CD160* and *TIM-3*) and transcription factors (*BLIMP1, FOXP3* and *DTX1*) at the mRNA level in immune cells of patients chronically infected with HCV.

5.1.7 Expression of Pro- and Anti-Apoptotic Molecules on PBMCs

Deletion and/or destruction of virus-activated and uninfected immune cells by apoptosis is one of the known strategies of many chronically-infecting viruses to facilitate persistence (Février, *et al.*,2011). Apoptosis has also been the driving force behind the depletion of CD4+ T-cells in HIV pathogenesis (Gougeon, 2003). Similarly, it also has been well documented that several other chronically-infecting viruses induce apoptosis via the extrinsic or intrinsic pathways to disrupt T-cell homeostasis leading to T-cell depletion (Lu *et al.*,2007; Cummins & Badley, 2010). Our study has revealed that the incidence of HCV-induced apoptosis in immune cells was prinicipally via the extrinsic pathway due to significant expression of pro-apoptotic molecules belonging to the TNF-R superfamily observed in CHC patients (Elmore, 2007). For instance, increased expression of pro-apoptotic ligands, *TNF-α, FasL* and *TRAIL* in CHC patients supports this observation (Malhi *et al.*,2010), which has also been confirmed in HIV disease (Sloand *et al.*,1997; Kumar *et al.*,2013).

TNF-α, FasL, and TRAIL induce apoptosis via binding to their cognate death receptors, TNFR, Fas, DR4 (TRAIL-RI) and DR5 (TRAIL-RII), respectively (Tomar *et al.*,2014). Ligation results in the trimerization of receptors followed by the assembly of death domains and adaptor molecules, Fas-associated death domain (FADD). This assembly forms the death-inducing signaling complex (DISC) (Gupta *et al.*,2004), which activates initiator caspases 8 and 10 that triggers the activation of executioner caspases 3, 6, and 7. Caspase 3 activation leads to cleavage of various death substrates responsible for the hallmark features of apoptosis such as DNA fragmentation, mitochondrial membrane alteration, membrane blebbing and formation of apoptotic bodies (Janicke*et al.*,1998; Kitazumi & Tsukahara, 2011; McIlwain *et al.*,2013). The genes responsible for these caspases were up-regulated in the immune cells of CHC patients compared to HCs. High level of cROS expression in PBMC cultures of patients suggests the potential role of HCV in the induction of apoptosome through caspase recruitment domain (CARD) and initiates the activation of the caspase cascade such as caspase 3, 6, and 7 via initiator caspase 9 causing morphological and biochemical damage with eventual apoptosis (McDonnell *et al.*,2003). Significant expression of apoptotic regulator, *CFLAR* in CHC patients speculates its unpredictable role in cell survival and cell death pathways (Faustman & Davis, 2010). It has been shown either to induce apoptosis or to reduce TNFRSF-triggered apoptosis (Scaffidi *et al.*,1999).

The significantly increased expression of anti-apoptotic genes, *BCL2L1, BCL2L2,* and *MCL-1* in immune cells of patients explain the inhibitory activities of BAX and BAK (Kroemer *et al.,* 2007). Importantly, BCL-2, a very important anti-apoptotic molecule was found to be down-regulated in CHC patients. However, adequate cellular levels of these anti-apoptotic molecules are necessary to counteract the deleterious consequences of activation of both cell death pathways.

5.2 Objective 2: T-Cell Senescence in Chronic HCV Infection

The accumulation of senescent T-cells in the peripheral blood and expansion of highly differentiated T-cells with short telomeres appears to be a key feature of immunosenescence (Chou & Effros, 2013). Aging in elderly people is commonly associated with an increased susceptibility to infectious diseases, weakened response to vaccinations, and increased incidence of cancer and autoimmunity due to changes in the secretion of numerous pro-inflammatory cytokines, chemokines, growth factors, and proteases (Aspinall *et al.*,2007; Derhovanessian *et al.*,2008). A similar trend was observed in individuals infected with CMV (Sansoni *et al.*,2014), HIV (Deeks *et al.*,2012), EBV (Brunner *et al.*,2011) and HBV (Walker *et al.*,2013), by which virus-activated T-cells have a tendency to undergo repetitive antigenic T-cell stimulation and CIA (Simpson, 2011). Accumulation of virus-induced senescent T-cells results in defective killing abilities, impaired responses to antigens, resistance to apoptosis and development of negative regulatory functions in bystander T-cells (Chou & Effros, 2013) Persistently-infecting viruses also may induce virus-specific T-cells to proliferate repeatedly throughout life. However, there is a paucity of knowledge on the potential role of immunosenescence as one of the contributing factors of chronic HCV infection. Hence, the frequency of senescent T-cell phenotypes potentially associated with exhaustion in HCV-specific and non-specific T-cells were studied in this objective. Since senescence may also occur in healthy elderly individuals henceforward to exclude this concern, participants <53 years were recruited (Barathan *et al.*,2015).

5.2.1 Surface Expression of Co-Inhibitory Receptors on HCV-Specific T-cells

Exhaustion is described as the progressive loss of T-cell functions (Zhang *et al.*,2015). *Ex vivo* studies on immune cells have shown that exhaustion of T-cells stem from CIA and replicative antigenic stimulation by persistent viruses, and to date, the most prominent classifying feature of T-cell exhaustion is the increased surface expression of the CD28 family member, PD-1 (Khaitan & Unutmaz, 2011). PD-1 expression has been found to be up-regulated during chronic experimental LCMV infection (Jin *et al.*, 2010). Meanwhile, virus-specific CD4+ and CD8+ T-cells also appear to up-regulate PD-1 during HIV-1 infection (Porichis *et al.*, 2014). In addition to as mentioned above, HCV also has been proposed to up-regulate PD-1 expression on both virus-specific CD4+ and CD8+ T-cells and may establish the causality of exhaustion and premature senescence across the T-cell compartment (Chou & Effros, 2013). Others have shown that HCV-specific CD8+ T-cells simultaneously express high levels of CD57, a senescence marker together with PD-1 on virus-infected CD8+ T-cells. This supports the findings of the present study where PD-1 and CD57 were significantly expressed on HCV-specific CD4+ and CD8+ T-cells of CHC patients (Golden-Mason *et al.*, 2007).

5.2.2 Expression of Chronic Immune Activation Markers on HCV-Specific and PHA-Expanded CD4+ and CD8+ T-Cells

CVI infection-induced CIA progressively drives to chronic inflammation eventually leading to accelerated aging of T-cells characterized by the expansion of functionally-impaired antigen activated cells and poor T-cell proliferation (Chou & Effros, 2013). It has been also reported that HIV-specific CD8+ T-cells express similar levels of two activation markers, CD38 and HLA-DR, which correlate with viral load in infected patients suggestive of elevated immune activation as the main pathogenetic mechanism involved in HIV infection (Paiardini & Müller-Trutwin, 2013). Similarly, an increase in the proportion of virus-specific T-cells of patients co-expressing HLA-DR and CD38

confirms the onset of CIA among peripheral virus-activated T-cells during chronic HCV infection. Under normal circumstances, healthy cells express low levels of CD38, and upon mitogenic simulation will up-regulate CD38 indicative of T-cell activation. A similar observation has also been reported to occur among virus-infected individuals in whom CD8+ T-cells express high levels of CD38 likely reflecting the breadth of immune activation. Accumulating lines of evidence also suggest that high levels of CD57 and low levels of CD127 in patients with CIA highly correlated with T-cell dysfunction and senescence describing the notion of potential association between CIA and immunosenescence, especially in T-cells (Strioga *et al.*, 2011). Our data has also highlighted the higher expression of CD38 and HLA-DR on HCV-specific T-cells, that also augmented the expression of PD-1 potentially leading to defective functional competence (Hua *et al.*, 2014).

5.2.3 Expression of Replicative Senescence Markers on HCV-Specific and PHA-Expanded T-Cell Subsets

Growing evidence suggests that a subpopulation of CD8+ T-cells expressing CD57 are proliferation incompetent and replicative senescent. CD57 is expressed not only on most terminally-differentiated (also known as nonproliferation-competent CD8+ T cells), but also on CD4+ T-cells under conditions of CIA, such as CVIs (Palmer *et al.*, 2005). In addition, CD8+ T-cells that express CD57 can carry out effector functions robustly, in contrast to exhausted CD8+ T-cells (Wherry, 2011). Accumulation of CD57+CD8+ T-cells upon activation by MTB has robust cytokine-secreting and cytolytic potentials (Pawlowski *et al.*, 2012). The rate of turnover of CD57+ CD8+ T-cells has also been shown to increase in cancer (Tsukishiro *et al.*, 2003) and autoimmune diseases (Mikulkova *et al.*, 2010). CD4+ T-cells expressing CD57 have been reported in several chronic pathological conditions such as TB, malaria, rheumatoid arthritis, and HIV-1 infections (Palmer *et al.*, 2005). However, the phenotype of virus-specific T-cells

expressing CD57 has not been well characterized, and the association of this cell population with HCV disease remains poorly understood. Hence, our current findings have demonstrated that HCV-specific CD4+ and CD8+ T-cells express increased levels of CD57 along with marked loss of membrane CD27 and CD28, suggesting the onset of premature T-cell replicative senescence. Up-regulation of CD57 on HCV-specific T-cells may be a physiological response to persistent antigenic exposure during chronic HCV infection (Barathan *et al.,* 2015). Future studies may be required to further elucidate the association of premature aging of virus-specific T-cells and their functional responses in chronic HCV infection.

5.2.4 Expression of T-Cell Survival Markers on HCV-Specific and PHA-Expanded T-Cell Subsets

Interleukin-7 alpha chain receptor (IL-7Rα), CD127 is highly expressed on immature B-cells, naïve T-cells, peripheral T-cells, and memory T-cells (Fry & Mackall, 2002). CD127 has been associated with activation, homeostasis, differentiation, and cell survival (Surh, 2011). IL-7−/− or IL-7Rα −/− mice display severe T-cell deficiency and poor TCR-mediated signaling (Xiong *et al.,* 2013). Diminished expression of CD127 may contribute to skewed maturation of HIV-specific CD8+ T-cells (Champagne *et al.,* 2001). Down-regulation of IL-7Rα during HIV infection has also been observed in CD4+ T-cells (Benito *et al.,* 2008). Likewise, decreased expression of CD127 on HCV-specific CD4+ and CD8+ T-cells of HCV-infected subjects were seen in the present study. Probably, the down-regulation of CD127 could be described as a functional status of terminal differentiation and a consequence of chronic antigenic stimulation during chronic HCV infection (Shankar, 2015). In addition, the loss of CD127 from the surface of these cells may dramatically reduce their proliferation and life span, thereby further weakening virus-specific responses (Mojumdar *et al.,* 2011). Down-regulation of CD127 and a

pronounced increase of CD57 would mark a cell population as senescent with limited regenerative and survival capabilities (Lim & Kim, 2007).

5.2.5 Surface Expression of Differentiation Markers on HCV-Specific and PHA-Expanded CD4+ and CD8+ T-Cells

CD28 is a major co-stimulatory receptor responsible for optimal antigen-mediated T-cell activation, proliferation, and survival. It enhances IL-2 production and augments cell cycle progression in response to TCR stimulation (Weng *et al.,* 2009). CD27 with its ligand, CD70 is another costimulatory molecule that induce co-stimulatory signals, which prevent antigen-induced cell death and promotes effector T-cell survival (Hendriks *et al.*, 2003). However, prolonged T-cell stimulation results in the loss of CD28 and CD27 expressions (Cui & Kaech, 2010). Loss of CD28 and CD27 on T-cells are reportedly the predictors of immune incompetence in the elderly (Warrington *et al.,* 2003). On the other hand, down-regulation of CD28 is regarded as a hallmark of chronic antigenic stimulation, especially in HIV infection (Gamberg *et al.,* 2004). The augmented frequency of circulating CD27− and CD28− T-cells is one of the features of CMV infection (Lewis & Speers, 2003). During differentiation, virus-specific CD8+ T-cells have been shown to initially experience the loss of surface CD28, followed by CD27 upon further differentiation (Gamadia *et al.,* 2004). HIV-1 infected subjects also show evidence of accumulation of intermediately-differentiated CD8+ T-cell (CD27+CD28-) subsets (Papagno *et al.,* 2004). Here, we observed that an increased proportion of HCV-specific CD27-CD28-T-cells in CHC patients representing yet another evidence for senescence in chronic HCV infection. Expansion of HCV-specific CD4+ T-cells with reduced expression of CD27 and CD28 has been associated with diminished proliferative capacity. Chronic HCV infection also appears to cause a contraction in early-differentiated CD27+CD28+ HCV-specific T-cells in comparison to HCs, probably, allowing the expansion of late-differentiated T-cell subsets. In contrast, there was no

significant progression of virus-specific CD4+ and CD8+ T-cells to the intermediate stage of differentiation (CD27+CD28-). Furthermore, CD27+CD28- T-cell was not observed among HCs. It has been proposed by others that these cells may have been deleted due to a lack of CD4+ T-cell help (Focosi *et al.,* 2010). In general, our data also has revealed that HCV infection enhances the skewing of CD8+ and CD4+ T-cells towards a late-phenotype to foster replicative senescence in chronic HCV infection.

5.3 Objective 3: Functional Exhaustion of HCV-Specific T-Cells in Chronic HCV Infection

T-cell exhaustion is a state of immune dysfunction that occurs in chronic viral infections and cancer (Pauken & Wherry, 2015). Exhausted T-cells express a group of inhibitory molecules (PD-1, TIM-3, CTLA-4, LAG-3, BTLA and TRAIL) and distinctive patterns of cytokines including anti- and pro-inflammatory (IL-2, TNF-α, IL-10 and TGF-β) besides transcription factors (BLIMP-1 and FOXP-3) (Schietinger & Greenberg, 2014). T-cell exhaustion was firstly described in the murine chronic LCMV clone 13 model when it was shown that LCMV-specific CD8+ T-cells were incapable of producing cytokines and killing virus-infected cells (Wherry *et al.,* 2007). In humans, HIV-specific CD8+ T-cells display dysfunctional features that includes loss of IL-2 production, T-cell proliferation, and impaired effector (Kim & Ahmed, 2010). Meanwhile, HIV-specific CD4+ T-cells are known to expand during initial stages of infection and are lost during the chronic infection stage (Porichis & Kaufmann, 2011). Interestingly SIV-specific CD8+ T-cells were found to display skewed phenotype and lack of survivability within the first few weeks of infection (Petrovas *et al*., 2007). In addition, altered inflammation and changes in immunoregulatory cytokines could also influence T-cell dysfunction. However, the immunopathogenesis of chronic HCV infection is believed to be multifactorial and complex (Raziorrouh *et al*., 2014), HIV (Peretz *et al*., 2012) and LCMV infection (Wilson & Brooks, 2010). Moreover, a recent study has concisely

documented that T-cells exhaustion is part of the natural history of chronic HCV infection (Sumida *et al*., 2013).

5.3.1 Expression of Co-inhibitory Receptors on HCV-Specific CD4+ T-Cells

CD4+ T-cells are known to promote antibody class switching, and enhance the development of CD8+ T-cells (Marshall & Swain, 2011). However, CD4+ T-cells tend to undergo exhaustion during persistent viral infections such as HBV (Raziorrouh *et al.*, 2014), HCV (Barrett *et al.*, 2005), LCMV infections (Uyangaa *et al.*, 2015) and HIV-1 infections (Porichis & Kaufmann, 2011). Yet, there is relative lack of data on the molecular factors associated with virus-specific T-cells exhaustion, especially in chronic HCV infection. Among the key features, are altered co-inhibitory and co-stimulatory receptor expression, impaired IFN-I signaling and inability to generate specific memory T-cells that maintain virus-specific immune responses (Kahan *et al.*, 2015). Exhausted CD4+ T-cells also show a loss of a strong Th1 cell-associated transcriptional signature but not an obvious skewing toward Th2 and Th17 cell. CD4+ T-cell depletion was first described in murine models, and has documented the crucial role of CD4+ T-cell help, in which adoptive transfer of LCMV-specific CD4+ T-cells into mice with chronic LCMV infection would reestablish the exhausted virus-specific CD8+ T-cells while reducing the viral loads significantly (Walton *et al.*, 2013). Findings from the present study are partially in agreement with others, providing evidence for exhaustion of virus-specific CD4+ T-cells by co-expressing co-inhibitory receptors on HCV-specific CD4+ T-cells during chronic HCV infection. High expression of CTLA-4 on CD4+ T-cell response could impair co-stimulatory signaling during early activation of productive immunity (Alegre *et al.*, 2001). Similarly, PD-1 was also found to be transiently co-expressed on non-specific CD4+ T-cells, and was highly expressed on HCV-specific CD4+ T-cells after chronic HCV infection. Both PD-1 and CTLA-4 can limit the immune function capabilities of virus-specific helper T-cells, thus diminishing viral control (Walton *et al.*,

2013). CD4+ T-cell dysfunction during chronic HBV infection has been linked to strong PD-1 expression on HBV-specific CD4+ T-cells (Raziorrouh *et al.,* 2014). Recent studies showed that, virus-specific CD4+ T-cells expressed increased levels of PD-1 on CMV-specific CD4+ T-cells during chronic infection (Antoine *et al.,* 2012). Inhibition of PD-1 was found to increase the proliferative responses of CMV-specific CD4+ T-cells during primary infection (Antoine *et al.,* 2012). Apart from PD-1 and CTLA-4, other co-inhibitory receptors have also been found to be upregulated on virus-specific CD4+ T-cells such as TRAIL, which induces cell death on activated T-cells (Falschlehner *et al.,* 2009). Our current data revealed a high expression of TRAIL on HCV-specific CD4+T-cells derived from CHC patients. The high expression of TRAIL could likely be due to expansion of Th2 cytokine-producing cells during chronic HCV infection (Zhang *et al.,* 2003). By contrast, our findings also showed that HCV-specific CD4+ T-cells expressed no significant levels of BTLA and TIM-3. BTLA has been known to potently inhibit CD4+ T-cell and B-cell functions (Hastings *et al.,* 2009). TIM-3 is specifically co-expressed on HIV-specific T-cells undergoing exhaustion (Hastings *et al.,* 2009). On the hand, BTLA was found to be upregulated on CMV-specific T-cells during acute infection and it has been down-regulated once virus is controlled (Serriari *et al*., 2010). Meanwhile, BTLA levels were downregulated in HIV-exposed DC-expanded T-cells *in vitro* (Shankar *et al*., 2011).

5.3.2 Expression of Co-Inhibitory Receptors on CD8+ T-Cells in Chronic HCV Infection

In addition to CD4+ T-cell exhaustion, severe exhaustion in CD8+ T-cell responses tend to develop during persistent virus infections (Yi *et al.*, 2010). The high viral load during chronic infection and lack of CD4+ T-cells are associated with severe exhaustion of CD8+ T-cells (Wherry *et al*., 2007). Furthermore, exhausted CD8+ T-cells also lose their cytokine-secreting abilities (Aubert *et al*., 2011). Several studies have shown that co-expression of inhibitory receptors are associated with exhaustion of virus-specific CTLs during chronic infections. CD8+ T-cell exhaustion was first designated with chronic LCMV infection of mice in which virus-specific CD8+ T-cells persist with lack effector functions (Wherry & Ahmed, 2004). For instance, *in vitro* culture of CMV-specific CD8+ T-cells could not produce IL-2 cytokines during activation (Wherry & Ahmed, 2004). In contrast to CD4+ T cells, we found that HCV-specific CD8+ T-cells expressed significantly higher levels of TIM-3 in CHC patients, which hints the potential role of TIM-3 with T-cell inhibition. TIM-3 is known to inhibit proliferative functions of cytotoxic T-cells (Schmidt *et al.,* 2013). Others have suggested that HIV-specific CD8+ T-cells also express high levels of TIM-3 (Sakhdari *et al.*, 2012). Meanwhile, TIM-3 has also been found to be increased in chronic HBV infection (Ye *et al*., 2015) and may contribute to inhibition of T-cells, which is in line with current our results on chronic HCV infection. Data from our current study also has shown that HCV-specific CD8+ T-cells display high levels of CTLA-4. Recent report shows that HIV-specific CD8+ T-cells had higher CTLA-4 as compared to CD4+ T-cells derived from HIV-infected individuals (Porichis & Kaufmann, 2011). High expression of CTLA-4 could also disrupt immune homeostasis and could lead to CD8+ T-lymphocytes losing their cytotoxic effector functions (Yi *et al.*, 2010). We also found that BTLA expression was down-regulated on CD8+ T-cells in the peripheral blood of CHC patients. Interestingly, our data also

indicated that TRAIL was not significantly increased on HCV-specific CD8+ T-cells. This could be because TRAIL expression could facilitate suppression of cytotoxic T-cells without initiating apoptosis since TRAIL-induced apoptosis occurs also via cytotoxic T-cells (Pan *et al.,* 1997).

5.3.3 Levels of Pro- and Anti-Inflammatory Cytokines in HCV-Specific T-Cell Cultures

Differential secretion of pro- and anti-inflammatory cytokines could likely influence virus-specific responses and T-cell exhaustion during CVIs (Walton *et al.,* 2013). For instance, abnormal functional profile has been reported in chronic HBV infection (Ye *et al.,* 2015). HIV-specific CD4+ T-cells has also been shown to aberrantly secrete IL-2 and IFN-γ during chronic HIV infection (Elrefaei *et al.,* 2010). A similar finding was reported in LCMV clone 13 infection where specific CD4+ T-cells showed poor abilities to proliferate and secrete IL-2 and TNF-α (Yi *et al.,* 2010). Our current findings have revealed that HCV may also down-regulate Th1 cytokines in patients. However, we also found that HCV-specific T cells of CHC patients secreted higher levels of IFN-γ compared to HCs upon *in vitro* stimulation with HCV peptides. High levels of IFN-γ in culture supernatants of HCV-specific T-cells possibly occurs due to persistent inflammation ongoing in chronic HCV infection. One study on HBV infection has stated that IFN-γ promotes hepatitis, and sustained release of this cytokine by exhausted T-cells could drive persistent inflammation associated with weak virus-specific T-cell responses in the periphery (Dunn *et al.,* 2007; Ye *et al.,* 2015). During viral infections, anti-inflammatory cytokines may overcompensate and prevent virus-specific responses. In this study, some of the anti-inflammatory cytokines, IL-4, IL-5 and IL-13 were expressed insignificantly in HCV-specific T-cells of CHC patients. The low expression of IL-4, IL-5 and IL-13 is probably due to ongoing persistence immune activation and inflammation (Zampino *et al.,* 2013). However, IL-10 and TGF-β1 were higher in HCV-specific T-cell

cultures of patients. Similar finding has also been observed in certain chronic infections (Brooks, 2011). Similar to IL-10, TGF-β1 also has also been reported to inhibit virus-specific T-cell expansion, cytokine production and interfere with Th1/Th2 cells differentiation (Oh & Li, 2013). Here, TGF-β1 was highly up-regulated on HCV-specific T-cells suggesting its likely involvement in T-cell exhaustion during chronic HCV infection. Another study on HCV infection has revealed that blockade of TGF-β1 would lead to improved secretion of IFN-γ by HCV-specific CD8+ T-cells (Alatrakchi *et al.*, 2007), although this functional aspect was not considered in our current investigation.

5.4 Objective 4: Peripheral Loss and Exhaustion of Mucosal-Associated Invariant T (MAIT) Cells in Chronic HCV Infection

Despite growing evidence on the significance of innate immunity in mediating host responses to chronic viral infections, the characterization of innate responses to chronic HCV infection is very limited. Only recently, has the importance of innate-like T-cells with antimicrobial properties, the mucosal-associated invariant T (MAIT) cells in persistent infections, been brought to the limelight. MR1-restricted MAIT cells are evolutionarily conserved components mounting immune responses against infectious pathogens (Howson *et al.,* 2015). Human MAIT cells are activated in an MR1-dependent manner by various group of bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Lactobacillus acidophilus, Staphylococcus aureus, Staphylococcus epidermidis,* and *Mycobacterium abscessus*) (Ussher *et al.,* 2014) and yeast (*Candida albicans, C. glabrata* and *Saccharomyces cerevisiae*) (Chua *et al.*, 2012), but not viruses. However, recent studies on HIV-1 infection have reported that although MAIT cells do not directly recognize viral molecules, indirect association of these cells in viral immunopathogenesis does occur (Leeansyah *et al.,* 2015). Significant advances have been achieved in describing the phenotypic and functional properties of MAIT cells in microbial infections especially HIV, HBV and TB infections (Cosgrove *et al.*, 2013).

There appears to be crucial changes in the levels of CD161++ T-cells in HCV disease, and hence the likely role of MAIT cells in chronic HCV infection remains less clear. Hence, we investigated the differential distribution as well as role of circulating MAIT especially in regards to senescence, immune activation, and exhaustion cells in chronically infected HCV individuals.

5.4.1 Phenotypic Characterisation of MAIT Cells

MAIT cells were characterized based on the expression of a semi-invariant Vα7.2 (+) TCR and high expression of NK cell receptor CD161 or NKR-P1A on CD8+ T-cells in peripheral blood by polychromatic flow cytometry (Saeidi *et al.*, 2015). Our findings revealed a reduction in the frequency of CD8+CD161++TCRVα7.2+ MAIT cells from the circulation of patients with CHC infection as compared to HCs. A similar reduction in circulating MAIT cells has also been observed in patients infected with HIV-1 (Leeansyah *et al.*, 2013), HCV (Billerbeck *et al.,* 2010) and active MTB (Gold *et al.*, 2015). Another observation found that severe bacterial sepsis was associated with a dramatic decline in MAIT cell levels (Gold *et al.,* 2010). A similar observation was also found in *Vibrio cholerae* infection (Leung *et al.,* 2014). On the other hand, individuals with inflammatory bowel disease also had lower numbers of circulating MAIT cells relative to controls as seen in certain inflammatory diseases, such as psoriasis (Johnston & Gudjonsson, 2014), rheumatoid arthritis (Cho *et al.,* 2014), multiple sclerosis (Miyazaki *et al.,* 2011), and experimental autoimmune encephalomyelitis (Miyazaki *et al.,* 2011). From our current findings, we speculate that the loss of MAIT cells from the peripheral blood of patients was probably owing to the tendency of MAIT to migrate to the inflamed liver during chronic HCV infection. An increased migration of MAIT cells from the blood to the gut was seen, may result depletion of peripheral blood MAIT cells of HIV-infected individuals (Cosgrove *et al.,* 2013). Another possible explanation for reduction in number of MAIT cells in peripheral blood of patients is ongoing persistent

inflammation during chronic HCV leads expansion of residual MAIT cells that are highly activated and the functionally exhausted cells eventually undergoing AICD (Leeansyah *et al.*, 2013). These mechanisms have also been suggested for the loss of MAIT cells from the blood in HIV infection, and that this increasingly deteriorates over time. Interestingly, chronic HCV infection seems to enhance the turn-over of functionally-defective peripheral MAIT cells (CD8+CD161-TCRVα7.2+) partly by down-regulating CD161 expression, which therefore results in functionally inefficient innate cells. Down-regulation of CD161 is also responsible for T cell proliferation and activation (Eberhard *et al.*, 2014). A change in the phenotype of MAIT cells associated with the loss these cells has also been reported by others in chronic HIV infection (Eberhard *et al.*, 2014) although this mechanism needs further investigation.

5.4.2 Characterisation of Homing Properties of Peripheral MAIT Cells

In the current study, we hypothesised that reduced MAIT cell frequency in CHC patients could likely be due to lack of expression of liver infiltrating chemokine receptor, CCR5 on peripheral MAIT cells during persistent infection upon homing to the liver, the main site of HCV replication (Heydtmann & Adams, 2009). This is also because chemokines and their receptors reportedly play a significant role in both lymphocyte recruitment and functions during liver inflammation (Oo *et al.*, 2010; Barashi *et al.*, 2013). Peripheral MAIT cells of patients expressing reduced CCR5 are believed to traffic to the inflamed liver during HCV infection causing in their apparent disappearance from the bloodstream. Our findings are suggestive of HCV-induced intra-hepatic chemokine ligand secretion facilitating the hepatic recruitment of CCR5-mediated hepatic infiltration of MAIT cells expressing CCR5 (Guidotti & Iannacone 2013). Furthermore, another study also suggests the selective migration of CD161++CD8+ T-cells to the liver during inflammation in HCV disease (Billerbeck *et al.*, 2010). In contrast, mucosal T-cell homing receptor, CD103 expression was relative low on MAIT cells from both the study groups. CD103 is

responsible for T-cell homing to the gut sites (Parekh *et al.,* 2014), which is probably not a HCV replication site, and hence there was no necessity for recruitment of MAIT cells. A similar observation was also found in HIV infection, in which circulating MAIT cells expressed non-significant levels of CD103 compared to HCs (Wong *et al.,* 2013).

5.4.3 Signatures of Immune Exhaustion Receptors on Circulating MAIT Cells

PD-1, TIM-3 and CTLA-4 are some of the well-known co-inhibitory receptors that have been implicated in the induction and maintenance of T-cell exhaustion (Chen & Flies, 2013). Conventionally, these receptors are not expressed on naive T-cells, but inducibly expressed following T-cell activation by pathogens (Chen & Flies, 2013). Our recent findings have demonstrated that expression of PD-1, TIM-3 and CTLA-4 was enhanced on HCV-specific CD8+ T-cells of patients compared to HCs (Shankar, E.M., unpublished data). Others have stated that the co-expression of multiple different co-inhibitory receptors was associated with severe T-cell exhaustion (Walker *et al.,* 2013). However, the role of co-inhibitory receptor expression on MAIT cells is still enigmatic. Here, in particular, for the first time, we have investigated the consequence of co-inhibitory receptor expression on peripheral MAIT cells to associate the already diminished frequency of MAIT cells in chronic HCV infection. Contrary to HCs, CHC patients showed elevated PD-1, TIM-3 and CTLA-4 expressions on circulating MAIT cells. Accordingly, these results speculate that patients display an exhausted phenotype of MAIT cells, which could be functionally defective as evident from our results, although the accurate mechanism still needs further validation. Meanwhile, we and others have reported similar findings, such as expression of PD-1 was highly observed among MAIT cells of HIV-infected and HIV/TB co-infected patients indicative of deteriorated mucosal defence against bacterial pathogens (Gold *et al.,* 2015; Saeidi *et al.,* 2015) and TIM-3 was highly expressed on peripheral MAIT cells derived from chronic HIV-infected patients (Leeansyah *et al.,* 2013). The degree of persistent functional impairment and exhaustion

of peripheral MAIT cells possibly is associated with the multifaceted negative regulation resulting from the multiple co-inhibitory receptor expression (Frebel & Oxenius, 2013).

5.4.4 Signatures of Senescence and Chronic Immune Activation on Circulating CD8+CD161++TCRVα7.2+ MAIT Cells

Although T-cell activation is essential for effector functions, persistent activation of the immune system is related to HCV disease progression (Gonzalez *et al.,* 2009). Here, we sought to examine if similar findings could be observed on MAIT cells of CHC patients. We observed higher frequencies of classical activated CD38+ or HLA-DR+ MAIT cells among CHC patients than HCs. A study involving HIV-infected patients revealed that MAIT cells express high levels of CD38 and HLA-DR compared to HIV-uninfected individuals, and has attributed this to persistent exposure of MAIT cells to HIV antigens (Leeansyah *et al.,* 2015). Our data also indicates that MAIT cells expressed elevated levels of CD57 in chronic HCV-infected patients and a latest report also has proven that MAIT cells from chronic HIV-1 infected patients express high levels of CD57 (Leeansyah *et al.,* 2013). The expression of CD57 on CD8+T-cells is related to replicative senescence, shortened telomere lengths, and chronic activation due to persistent virus exposure (Dock & Effros, 2011). Hence, we speculated that the disappearance of MAIT cells from peripheral blood worsens over time probably due to high expression of CD57 on MAIT cells, although this warrants further investigation.

CHAPTER SIX

CONCLUSIONS

CHAPTER 6: CONCLUSIONS

In this study, several concepts related to potential persistence of HCV in chronic infection were proposed and examined in a cross-sectional cohort investigation that consisted of chronically-infected HCV individuals and healthy controls. The results from our first objective have revealed that chronic HCV infection showed signs and signatures of apoptosis along with oxidative stress, a potential inducer of mitochondrion-mediated apoptosis in virus-infected immune cells. Remarkably, significant expression of inhibitory molecules, transcription factors and immunoregulatory enzymes were clearly evident among CHC patients indicating the possible association between T-cell inhibition associated with immune exhaustion and apoptosis of immune cells. Findings from this objective also has demonstrated the elevated expression of pro-apoptotic factors that induce spontaneous apoptosis involved in extrinsic and intrinsic apoptotic pathways collectively suggests their utility for intracellular viral persistence, which however, further studies are necessary to increase the understanding of the exact roles of co-inhibitory receptors in T-cell apoptosis remains to be investigated.

The findings from the second objective have clearly shown that chronic HCV disease leads to loss of early-differentiated T-cells, and progressive accumulation of premature senescent cells, that were persistently activated and late-differentiated. Accumulation of virus-induced premature senescence in virus-specific T-cells potentially results in defective killing abilities, impaired responses to antigens and resistance to apoptosis. Overall, our observations indicate certain immunological changes occurring on CD4+ and CD8+ T-cells eventually driving them towards the end stage of senescence likely favoring viral persistence. Future studies are warranted to elucidate the predictive importance of these abnormalities on the clinical outcomes as well as the impact of these aberrations on CD4+ and CD8+ T-cell functions in chronic HCV disease.

Data from third objectives have shown that the *in vitro* differential expression of co-inhibitory receptors PD-1, CTLA-4, TRAIL on HCV-specific CD4+ T-cell responses and CTLA-4, TIM-3 on HCV-specific CD8+ T-cell responses respectively, can be related to exhaustion and immune dysfunction stage during chronic HCV infection. Expression of these co-inhibitory receptors also would play role in the upregulation in secretion of immunoregulatory cytokines and down-regulation of most of Th1 cytokines in virus-specific T-cells culture in CHC patients. As a consequence, newer immunotherapeutics will have to be developed to overcome HCV-specific CD4+ and CD8+ T-cell exhaustion and impairment pathways in chronic HCV infection. Thus, dual blockade of inhibitory receptors pathways may allow for a more comprehensive reversal of T-cell exhaustion, potentially leading to potent combination therapies.

Fourth objective demonstrated that MAIT (CD8+CD161++TCRVα7.2+) cell compartment was severely compromised meanwhile less-functional MAIT (CD8+CD161-TCRVα7.2+) T-cells were expanded in the peripheral blood of chronic HCV patients. Furthermore, the expressions of signatures of immune exhaustion, senescence, persistent immune activation on peripheral MAIT cell subsets of CHC patients suggests that these cells may functionally undergo exhaustion and CIA resulting in the possible loss of these cells from the systematic circulation. Future studies should be aimed at addressing this possibility to examine the role of tissue-homing abilities of MAIT cells. Newer strategies to effectively overcome MAIT cell exhaustion and impairment during chronic HCV infection may urgently be required.

Future work will be aimed at addressing some of the shortcomings of the current research and extending the ideas explored here to address whether if similar immunological mechanisms occur among patients with acute HCV infection. It will be needed to differentiate how these mechanisms works in aspect of phenotypic and functional of immune cells and the response in the course of acute and chronic infection.

It would also be interesting to recruit HCV patients with spontaneous viral clearance into this study to better define the factors that predict viral clearance. This could be possibly unravel the concepts that underlie the pathogenesis of HCV infection. Future research should also take in account the duration of infection among the chronic HCV patients because it may influence the findings in terms of T cell kinetics and activation. On the hand, possible association of increased liver stiffness with HCV genotype 3 infection may occur. Genetic variation in IL28B may influence fibrosis development in HCV genotype 3 infected individuals but further studies are required to confirm this.

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APPENDIXES

APPENDIX

Preparation of Tissue Culture Medium and Reagents:

A) Phosphate buffer saline (PBS) solution

Sodium chloride – 8 g
Potassium chloride – 0.2 g
Disodium phosphate – 1.44 g
Potassium dihydrogen phosphate – 0.24 g
Distilled water – 1000 ml

Mixed well and until dissolved later autoclaved. Stored it at 4°C in order to obtain ice-cold PBS solution

B) RPMI complete growth medium

RPMI powder with 25mM HEPES– one pack
Sodium bicarbonate – 2g
Fetal Bovine Serum (FBS) – 100ml
2mM L-glutamine – 10ml
1mM of penicillin-streptomycin – 5ml
Autoclaved ultrapure water - Top up bottle to 1000 ml

Mixed well and until dissolved then sterile filtered. Stored it at 4°C and warmed the media before use

C) Freezing medium

Sterile FBS – 80% (8ml)
Sterile dimethyl sulfoxide (DMSO), tissue culture grade – 20% (2ml)

Mixed well and stored it at -20°C.

D) Human Interleukin-2 (IL-2) working solution, 18000 Units (U)/ml

Il-2 stock solution - 10µl
Buffered solution (10% FBS in PBS) – 990µl

To prepare stock solution: lyophilized hIL-2 was reconstituted in 100mM acetic acid to 1mg/mL and stored at -20°C
To prepare hIL-2 working solution: 10µl of stock solution was diluted with 990µl of buffered solution. Apportioned into working solutions and stored at -20°C. Buffered solution was prepared by mixing 1ml of sterile FBS into 10ml of sterile PBS and the solution was stored at 4°C.

E) HCV peptide pools working solution

To prepare HCV peptide stock solution: the commercially available lyophilized peptide pool was reconstituted by dissolving 30 nmol PepTivator® Peptide Pool with 1000 µl of sterile water using sterile syringe. The water from sterile needle was slowly injected into the vial containing the lyophilized peptide pool. The solution was vortexed until the lyophilized peptide pool is completely dissolved. The concentration of the stock solution of HCV peptide pools is 30 nmol (approximately 50 µg) of each peptide per ml. The stock solution was aliquoted into several working solution to avoid repeated freeze- thaw cycle and the working solutions were stored at -20°C until further use. To prepare HCV peptide working solution: the aliquoted working solution 0.6 nmol in 20µl of total stock peptide pool was added into PBMC culture.

PBMC isolation protocol

A) About 7ml of blood was drawn from participants in lithium heparin blood tube

B) The blood was mixed well with additive by inverting the tubes several times

C) The blood was then transferred into 50ml centrifuge tube and diluted with sterile PBS

D) The blood mixture was mixed well by pipetting up and down several times

E) About 5ml of Ficoll-Paque solution was added in a separate clean 50 ml centrifuge tube. The blood mixture was then carefully overlaid onto Ficoll-Paque layer

F) The overlaid blood mixture was centrifuged for 22 minutes at speed of 2200 rpm with acceleration 9 and no brake

G) The four layers were formed after the centrifugation. The buffy coat layer was carefully removed and added into a clean 50ml centrifuge tube. the buffy coat contains of immune cells such as lymphocytes, monocytes and etc

H) The collected buffy coat was then mixed with 30ml of PBS and centrifuged at 1800rpm for 10 minutes

I) The supernatant was discarded and fresh 30ml of PBS was added followed by centrifugation at speed of 1500rpm for 10 minutes

J) The viability of cells was checked using trypan blue solution. The cells were also counted by same the assay

K) The cells were finally stored in freezing medium at -20°C for several hours before transferring to -80°C for 3 days. The cells were permanently stored in liquid nitrogen until further use

Table 3.2: Patient demographic data

Patient No	Age	Sex	ALT	AST	HCV PVL (IU/ml)	TLC (%)	HCV genotype
1	41	M	198	73	180000	36.2	G3
2	50	F	28	26	156000	32.3	G3
3	44	M	184	86	202000	31	G3
4	67	M	64	64	15	38	G3
5	60	M	42	24	15	30	G1
6	74	M	48	35	43	53	G3
7	50	M	223	94	287	50	G1
8	45	M	82	105	26700	41.9	G3
9	63	F	68	62	86740	20.6	G1
10	65	F	111	87	1310000	40.9	G3
11	40	F	27	28	15	44.6	G1
12	57	F	102	118	7214	34.8	G3
12	27	F	84	99	7058	24.9	G1
13	80	M	21	19	15	38.4	G3
14	25	F	144	109	202000	38	G1
15	67	F	48	41	15	31	G3
16	63	M	36	17	143	22.5	G1
17	38	M	69	67	377860	32.8	G3
18	66	F	74	103	15	26	G1
19	36	F	51	61	1230000	20	G3a
20	73	M	33	45	198	58	G1
21	60	F	57	114	300	19.2	G3
22	59	M	50	100	320	19.5	G1
23	62	F	32	115	356	40.2	G3
24	61	F	56	100	15	40	G3
25	58	F	44	89	1070	40	G1
26	62	M	45	87	11569	45	G1
27	60	F	35	88	11200	40.5	G3
28	54	M	50	85	1100	47.3	G3
29	76	F	50	36	151520	18.1	G1
30	60	M	39	81	1520	19.6	G1
31	70	M	54	82	2014	41.2	G3
32	90	M	45	80	15	43.6	G3
33	64	F	45	74	15	45.5	G3
34	65	F	50	75	320	50.2	G3
35	66	F	51	60	140	60.2	G1
36	42	F	54	37	1154000	11.2	G3
37	45	F	46	119	15	28.5	G1
38	40	M	39	37	320	30.6	G3
39	50	M	51	36	300	48.9	G3
40	52	M	50	63	310	45.7	G1
41	56	F	40	48	258	58.6	G1
42	45	F	47	71	15	57.1	G1
43	60	F	41	77	15	68.5	G1
44	64	F	45	20	15	41.5	G3
45	50	M	50	84	200	47.6	G3

46	29	F	50	64	898000	35.6	G3
47	30	F	58	24	450000	20.5	G3
48	34	M	59	36	403	23.6	G3
49	30	F	60	91	40	28.9	G1
50	58	M	60	99	8904	24.8	G3
51	37	F	60	78	7052	45.6	G1
52	54	F	60	87	70	40.3	G1
53	45	F	112	35	15	45	G3
54	46	F	48	118	191400	15.9	G3
55	32	F	40	19	15	14.8	G3
56	42	F	100	109	30	20.7	G3
57	41	M	35	41	30	21.2	G3
58	48	M	27	17	15	22.6	G3
59	50	F	48	67	198700	20.6	G1
60	51	F	48	113	1087	35	G1
61	52	M	100	51	15	45	G1
62	39	F	54	60	368	58.9	G1

LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications

1. **Barathan M**, Gopal K, Mohamed R, Ellegård R, Saeidi A, Vadivelu J, Ansari AW, Rothan HA, Ravishankar Ram M, Zandi K, Chang LY, Vignesh R, Che KF, Kamarulzaman A, Velu V, Larsson M, Kamarul T, Shankar EM (2015) Chronic hepatitis C virus infection triggers spontaneous differential expression of biosignatures associated with T cell exhaustion and apoptosis signaling in peripheral blood mononucleocytes. ***Apoptosis*** 20: 466-480. (**Tier 2**)

2. **Barathan M**, Mohamed R, Saeidi A, Vadivelu J, Chang LY, Gopal K, Ram MR, Ansari AW, Kamarulzaman A, Velu V, Larsson M, Shankar EM (2015) Increased frequency of late-senescent T cells lacking CD127 in chronic hepatitis C disease. ***Eur J Clin Invest*** 45: 466-474. **(Tier 1)**

3. **Barathan M**, Mohamed R, Vadivelu J, Chang LY, Saeidi A, Yong YK, Ravishankar Ram M, Gopal K, Velu V, Larsson M, Shankar EM (2015) Peripheral loss of CD8(+) CD161(++) TCRVα7·2(+) mucosal-associated invariant T cells in chronic hepatitis C virus-infected patients. ***Eur J Clin Invest*** 46: 170-180. (**Tier 1**)

4. **Barathan M**, Mohamed R, Vadivelu J, Larsson M,Velu V, Saeidi A,Chang LY, Shankar EM (2014) Hepatitis C virus infection contributes to impregnation of markers of immune inhibition: potential preludes underlying viral latency and persistence. ***BMC Infect Dis***, 14: (Supplement 3) P3.

Submitted

1. **Barathan M**, Mohammed R, Vadivelu J, Chang LY, Saeidi A, Larsson M, Shankar EM (2015) HCV-specific CD4+ and CD8+ T cells responses in chronically HCV-infected individuals. (Manuscript submitted to *Medical Microbiology & Immunology*).

Posters

1. **Barathan M**, Mohammed R, Vadivelu J, Chang LY, Saeidi A, Ellegård R, Larsson M, Shankar EM. (2014). Persistent hepatitis C virus infection accentuates spontaneous immune exhaustion and biosignatures of mitochondrial and extrinsic cell death pathways in immune cells. 2nd International HIV Science and Infectious Diseases Congress (HIV Science 2014). 30th January – 1st February 2014, Accord Metropolitan Hotel, T Nagar Chennai, India.

2. **Barathan M**, Mohammed R, Vadivelu J, Larsson M, Chang LY, Shankar EM. (2014). Chronic Hepatitis C virus infection leads to accumulation of late-senescent CD8+ T cells that simultaneously express CD38, HLA-DR, PD-1 and CD57 on HCV-specific CD4+ and 8+ T cells. 19th Biological Sciences Graduate Congress (19th BSGC). 11th -14th December 2014, National University of Singapore, Singapore.

Awards & Recognitions

1. **Travel Award,** The 19th Biological Sciences Graduate Congress, National University of Singapore, Singapore (2014)

Other Publications

1. Saeidi A, Chong YK, Yong YK, Tan HY, **Barathan M**, Rajarajeswaran J, Sabet NS, Sekaran SD, Ponnampalavanar S, Che KF, Velu V, Kamarulzaman A, Larsson M, Shankar EM (2015) Concurrent loss of co-stimulatory molecules and functional cytokine secretion attributes leads to proliferative senescence of CD8(+) T cells in HIV/TB co-infection. ***Cell Immunol*** 297: 19-32.

2. Saeidi A, Tien Tien VL, Al-Batran R, Al-Darraji HA, Tan HY, Yong YK, Ponnampalavanar S, **Barathan M**, Rukumani DV, Ansari AW, Velu V, Kamarulzaman A, Larsson M, Shankar EM (2015) Attrition of TCR Vá7.2+ CD161++ MAIT cells in HIV-tuberculosis co-infection is associated with elevated levels of PD-1 expression. ***PLoS One*** 10: e0124659.

3. Boppana NB, Devarajan A, Gopal K, **Barathan M**, Bakar SA, Shankar EM, Ebrahim AS, Farooq SM (2014) Blockade of CXCR2 signalling: a potential therapeutic target for preventing neutrophil-mediated inflammatory diseases. ***Exp Biol Med*** *(Maywood)* 239: 509-518.

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