In vitro Evaluation of Anti-Apoptotic Potential of IL-6 in Primary Culture of Peripheral Blood Mononuclear Cells Derived from Chronic HCV Patients

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2016

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A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Philosophy in **Healthcare Biotechnology**

By

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Dedicated to

My Parents …

For their unconditional love and prayers

ACKNOWLEDGEMENTS

All Praises to Allah Almighty, the Creator of opportunities and Authority of knowledge, for His help and blessings that enabled me to complete this dissertation. All regards to Holy Prophet (SAW), the everlasting source of guidance and knowledge for humanity.

First and foremost, I would like to express my sincerest gratitude to my research supervisor Dr. Sobia Manzoor for her valuable guidance, constant support and scholarly supervision throughout the project that helped me to accomplish this work. Her thought provoking ideas, kind suggestions and motivation throughout the experimental and thesis work has been a major contribution for this research work. It has been an honor to work under her supervision.

I would also like acknowledge my institution National University of Sciences and Technology (NUST) for providing me the required resources and environment for completion of this project. I am grateful to my dean Dr. Peter John and Head of Department Dr. Attiya Bhatti for their support and efforts to create a better research environment. A special thanks to Rector NUST for promoting the vision of science and technology.

I pay my sincere gratitude to my thesis committee members, Dr Aneela Javed, Dr Rumeza Hanif and Dr Usman Ali Ashfaq for their guidance and intellectual support. I am extremely thankful to Dr Aneela Javed for her kind suggestions and support throught the thesis work. I am also thankful to Col. Ejaz Ghani (AFIP), Virology department and the entire technical staff of AFIP laboratories for providing us blood samples and letting us interact with patients. I am indebted to Dr Saadia Zahid, Assistant Professor, ASAB and Miss Yasmin Badshah, Lecturer, ASAB for helping me in performing experiments.

I would like to thank all seniors Madiha Khalid, Saadia Nazeer, Uzma Malik, Noreen Ehsan and my lab fellows Sidra Tariq, Bukhtawar Fatima, Tahira Khan, Ayesha Khalid, Maliha Malik, Ameema Tariq, Saif Khan, Bisma Sarfraz and Sadaf Abbas for their helping attitude and co-operative behavior. I am also thankful to my friends Amnah Mahroo, Misha Mazhar, Sana Javed and Fatima Darakhshan for their expert advice and cheerful company that has always been a blessing in difficult times.

Finally, I would like to convey my extreme gratitude to my family, especially my parents for their prayers and support throughout my studies without which I would have never been able to do so. Their love and guidance always provide me the inspiration and motivational force; I owe them the most of my gratitude.

SIDRAH NASEEM

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LIST OF ABBREVIATIONS

µg	microgram
µM	micro Molar
EDTA	Ethylenediaminetetraacetic Acid
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
IFN-γ	Interferon Gamma
IL	Interleukin
PBMCs	Peripheral Blood Mononuclear Cells
BCL-2	B-cell lymphoma 2
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
STAT3	*Signal transducer and activator of transcription 3*
SOCS3	*Suppressor of cytokine signaling 3*
CTL	Cytotoxic T lymphocytes
DEPC	Diethyl Pyrocarbonate
Th1	Type 1 T helper cells
Th2	Type 2 T helper cells
PBS	Phosphate Buffered Saline
FACS	Fluorescence activated cell sorting
BIM	Bcl-2-like *protein* 11
MCL-1	Myeloid leukemia cell differentiation protein

ABSTRACT

Chronic HCV infection appears to trigger the impairment and exhaustion of immune cells, to potentially assist viral persistence and disease progression. In individuals where HCV infection is not resolved, the virus promotes functional exhaustion of HCV specific T cells, anergy and deletion owing to lack of positive stimulation, upregulation of inhibitory receptors and death markers, and rapid expansion of regulatory T cells (Tregs). Continuous evidence has suggested that the spontaneous apoptosis of peripheral blood mononuclear cells (PBMCs) could be one of the mechanisms responsible for diminished immune response in chronic viral infection (Barathan et al., 2015).

Interleukin 6 is a pleiotropic cytokine which plays an essential role in immune responses against infection. It acts both as a pro-inflammatory and anti-inflammatory cytokine. IL-6 is a prominent regulator of transition from non-specific innate immunity to a more specialized and highly specific adaptive immunity against infection. Furthermore, T cell homeostasis and functionality is critically monitored by IL-6 which ensures an efficient and sustained adaptive immunity. The present study investigates protective effect of Interleukin-6 in rescuing PBMCs from apoptosis and exhaustion induced by chronic HCV infection. Resistance to apoptosis by upregulating anti-apoptotic proteins can allow long lasting survival of T cells even in the absence of nutrients and growth factors. T cell death due to exhaustion or deprivation of cytokine and growth signal is strictly controlled by Bcl-2 family members such BIM and MCL-1.

In this study, we identified the pro-inflammatory cytokine IL-6 as a potential homeostatic cytokine that expands T cell space by inducing expression of anti-apoptotic genes, such as Mcl-1, Bcl-2 (3 folds and 2 folds' change in gene expression respectively) and by downregulating expression of T cell inhibitory receptor TIM-3. Similarly, the capacity of PBMCs to produce IFN-γ was also significantly increased ($p<0.001$) depicting

the promising nature of IL-6 in enhancing lymphocyte effector function. Overall the study concludes that IL-6 has an immense potential in rescuing PBMCs population, however IL-6 alone is not sufficient to sustain the adaptive immunity. It could be used as a potent candidate in combination with other regulatory factors for ex-vivo enhancement of lymphocyte and may help in moving one step towards adoptive T cell therapy in chronic HCV infection.

Chapter 1

INTRODUCTION

Hepatitis C virus (HCV) has created a huge burden worldwide with 185 million people suffering from hepatitis C infection, among which 80% are residing in low income and underdeveloped countries (Messina et al., 2015). About 15%–45% of infected persons naturally clear the virus within 6 months of infection and do not need any treatment. The remaining 55%–85% of persons will develop chronic HCV infection. Among those with chronic HCV infection, the risk of cirrhosis of the liver is 15%–30% within 20 years. These chronic infections result in great stress on the community both economically and socially. In a low-income country like Pakistan, the standard care of antiviral regimes including interferon and direct acting antiviral therapies resulted in enormous burden on government budget as well as community (Afzal, 2016).

Chronic infection especially persistent viral infection is often accompanied by a progressive loss of T cell efficiency, reduced polyfunctionality and diminished cytokine production, a state termed as ‘’T cell exhaustion’’. Viral antigen specific cytotoxic T lymphocytes (CTLs) play a leading role in overcoming infection (Lechner, Sullivan, et al., 2000). Unfortunately, during persistent infection, continuous exposure to antigen renders the T cells inefficient by impeding their effector functions and allowing viral evasion of the immune system (Wedemeyer et al., 2002). In chronic HCV infection, HCV specific CD8 are depleted by Bcl-2-like protein 11 (BIM) mediated signaling, and the remaining cells are functionally impaired.

Apoptosis is regulated by two major mechanisms: intrinsic and extrinsic pathway. The intrinsic or mitochondrial pathway is triggered via non-receptor mediated intracellular signalling inside the cell and induces activities in the mitochondria that initiate apoptosis (Larrubia, Lokhande, Garcia-Garzon, Miquel, Gonzalez-Praetorius, et al., 2013). Death

involves permeabilization of mitochondrial outer membrane and is regulated by the BCL-2 family of proteins (Kuwana & Newmeyer, 2003). Bcl-2 family of proteins include three distinct subfamilies i) pro-apoptotic members Bak and Bax ; ii) anti-apoptotic members Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A1 and iii) pro-apoptotic BH3 only protein (BIM), which supports apoptosis either by binding and oligomerization of Bax, Bak or indirectly by neutralizing anti-apoptotic members of Bcl-2 family (Lomonosova & Chinnadurai, 2008). The procedure of T cell death during chronic viral infection is carefully determined by a fine balance between group of pro- and anti-apoptotic proteins of the Bcl-2 family, such as Bim and Mcl-1 (Larrubia, Lokhande, Garcia-Garzon, Miquel, Gonzalez-Praetorius, et al., 2013).

Interleukins are cytokines that act mainly on leukocytes. For a long time, since the cloning of Interleukin-6 (IL-6) in 1986 (Hirano et al., 1986), it has been recognized as a pro-inflammatory cytokine that is found elevated in numerous malignancies and inflammatory diseases. The continuous production of IL-6 in distinct cell population has been implicated in disease progression and tissue damage (Tanaka & Kishimoto, 2014). It has been presumed that patients with elevated serum IL-6 have shorter life span and poor prognosis (Guo, Xu, Lu, Duan, & Zhang, 2012). Previous studies have suggested the involvement of IL-6 in inflammation associated carcinogenesis and innate-adaptive immunity interface (Naugler & Karin, 2008; Rath, Billmeier, Waldner, Atreya, & Neurath, 2015). Besides its detrimental role in inflammation and cancer, IL-6 also has regenerative and anti-inflammatory potential (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). Moreover IL-6 regulates acute phase protein production in the liver as well as survival and functioning of leukocytes (Kamimura, Ishihara, & Hirano, 2003).

IL-6 is a prominent regulator of transition from non-specific innate immunity to a more specialized and highly specific adaptive immunity against infection. T cell homeostasis and functionality is critically monitored by IL-6 which ensures an efficient and sustained adaptive immunity (Rath, Billmeier, Waldner, Atreya, & Neurath, 2015). Several lines of evidence support the notion that IL-6 markedly influences the proliferation and survival of T cells by IL-2 secretion followed by activation of STAT3 and Bcl-2 (Atreya et al., 2000; Teague, Marrack, Kappler, & Vella, 1997; H. Yoshida, Hashizume,

Suzuki, & Mihara, 2010). IL-6 has an immense potential in differentiating CD8 T cells to cytototoxic T lymphocyte (CTL) subsets (M. Okada et al., 1988).

As discussed previously, effective CD8 responses play a major role in clearing HCV infection and associated liver cancer. However, during chronic HCV infection, CD8 cells are deleted via BIM mediated apoptosis pathway and rest of the cells are exhausted. The induction of T lymphocyte apoptosis is strictly regulated by balance between pro and antiapoptotic proteins of Bcl-2 family, Mcl-1 and Bim (Holz, 2010). Restoration of T cell function could occur by correcting the levels of BIM and Mcl-1 expression. Recently, IL-6 has been shown to up regulate Mcl-1 messenger RNA (mRNA) expression and prolong Mcl-1 protein half-life in cholangiocarcinoma cell lines. Taken together, previous literature suggests that T cell dysfunction and apoptosis play a major role in virus immune evasion and establishment of tumor. Additionally, co-stimulation by Interleukin-6 could contribute towards revival of T cells that progress towards BIM mediated intrinsic apoptotic pathway. Therefore, present study was designed to investigate the anti-apoptotic potential of Interleukin-6 in rescuing PBMCs derived from chronic HCV patients. In vitro expansion and inhibition of apoptosis in lymphocytes will help in inducing an effective immune response against chronic HCV infection and hence moving one step towards better survival and immunotherapy of these patients.

Chapter 2

LITERATURE REVIEW

2.1 Hepatitis C Virus

2.1.1 History, Origin and Epidemiology

Hepatitis C virus (HCV) has created a huge burden worldwide with 185 million people suffering from hepatitis C infection, among which 80% are residing in low income and underdeveloped countries (Waheed, 2015). It is the major etiological factor of chronic hepatitis, cirrhosis, fibrosis and liver cancer (Echeverria, Moratorio, Cristina, & Moreno, 2015). In Pakistan more than 17 million people are currently infected with HCV leading to high morbidity and mortality rates (Umer & Iqbal, 2016).

HCV is a member of genus *Hepacivirus* and belongs to family *Flaviviridea* (Alter, 1999*).* The virus was first discovered in 1989 as a pathogen causing non A and non B hepatitis (Choo et al., 1989). HCV can only infect humans and chimpanzees. The majority of humans infected (60-80%) develop chronic infection, which ultimately leads to cirrhosis and hepatocellular carcinoma (Micallef, Kaldor, & Dore, 2006) (Khan et al., 2014). Genotype 3a is most common in Pakistan, and is present in about 80% of chronic hepatitis C (CHC) patients.

HCV mainly infects the hepatocytes (liver parenchymal cells) and cause chronic hepatitis that may lead to cirrhosis and liver cancer (González-Grande, Jiménez-Pérez, Arjona, & Torres, 2016). HCV transmission occurs primarily through contact with infected blood, in the form of injection drug use (IDU), organ transplantation and blood transfusion, occupational exposure to infected blood, and birth to infected mother. Sexual transmission of HCV occurs at lower rates (Umer & Iqbal, 2016).

2.1.2 Virus Structure and Genome

HCV has a single positive stranded RNA genome comprising of 9600 nucleotides that encodes a single polyprotein of 3000 amino acids (Reed & Rice, 2000). This polyprotein is further processed by cellular and viral enzymes to produce three structural proteins, core (C), envelope glycoproteins (E1 and E2), and seven non-structural proteins designated as (P7, NS2/3, NS3, NS4A, NS4B, NS5A and NS5B) **(Fig.2.1)**. HCV has 6 major genotypes which differ in geographical distribution and response to alpha interferon therapy (Fried et al., 2002). HCV generally exists as variable quasispecies that differ in host cell range and susceptibility to immune recognition (Shoukry, Cawthon, & Walker, 2004).

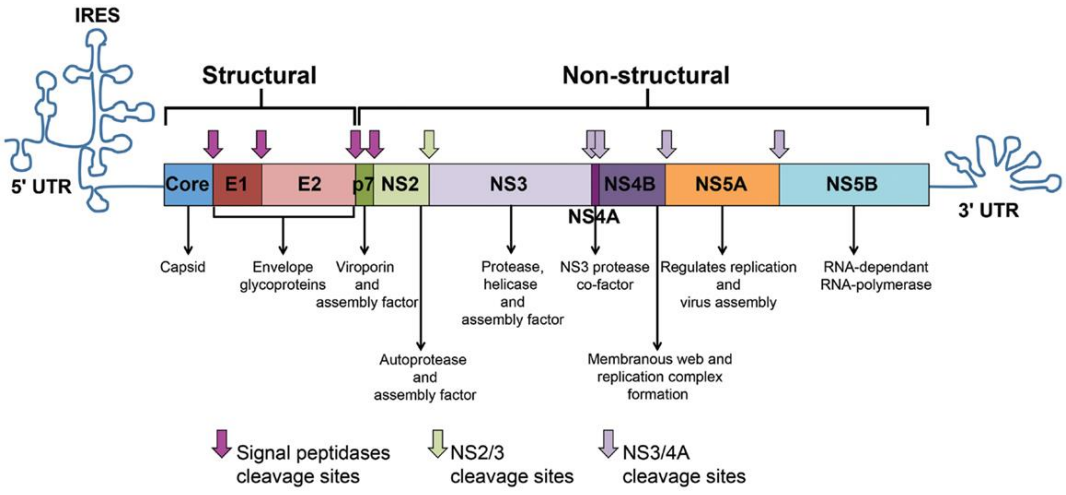


Figure 2.1- HCV genome and polyprotein. The HCV genome comprises of an open reading frame (ORF) region enclosed by 5' and 3' untranslated regions (UTRs). Translation of ORF generates a polyprotein structure, that is further processed into 10 HCV proteins (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B).

2.1.3 HCV Treatment and Treatment Response

HCV treatment is only efficacious for 1-6 months unless it turns in to chronic infection. Interferon alpha is highly effective for 24 weeks period to controlling the HCV virus (Nouroz, Shaheen, Mujtaba, & Noreen, 2015). Previously PEGylated interferon in combination to ribavirin was used as a therapy for HCV infection (Gokhale, Vazquez, & Horner, 2014). Four types of Vaccines against HCV infection are also under clinical trials

including peptide vaccines, DNA vaccine, recombinant protein vaccines and vector vaccines (Nouroz et al., 2015). Recently major advances have been done in the field of HCV therapy and many direct acting antivirals are available in market (Gokhale *et al.,* 2014).

HCV genotypes 1, 4, 5 or 6 are being treated with a combination of PEGylated interferon, Sofosbuvir and ribavirin which is productive in 90% of viral infections and produce a sustainable virological response. Patients infected with genotype 2 or 3, undergo standard HCV treatment of PEGylated interferon plus ribavirin (PEG-IFN/RBV) and patients infected with genotype 1 are treated with one of the two recently approved protease inhibitors, boceprevir and telaprevir, to PEG-IFN/RBV for 48 weeks (Gane et al., 2014). Sofosbuvir, NS5B polymerase inhibitor, is a nucleotide-analogue which is approved for all HCV genotypes 1-4. In a phase 3 clinical trial, conducted on treatment naïve category patients infected with HCV genotype 1, 89 % individuals achieved SVR after 12 weeks' treatment with sofosbuvir and peginterferon/ribavirin (Lawitz et al., 2013). The newly developed drug RNA-dependent RNA-polymerase inhibitor, Sofosbuvir, is incredibly effective but it has prohibitive cost of treatment and viral resistance may develop against this drug (Gokhale *et al.,* 2014). There are certain side-effects of the drugs used for treatment of HCV infection. Flu like symptoms may occur in the initial stages of IFN treatment. Further side effects include headaches, fever depression, arthralgia and irritability. Haemolytic anaemia is the major side-effect of ribavirin. Approximately 75% of Pakistan's population are unable to use the medical facilities. Around 80 different brands of interferon are available in Pakistan but these brands are not accessible to common man due to their high cost (Nouroz *et al.,* 2015).

2.2 Virus- Host Interactions

Hepatocytes are the major target cells for HCV. It has been suggested that approximately less than 10% of hepatocytes become infected with HCV (Lanford & Bigger, 2002). HCV enters these cells through a receptor-mediated endocytosis. Several receptors and co-receptors are identified to be involved with cellular entry of HCV, including tetraspanin CD81 (Mazzocca et al., 2005), the scavenger receptor (SR-BI) (Scarselli et al., 2002), the tight junction proteins occluding and claudin-1 (Ploss et al., 2009), the LDL receptor, and

the lectins L SIGN and DC-SIGN (Pohlmann et al., 2003). Once the virus enters the cell, it uncoats and releases its RNA into the cytoplasm. The released RNA acts as a template to generate the viral proteins. These proteins along with newly replicated viral RNA are assembled to produce new virions (Thimme, Lohmann, & Weber, 2006).

The initial response to HCV infection is induction of interferon stimulating genes in the liver which helps in controlling viral replication to some extent. Besides activation of numerous interferon stimulating genes (ISG), little antiviral effect is shown by endogenous interferon system. There is no significant reduction in viral load and HCV remains to persist for decades in hepatocytes. Various studies have revealed the involvement of HCV proteins in inhibiting JAK-STAT pathway and ISGs production, however the mechanism of interference needs to be elucidated (Heim & Thimme, 2014).

2.3 HCV: Natural Course of Infection

2.3.1 Acute Infection: Clinical and Immunological Events

HCV RNA is detectable in patients' blood within 1 to 3 weeks after exposure. Antibodies to HCV are only recognized in 50 to 70% of patients after the appearance of symptoms. Within a period of 8 to 12 weeks, elevation in the level of serum alanine aminotransferase (ALT) is detected. It is during this stage that T-cells against HCV are encountered in the blood. This delayed immune response, despite an increase of viremia, is one of the characteristics of HCV infection. Symptoms usually diminish as ALT levels reduce after several weeks' post infection (Rehermann, 2009). Some HCV infected patients spontaneously resolve the infection during the acute phase, while the majority (around 75%) continue to maintain persistent viremia that leads to chronic infection (Lauer & Walker, 2001). Antibody responses against HCV are mounted during the acute phase of infection; however, these responses are delayed and limited by the emergence of virus escape mutants. Unlike neutralizing antibodies which fail to clear HCV in this stage, HCV specific CTLs are essential for viral clearance.

Early studies signified the role of HCV specific CD4+ T cells in controlling the infection (Missale et al., 1996). Although resolution of HCV infection during the acute phase was shown to be linked with sustained HCV specific CD4+ T cell response (Lechner, Wong, et al., 2000), follow-up studies revealed that these responses are not missing in all acute infections that undergo persistence (Gerlach et al., 1999). Infected individuals who are unable to produce HCV specific CD4 cell response usually develop chronic disease. Some individuals on the other hand, who initially clear the virus from their blood, show strong CD4+ T cell responses. However, these responses contract just before a reappearance of viremia and the infection continues to become chronic (Gerlach et al., 1999). Furthermore, expansion of HCV specific CD8+ T cells in the blood is also correlated with the clearance of acute infection (Shoukry et al., 2003). In individuals who are unable to limit the viral infection in acute stage, HCV specific CD8+ T cells appear impaired with decreased proliferation and cytokine production. Studies on CD4+ T-cell depletion in chimpanzees, signified the value of CD4+ T helper cells for generation and regulation of CD8+ T-cells (Grakoui et al., 2003).

2.3.2 Chronic Infection: Clinical and Immunological Events

Persistence of HCV infection is defined as the appearance of detectable level HCV RNA in patients' blood for up to six months of infection. Various studies have confirmed the fact that 60-85% of the infected individuals develop chronic HCV infection. The clinical outcome of the disease is manifested by progressive liver fibrosis, cirrhosis, hepatocellular carcinoma and end-stage liver disease. Chronic HCV infection is accompanied with a considerable loss of virus specific CD4+ T-cells. The reasons behind the lack of CD4+ T-cell responses during the chronic phase of HCV infection is still a topic of speculation. Escape mutations take place among some MHC class II HCV epitopes, which could be one of the mechanisms behind the weak CD4+ T-cell responses (Wang & Eckels, 1999). However, accumulating evidence suggests that CD4+ T cell escape mutations are rare and may not play an important role in the silencing of CD4 responses in chronic phase of infection.

CD8+ T-cell activity against HCV is also impaired in this stage and insufficient to control the virus. The failure of CD8+ T-cells in controlling viral infection could also be related to emergence of escape mutants in MHC class I epitopes (Erickson et al., 2001), but effector anergy of these cells is also an involved mechanism. These mechanisms however, are probably secondary to the failure of help from HCV specific CD4+ T cells (Kalams & Walker, 1998). The functional exhaustion of HCV specific T lymphocytes thus lead to impaired viral clearance in chronically infected patients (Rehermann, 2009) **(Fig.2.2)**.

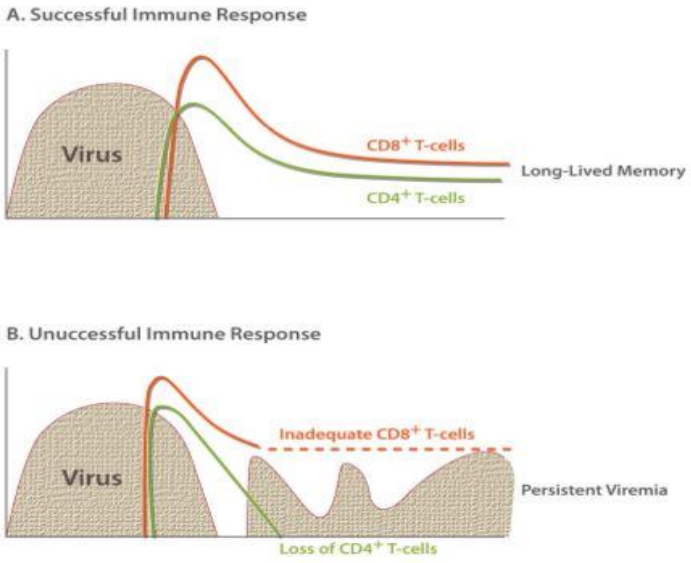


Figure 2.2- Models of Immune Responses to HCV Infection.

(A) An immune response to HCV is considered successful when robust virus-specific CD4+ and CD8+ T-cells are generated. The maintenance of these T cells beyond this point results in permanent clearance of the infection.

(B) A transient immune response becomes unsuccessful. Partial control of viral replication happens through a short-lived generation of virus-specific CD4+ and CD8+ T-cells. Sudden loss of the CD4+ responses correlates with the recurrence of viremia, which also leads to inadequacy of virus-specific CD8+ T-cells and the persistence of viremia. Adapted from: Shoukry, N.H., Annu. Rev. Microbiol. 2004

2.4 T Cell Apoptosis and Exhaustion in Chronic Viral Infection

HCV may persist in the host microenvironment besides the presence of neutralizing antibodies and T cell responses to establish chronic infection. Several mechanisms have been proposed for the failure of adaptive immune response in viral infection. These include viral evasion from neutralising antibodies and T cells with the emergence of quasi-species, direct cell to cell transfer of virus and interaction of HCV glycoproteins with high-density lipoprotein (HDL) and scavenger receptor B1 (SCARB1) to acquire protection from neutralizing antibodies. Majority of the events occurring in HCV specific T cell failure are caused by T cell exhaustion and due to production of viral escape mutants (Heim & Thimme, 2014).

During chronic infection, HCV specific CD8 cells account for 0.05-2% of circulating CD8+ T-cells (Abel et al., 2006). These CD8+ T-cells have been the most thoroughly investigated type of CD8+ T-cells in HCV infection. In general, HCV specific CD8+ T cells produce less perforin, have reduced CTL function, and reduced ability to produce IL-2, TNF-α and IFN-γ (Rehermann, 2007). Virus specific CD8 T cells with these attributes are termed as exhausted T cells. When stimulated with their cognate antigen, fewer HCV-specific CD8+ T-cells produce IFN- γ, the cells proliferate less than flu-specific cells (Wedemeyer et al., 2002), and they produce less perforin than CMV-specific cells in the same individual. Therefore, the function of HCV CD8+ T cells is decreased compared to other antigen-specific CD8+ T-cells within a given individual. Furthermore, this state of T cell exhaustion is accompanied by upregulation of inhibitory receptors PD1, TIM-3, LAG-3 etc and low expression of CD127. Accumulating evidence has suggested that upregulation of inhibitory receptors can be correlated to lymphocyte apoptosis. Consistently, intrahepatic HCV specific CD8 T cells with increased PD-1 expression are more prone to apoptosis. However, it is important to note that only a single inhibitory receptor does not regulate T cell exhaustion but involves combined expression of one or more inhibitory receptors whose affect can be reversed by blocking the receptors (Nakamoto et al., 2009)

On the other hand, HCV infection also favours pro-apoptotic pathways to induce T cell death which further weakens the immune system. HCV specific CTLs are associated with an upregulation of pro-apoptotic member Bcl-2 interacting mediator (BIM) and downregulation of induced myeloid leukemia cell differentiating protein (MCL-1) of the Bcl-2 family of apoptotic proteins. Mcl-1 sequesters and blocks the activity of Bim. The induction of apoptosis is strictly monitored by the imbalance between Bim and Mcl-1 (Larrubia et al., 2014).

2.5 Apoptosis

The term apoptosis is explained as a physiological process of programmed cell death and deletion of unwanted cells. Apoptosis is necessary for maintaining cell and tissue homeostasis, morphogenesis and host defense in various organisms. Cells that undergo apoptosis, die without damaging other neighbouring cells or their environment. Apoptosis involves various signals and a cascade of signaling events to activate caspases and other pro-apoptotic proteins. This leads to shrinkage and blebbing of cellular membrane and cytoplasm along with condensation of nucleus. Phagocyte cells then pick up the apoptotic bodies, to avoid release of cellular content spread of inflammation in the microenvironment (Rathmell & Thompson, 1999).

Apoptosis is regulated by two major mechanisms: intrinsic and extrinsic pathway. Binding of death receptors FAS, TNF to their ligands lead to activation of extrinsic pathway of apoptosis. The intrinsic (mitochondrial pathway) is triggered via non-receptor-mediated intracellular signalling inside the cell that induces such activities in the mitochondria to initiate apoptosis (Larrubia, Lokhande, Garcia-Garzon, Miquel, Subira, et al., 2013). Death involves permeabilization of mitochondrial outer membrane and is regulated by the BCL-2 family of proteins (Kuwana & Newmeyer, 2003). Overall the mechanism of apoptosis involves a family of cysteine proteases, the caspases. These molecules are internally synthesised inside the cell as inactive pro-caspases which become active upon cleavage. Active caspases are then ready to target other proteins and effector caspases (Rathmell & Thompson, 1999).

2.5.1 Intrinsic Pathway

The intrinsic or mitochondrial pathway is a non-receptor mediated intracellular signaling pathway that is triggered within the cell. It is usually initiated by DNA damage, stress or loss of other cell survival factors. The mechanism of mitochondrial pathway is regulated by a balance of pro-apoptotic and anti-apoptotic signals initiated inside the cell (B. Mayer & Oberbauer, 2003). Following activation and permeabilization of mitochondrial membrane, the pathway is split into two routes i) Apoptosis protease activating factor 1 (Apaf-1) dependant pathway and ii) Apaf-1 independent pathway. Apaf-1 dependant pathway follows activation of pro-apoptotic Bcl-2 members followed by release of cytochrome c from mitochondria and formation of apoptosomes. Apoptosomes then facilitate activation of pro-caspase 9 which then triggers the caspase cascade (P. Li et al., 1997). On the other hand, the Apaf-1 independent pathway involves the release of DIABLO like proteins from the permeabilized membrane of mitochondria which then activates effector caspases and allows initiation of caspase cascade (H. Okada et al., 2002).

Bcl-2 family of proteins include three distinct subfamilies i) pro-apoptotic members Bak and Bax ; ii) anti-apoptotic members Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A1 and iii) pro-apoptotic BH3 only protein (BIM), which supports apoptosis either by binding and oligomerization of Bax, Bak or indirectly by neutralizing anti-apoptotic members of Bcl-2 family (Lomonosova & Chinnadurai, 2008).

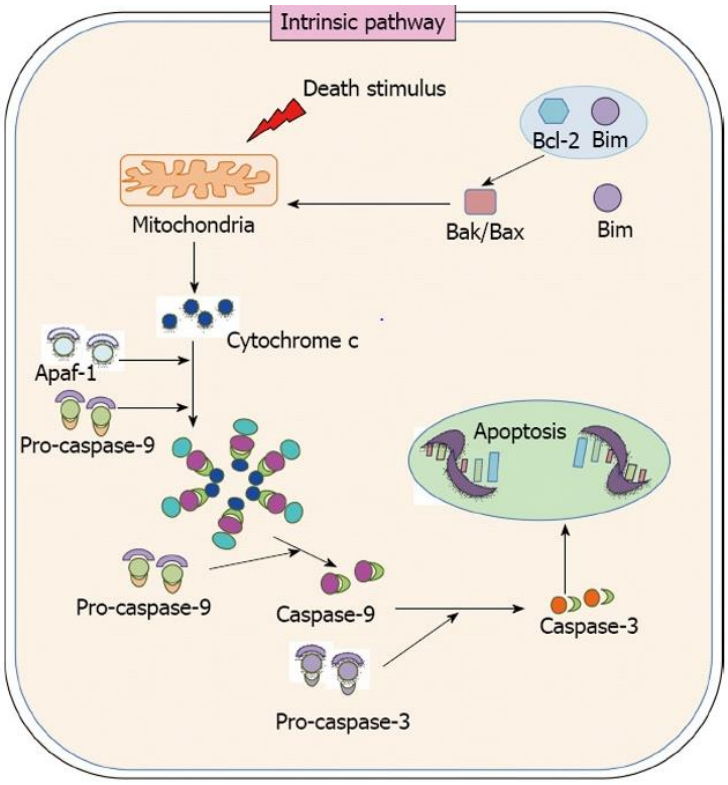


Figure 2.3. **Intrinsic pathway of mitochondrial apoptosis.** Upregulation of Bim favours activation of Bax, Bax which leads to permeabilization of membrane and the release of cytochrome c from mitochondria. Cytochrome c along with Apaf-1 and procaspase 9 form a structure known as apoptosomes, which then activates caspase 9. Caspase 9 then activates caspase 3 which triggers the signaling cascade and ultimately cell death.

2.5.2 Bcl-2 interacting mediator of cell death (BIM)

BIM is a pro-apoptotic protein that belongs to BH3 only group of Bcl-2 family and is known as the ‘’suicidal molecule’’, which enables the cell to expire gracefully. BIM initiates apoptosis by binding anti-apoptotic proteins of the Bcl-2 family and antagonize their effect. Its interaction has been observed with Bcl-2 family members, Bcl-xl, Bcl-2, Bcl-w and Mcl-1 (O’Connor, 1998). BIM has three major isoforms, BimEL (extra-long), BimL (long) and BimS (short). The short form is the most important and majorly involved in apoptosis (O'Connor et al., 1998).

During chronic viral infections, BIM levels are upregulated owing to deprivation of growth factor or stress in endoplasmic reticulum. These changes in transcriptional level are regulated by class O fork-head box transcription factor (FOX03A) and transcriptional factor CEPB-α (Dijkers, Medema, Lammers, Koenderman, & Coffer, 2000). The signaling adaptor TNFR associated factor 1 (TRAF1) correlates negatively with Bim and contributes in maintaining CD8 T cell mediated immune response against infection (Sabbagh, Pulle, Liu, Tsitsikov, & Watts, 2008). BIM plays a vital role in inducing apoptosis in lymphocytes. Bim-/- thymocytes were more resistant to apoptosis after treatment with ionomycin, taxol and γ irradiation (Bouillet et al., 1999).

As discussed previously, robust CD8 responses are necessary to counter HCV infection. However, in chronic infections CD8 T cells are deleted via Bim mediated signaling and the remaining cells are functionally impaired (Opferman et al., 2003). T cell apoptosis during chronic viral infections is strictly regulated by a balance between pro- and anti-apoptotic proteins such as Bim and Mcl-1. Furthermore, the activity of Bim could be blocked by anti-apoptotic members, Mcl-1 and Bcl-2 (Jamil, Wang, Bondy, Mojtabavi, & Duronio, 2010).

2.5.3 MCL-1

Myeloid leukaemia cell 1 (MCL1) protein was introduced by Ruth Craig and his colleagues. As the name indicates it is an immediate early gene which is activated upon myeloid cell differentiation and contributes both in cell viability as well as proliferation. Mcl-1 inhibits apoptosis by stopping Ca2+ signals inside the mitochondria. Mcl-1 transcripts exist in the form of two variants, Mcl-1 (long) and Mcl-1 (short). Mcl-1L (long) promotes cell survival by inhibiting apoptosis, whereas Mcl-1S (short) supports apoptosis (Bae, Leo, Hsu, & Hsueh, 2000). Deletion of Mcl-1 in peripheral T and B cells resulted in rapid loss of these cells. Mcl-1 sequesters and inhibit BIM, for proper development of lymphoid cells (Opferman et al., 2003).

2.6 Interleukin-6 in Chronic HCV infection

Interleukin 6 is a pleiotropic cytokine that plays an essential role in immune responses against infection. Elevated serum levels of IL-6 are usually correlated with inflammation and severity of disease (K. Li et al., 2012). High serum and intrahepatic levels of IL-6 have been observed in acute and chronic hepatitis (Malaguarnera et al., 1997; Sun, Tokushige, Isono, Yamauchi, & Obata, 1992) , HCV associated liver cirrhosis and hepatocellular carcinoma (HCC) (Malaguarnera et al., 1996).

In context of chronic liver diseases, IL-6 has a significant role in determining the outcome of hepatitis C patients. A study highlighted the importance of IL-6 in anticipating treatment response to interferon therapy (Huang et al., 2007). Besides interfering with IFN response pathway, IL-6 also activates acute phase proteins in the hepatocytes (Ramadori & Christ, 1999). It was concluded in a study that high serum levels of IL-6 can be correlated with increased risk of developing HCC (Sheng et al., 2015). Moreover, zebrafish model of hepatic tumorigenesis further validates the direct impact of IL-6 in inflammation associated hepatocarcinogenesis (Jung et al., 2015).

Focusing on the intracellular signaling, HCV core protein inhibits JAK-STAT pathway under IL-6 stimulation supporting its involvement in disease pathogenesis (Hosui et al., 2003). Contrary to this, some investigators have demonstrated low serum levels of IL-6 in HCV infected individuals as compared to healthy controls and the cytokine levels were high in IFN responders (Lee et al., 2002). STAT3 activation via IL-6 can induce an efficient IFN response and inhibit HCV activity. Furthermore STAT3 activation also impairs HCV RNA replication and promotes the synthesis of potent antiviral genes (Zhu, Shang, Terada, & Liu, 2004). Considering these observations, it is still debatable that whether IL-6 exacerbates HCV associated pathogenesis or acts as a blessing in disguise for improving prognosis of these patients?

2.6.1 IL-6 Receptor and Signalling

The origin of IL-6 dates to 1985 when it was initially termed as B cell stimulatory factor-2 (BSF-2) due to its ability to differentiate B cell population. Later on it was given various names such as hepatocyte stimulating factor (HSF) and hybridoma growth factor (HGF) owing to its ability in acute phase protein synthesis and enhancing growth of hybridoma (Tanaka, Narazaki, & Kishimoto, 2014). Although IL-6 has been linked to various hematopoietic and non-hematopoietic cells; the primary sources of its production are monocytes and macrophages (Naugler & Karin, 2008). IL-6 is also released by fibroblasts, endothelial cells and mesenchymal cells in response to distinct stimuli (Kyurkchiev et al., 2014). Ubiquitous nature of IL-6 and its receptor, allows it to bind to numerous cells in different tissues. However, the interaction may vary depending upon the type of IL-6 signalling and receptor system(Kumari, Dwarakanath, Das, & Bhatt, 2016).

IL-6/IL-6 receptor system comprises of two important molecules, IL-6R (CD126) and gp130 (CD130) (Skiniotis, Boulanger, Garcia, & Walz, 2005). IL-6 receptor is necessary for ligand binding but does not play a very significant role in signal transduction as it is expressed only on hepatocytes and certain immune cells (monocytes, neutrophils, B and T lymphocytes) (Heinrich et al., 2003) . However, gp130 is a major signalling unit for development, survival and growth induced by IL-6 related cytokine (Heinrich et al., 2003). Gp130 is promptly expressed on immune and non-immune cells, and disruption of gp130 in mice resulted in myocardial and haematological disorders (K. Yoshida et al., 1996). IL-6 binds to its receptor on plasma membrane, followed by association and homodimerization of gp130 which leads to formation of an activated IL-6 receptor complex. IL-6 then executes its desired biological effect by phosphorylating transcription factors and activating multiple signalling events (Kumari et al., 2016).

Several transcription factors including NF-kB, enhancer binding protein *a* and activator protein 1 have been shown to regulate IL-6 production (Tanaka et al., 2014). IL-6 mediates its biological activity by engaging in classical or alternative signalling mechanism. Direct/classical signalling requires the expression of both IL-6R and gp130 on cells to allow initiation of biological effect. In contrast the alternative signalling mechanism eliminates the requirement of IL-6R receptor on target cells and allows binding

of secreted IL-6 to a soluble IL-R which enhances its half-life and promotes bioavailability. IL-6/sIL-6R then forms a complex with gp130 and thus initiate trans-signaling. In this way cells that do not express IL-6 R but only gp130 are also privileged to show responsiveness to IL-6 signalling (Rose-John & Heinrich, 1994). Classical signalling usually indulges in homeostasis process and immunological activities such as acute phase protein production, haematopoiesis, regulation of endocrine system and monitoring glucose metabolism (Schobitz et al., 1995). Contrarily, IL-6 trans signalling is necessary for inflammatory activities, sustaining T cell effector function and regulating recruitment and apoptosis of leukocytes (Jones, 2005) .Taken together, it has been observed that IL-6 trans-signaling is pro-inflammatory whereas the classic IL-6 signaling is required for anti-inflammatory or regenerative activities of the cytokine **(Fig.2.4)**.

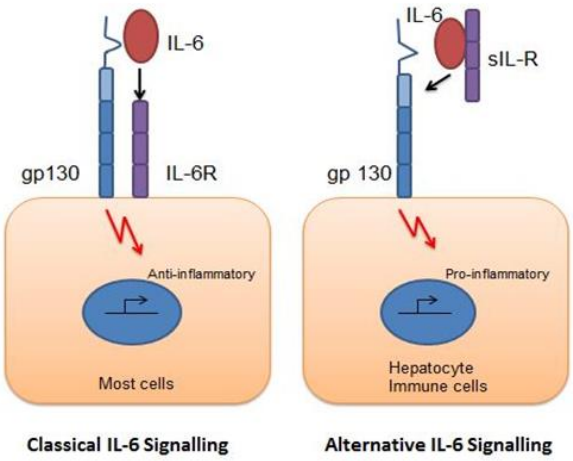


Fig.2.4- IL-6 classic- signalling and IL-6 trans-signalling. IL-6 Classical signaling requires its membrane bound receptor IL6R, hence restricted only to hepatocytes and few other cells. On the other hand, IL-6 trans-signaling can bind to soluble sIL-6 and can initiate signaling in a variety of cells that express gp130 molecule.

2.6.2 IL-6 Mediated JAK-STAT3 Pathway

The association of IL-6/IL 6R complex and signal transducer gp130 leads to activation of Janus Kinase pathway. IL-6 is a potent activator of STAT3 and exerts is biological action by binding to its receptor (IL-6R) and associated signal transducer gp130. The complex comprising of IL-6R and two gp130 molecules then relays signal to subsequently activate gp-130 associated tyrosine kinases JAK1, JAK2, TYK2 and STAT3 (Schindler & Darnell, 1995). JAK1 (Janus kinase) is recruited to the intracellular portion of gp130 which in turn activates STAT3 (signal transducer and activator of transcription) by phosphorylation (Vanden Berghe et al., 2000). STAT3 forms a dimer and is translocated to the nucleus, where it initiates transcription of multiple genes that are involved in growth, differentiation and inhibition of apoptosis (Taub, 1996). Suppressor of cytokine signaling 3 (SOCS3) is an important component of IL-6/STAT3 negative feedback loop whose expression is dependent upon STAT3 activation (Kubo, Hanada, & Yoshimura, 2003) **(Fig.2.5).**

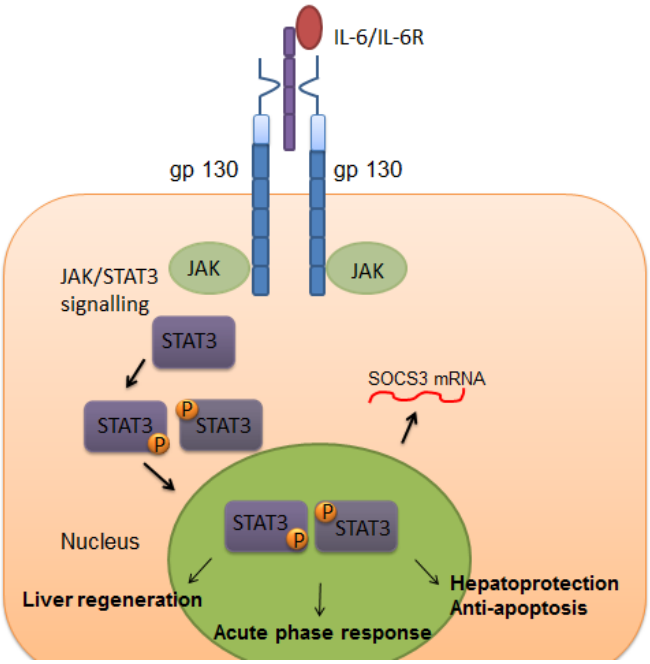


Fig.2.5- Model for IL-6/Stat3 signalling pathway. IL-6 associates with its soluble receptor sIL-6R and binds to gp130 receptor, which then results in activation of Janus kinase (JAK). This leads to phosphorylation and activation of Stat3. Stats then dimerize and are translocated to the nucleus where they activate gene transcription. In liver, this type of signaling promotes regeneration, acute-phase response and hepatoprotection.

2.6.3 Role of IL-6 in Framing Adaptive Immunity

Chronic infection especially persistent viral infection is often accompanied by a gradual loss of T cell efficiency, reduced polyfunctionality and diminished cytokine production, a state termed as ‘’T cell exhaustion’’. Exhausting T cells are able to recognize foreign antigen but they remain in a state of hypo responsiveness with diminished cytotoxic potential and increased expression of inhibitory receptors (Jiang, Li, & Zhu, 2015). Viral antigen specific CTLs play a leading role in overcoming infection (Lechner, Wong, et al., 2000). Unfortunately during persistent infection, continuous exposure to antigen renders the T cells inefficient by impeding their effector functions and allowing viral evasion of the immune system (Wedemeyer et al., 2002). Moreover chronic inflammation in liver, associated with viral hepatitis, autoimmune and non-alcoholic fatty liver disease also creates a tolerogenic environment that favours exhaustion of T cells (Kassel et al., 2009). HCV specific CD8 T cells are depleted in peripheral blood of patients who have chronic infection and express a myriad of inhibitory receptors PD-1,LAG-3,TIM-3, and 2B4 on their surface (He et al., 1999; Radziewicz et al., 2007).

IL-6 is a prominent regulator of transition from non-specific innate immunity to a more specialized and highly specific adaptive immunity against infection. T cell homeostasis and functionality is critically monitored by IL-6 which ensures an efficient and sustained adaptive immunity (Rath et al., 2015). Several lines of evidence support the notion that IL-6 markedly influences the proliferation and survival of T cells by IL-2 secretion followed by activation of STAT3 and Bcl-2 (Atreya et al., 2000; Teague et al., 1997; H. Yoshida et al., 2010). IL-6 has an immense potential in differentiating CD8 T cells to cytotoxic T lymphocyte (CTL) subsets (M. Okada et al., 1988). Surprisingly, IL-6 trans-signaling works in combination with TCR signalling to induce a rapid expression of granzyme B (GzmB) and thus contributing in development of effector T cell function (Bottcher et al., 2014). IL-6 triggers phosphorylation of STAT3 in antigen specific CD4 T cells and also support production of IL-21 by these cells (Beltra & Decaluwe, 2016). This indicates that IL-6 acts as an important factor to overcome chronic viral infection. IL-6 acts directly via its receptor IL-6Rα and gp130 to phosphorylate signal transducer which then leads to activation of STAT1/STAT3 and hence promoting cell survival(Teague et al.,

1997). In addition, the cytokine also acts as an important regulator of CD4 T cell population thereby shaping the cell mediated immunity. CD4 T cells expanded more robustly *in vivo* when immunized with exogenous IL-6 treatment (Rochman, Paul, & Ben-Sasson, 2005). Regarding the role of IL-6 in strengthening adaptive immunity, the cytokine acts as a mediator in inducing differentiation of naïve T cells to Th17 cells (Bettelli et al., 2006). Despite its overwhelming response in cell proliferation and survival, IL-6 alone is not sufficient to sustain the adaptive immunity. However, when IL-6 is used in combination with other cytokines like IL-7 and IL-15 it tremendously increases granzyme B and cytolytic capacity of effector cells (Gagnon et al., 2008). In accordance with this finding, combined action of IL-6 and TGB-B provoked differentiation of naïve CD4 T cells to Th17 instead of developing into suppressor Treg population. This implies that IL-6 acts as a decisive factor that can either convert T cell to become regulatory T cells (Treg) or stimulator of adaptive immunity (Th17) cells(Mangan et al., 2006) . IL-6 also influences Th1/Th2 balance and allows the shift in balance from Th1 to Th2 (Murphy et al., 2000). It was observed in a study that recombinant IL-6 negatively influences Th2 cell differentiation in *in vitro* experiments (A. Mayer et al., 2014) (**Fig.2.6**).

Resistance to apoptosis by upregulating anti-apoptotic proteins can allow prolonged survival of T cells in the absence of nutrients and growth factors (Michalek & Rathmell, 2010). T cell death due to exhaustion or deprivation of cytokine and growth signal is controlled by Bcl-2 family members such Bim and Mcl-1. Along with other pro-apoptotic members, BIM promotes cell death via mitochondrial/intrinsic apoptosis and its action on intrahepatic T cells is counteracted by Mcl-1(Herrant et al., 2004; Holz, Bowen, & Bertolino, 2010). Another appealing aspect of IL-6 in framing adaptive immunity is by inhibiting deletion and demise of lymphocyte subsets occurring via programmed cell death. IL-6 trans-signalling has been shown to rescue T lymphocytes by upregulating anti-apoptotic mediators and regulating Fas surface expression (Curnow et al., 2004). IL-6 promotes anti-apoptotic Bcl-2 family proteins to prolong survival and life span of T cell cultures (Teague et al., 1997). Likewise it has been demonstrated that IL-6 guards CD4 T cells from Activation induced cell death (AICD) via inhibition of Fas/FasL death signals (Ayroldi et al., 1998; Kishimoto & Sprent, 1999). Keeping in view its proliferating and

lymphocyte activation abilities, IL-6 has been considered as a co-stimulatory factor for T cells.

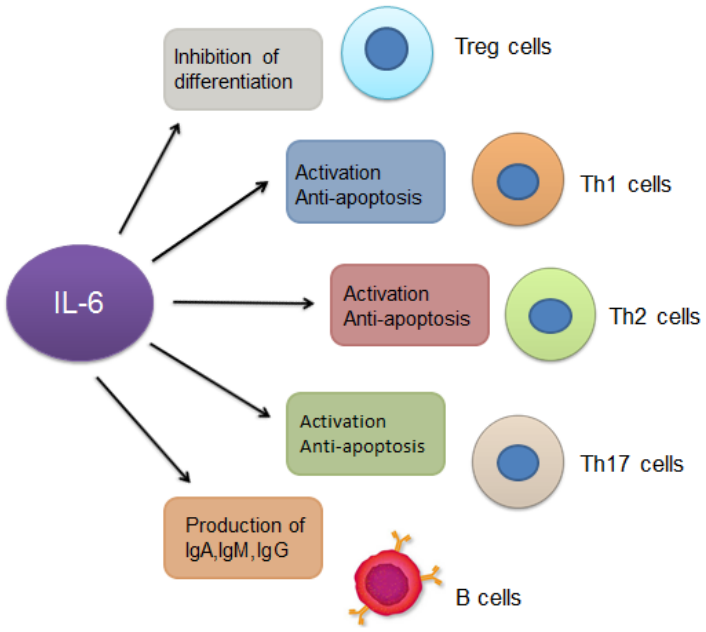


Fig.2.6. Effects of IL-6 on immune cell homeostasis. IL-6 induces the generation of Th1, Th2, Th17, effector T cells and B lymphocytes, while it inhibits the differentiation of regulatory T cells (Tregs), thereby promoting a robust immune response.

2.6.4 Role of IL-6 in upregulating anti-apoptotic protein MCL-1

Interleukin 6 is a proinflammatory cytokine expressed by antigen presenting cells (APCs) and other cells of hematopoietic and non-hematopoietic origin (Kamimura, Ishihara, & Hirano, 2003). IL-6 initially binds to IL-6 receptor, which further activates JAK and STAT3 mediated signalling pathway. (Isomoto et al., 2005). IL-6 is known to be a co-stimulatory molecule for T cell activation and proliferation independently of IL-2 expression (Dienz & Rincon, 2009). Antigen specific CD4 T cells expand rapidly in vivo when supplemented with IL-6 due to reduced apoptosis and increase in effector/memory T cell population (Rochman et al., 2005) . Previously it has also been reported that IL-6 may upregulate Mcl-1 protein half-life cholangiocarcinoma cell lines (Isomoto et al., 2005).

Taken together, previous literature suggests that T cell dysfunction and apoptosis play a major role in virus immune evasion and establishment of tumor. The pro-inflammatory cytokine IL-6 along with other cytokines is known to promote T cell survival in vitro. Notably, IL-6 increases lymphocyte number and has great potential in inhibiting apoptosis. Keeping in view previous findings, the present study was designed to investigate anti-apoptotic capacity of IL-6 in rescuing PBMCs derived from chronic HCV patients. Although the pro-inflammatory and anti-apoptotic role of IL-6 has been well established, its effect on depleting PBMCs in chronic viral infection has not been exploited in the past.

Objectives of the study:

- To investigate the potential of IL-6 in in vitro expansion of lymphocytes derived from chronic HCV patients
- To explore the anti-apoptotic capacity of IL-6 in upregulating pro-survival factors (Mcl-1, Bcl-2) of mitochondrial apoptosis
- To investigate the extent of cell death in patient PBMCs and evaluating the role of IL-6 in minimizing lymphocyte apoptosis
- To study the correlation of IL-6 treatment and expression of T cell inhibitory receptor (TIM-3)

Chapter 3

Material and Methods

3.1 Study Participants

Blood samples of 20 chronic HCV patients were collected from the Virology department of Armed Forces Institute of Pathology Rawalpindi (AFIP), Pakistan after prior approval of the study by the Commandant AFIP, Head of Virology Department and Institutional Ethics Committee. Equal numbers of male and female candidates, age ranging from 25 to 60 years were enrolled in the study. Written and informed consent was also taken from all individuals. 10 ml of peripheral blood was withdrawn by trained laboratory personnel's in EDTA coated BD vacutainers. Samples were processed within 3-4 hours of blood collection.

3.2 Patient selection criteria:

3.2.1 Inclusion criteria:

The patients who were non-responders to standard HCV treatment (Ribavirin + PEGylated interferon alpha) with detectable levels of viral RNA in blood up to 6 months of completion of therapy.

3.2.2 Exclusion criteria:

Patients who were co-infected with other viruses i.e HBV and HIV or had any co-morbidity were excluded from the study. Moreover, patients with incomplete data or those currently undergoing treatment were ruled out from the study.

3.2 Separation of PBMCs from Blood Samples

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by Ficoll density gradient centrifugation. It separates the different components of blood based on their density. Histopaque-1077 (Sigma) layered blood was centrifuged at 450xg for 45 min at 15^{o}C in 15 ml falcon tubes. The buffy coat at the interface containing lymphocytes was

collected and twice washed with 5ml PBS by centrifugation for 10min at 300xg at 4^{o}C with acceleration and deceleration reduced to minimum. Blood samples were fractioned into distinct layers after centrifugation. Erythrocytes and granulocytes were deposited at the bottom. Above this layer was a layer of Histopaque on top of which was a layer of plasma. Right at the interface of plasma and Histopaque, there is a buffy layer of PBMCs. The white layer containing lymphocytes was carefully collected and washed in 5ml PBS by centrifugation for 10 min at 350xg. PBMCs were cryopreserved in 90% FBS and 10% DMSO and stored at -80 degree Celsius until further use.

3.3 Trypan Blue Exclusion Assay and cell counting

Trypan Blue is one of the most recommended stains used in viable cell counting. It is basically used as an exclusion dye to differentiate between viable and non-viable cells. The principal of assay is that live (viable) cells will not take up dye and will remain translucent under the microscope and the dead (non-viable) cells will take up dye and shall be stained as blue and staining of cells facilitate the visualization of cell morphology.

While performing the assay, 10ul of cells and trypan blue were taken in equal ratio (1:1) and allowed to stain for few minutes. With the cover slip in place, 10ul of trypan blue-cell suspension mixture was transferred to one of the chambers of haemocytometer. Starting with chamber 1 of the haemocytometer, all the l in 1mm centre square and four 1 mm corner squares were counted. Non-viable cells stained blue. The cell count was determined after viewing the cells under microscope at 20X magnification.

Cells per ml= the average count per square x dilution factor x 10^4

Total cells = cells per ml x the original volume of sample

Cell viability (%) = total viable cells (unstained cells) + total cells (stained + unstained) /100

3.4 In vitro stimulation of PBMCs with Interleukin-6

PBMC's were collected washed and resuspended in RPMI 1640 media (Sigma) supplied with 10% heat inactivated Fetal Bovine Serum (FBS) (Sigma) and 1% penicillin and streptomycin (Pen-Strep Sigma). Sodium bicarbonate was added to adjust pH of media. Cells were then plated in 6 wells plate at a density of 4x10^6 cells/2ml/well and were stimulated with Interleukin 6 (IL-6) at concentrations of 5ng, 10ng, 20ng /ml. In parallel experiment, unstimulated PBMC's were used as controls. Cells were then allowed to culture for three hours at 37℃ with 5%CO2.

3.5 Time and Dosage Optimization of IL-6 stimulation

For time and dosage optimization of IL-6, PBMC's were simulated at three different concentration 5ng, 10ng, 20ng/ml and time points 3hr, 6hr and 24 hr. Optimum dose and time point was selected based on qualitative and quantitative expression of our target gene MCL-1.

3.6 Extraction of Cellular RNA

After stimulating the culture for required time, cells were harvested and PBMCs pellet washed with PBS. Cells were then mixed and homogenized thoroughly in 1000 uL TRIzol reagent. The micro centrifuge tubes were then kept at room temperature for 5 min. Cell lysis was further enhanced by adding 20-25 uL of freshly prepared dilution of glacial acetic acid (GAA). Sample was again left at room temperature for 3 mins. Next we added 200 uL of chloroform and the sample was mixed and vigorously shaked for 15 seconds. This step was important for phase separation. After that centrifugation was performed 12000 x g for 20 minutes at 4^{o}C. At this step sample is separated into three layers; upper aqueous layer containing RNA, white coloured interface containing DNA and red phenol-chloroform layer at the bottom comprising proteins and debris. The aqueous layer containing RNA is carefully aspirated with micropipette and transferred into new micro centrifuge tube. 500uL of chilled isopropanol is then added in this tube and left for 10 min at room temperature. RNA was precipitated after centrifugation at 12000 x g for 20 minutes at 4^{o}C. The supernatant was discarded and RNA sample washed with 1mL of 85% ethanol

(chilled). Sample was centrifuged at 12000Xg for 10 min and RNA pellet air dried in laminar flow hood for 5 min. Lastly pellet was dissolved in 20uL DEPC water and stored in -80 degree for later use.

3.7 Quantification of RNA

The quantity and quality of extracted RNA was determined by using Nanodrop. Absorbance at a wavelength of 260nm shows the concentration of RNA and ration of 260/280nm displays the quality to product being analysed. Quantification of RNA was assessed by using only 1uL of RNA sample previously suspended in DEPC water. Pure DEPC water was used for blank to ensure accuracy of procedure.

3.8 cDNA Synthesis

The complementary cDNA strand was synthesized from this extracted RNA by using oligo (dT) 18 primer (Fermentas) and RevertAid™ H minus reverse transcriptase (Fermentas) in the presence of Ribolock RNase Inhibitor (Fermentas). Oligo (dT) primer binds specifically to the polyA tail of mRNA and allows synthesis and elongation of cDNA from RNA sample. The process of cDNA synthesis involves two major steps. The first step comprises of following components in reaction mixture.

Step 1

RNA sample	1µg
Oligo (dT) primer	1µL
DEPC water	up to 12µL

The components were mixed and incubated at 70°C for 5 min and then quickly chilled at ice for next 5 minutes. Following components were added in the next step.

Step 2

Mixture of Step 1	12µL
5X Reaction Buffer	4µL
Ribolock (RNase Inhibitor)	1µL

dNTP Mix (10mM)	2µL
Revertaid H minus Reverse Transcriptase	1µL
Total Quantity	20µL

Reaction mixture was homogenized by brief centrifugation and then incubated at 42°C for 60 minutes. This temperature is optimal for efficient working of reverse transcriptase enzyme. Lastly the enzyme activity was terminated by heating the sample at 70°C for 10 minutes. cDNA synthesized was then stored at -20°C till further use.

All the equipment including micro centrifuge tubes, pipette tips and glassware used in the procedure were treated with 1% DEPC water and autoclaved before use. cDNA synthesis was confirmed by PCR amplification of Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH), a house keeping gene that is constitutively and continuously expressed in almost all cells.

3.9 Primer Designing

For the quantitative analysis of target set of genes, gene specific primers were designed using online Primer 3 software (http://primer3.ut.ee/). The sequences were obtained from NCBI gene bank. The specificity of the primer sequences was checked using BLAST (http://blast.st-va.ncbi.nlm.nih.gov/) of NCBI and Human BLAT of UCSC (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start). The primer sequences are given in Table 1.

Gene	Primer Sequence	Product Size
TIM-3 F	GAGGTTCTGTCCACTGCTATG	196 bp
TIM-3 R	CCCACGTCAAGAATTCCTAGTG	
BIM-F	TGGCAAAGCAACCTTCTGATG	70 bp
BIM-R	GCAGGCTGCAATTGTCTACCT	
MCL-1 F	TAA GGA CAA AAC GGG ACT GG	300 bp
MCL-1 R	CCT CTT GCC ACT TGC TTT TC	
INF-γ F	TGACCAGAGCATCCAAAAGA	200 bp
INF-γ R	CTCTTCGACCTCGAAACAGC	
Caspase-3 F	AGCTTCTCCAACACATCGGA	150 bp
Caspase-3 R	CTCTTTGATCTGCGCCTTGG	
BCl-2 F	TGTGGCCTTCTTTGAGTTCG	150 bp
BCL-2 R	GTACAGTTCCACAAAGGCATCC	

3.10 Optimization of the Amplification Conditions on Conventional PCR

Primers and reaction conditions were first optimized for on conventional PCR (Model No. 2720 Applied Biosystems). cDNA of 1µg - 5µg from healthy individual was used as template for optimization purpose with GAPDH primer acting as positive control. While reactions without template served as negative control.

The reaction mixture for conventional PCR was made up of following components

10X Taq Buffer with $(NH_4)_2SO_4$	2µL
$MgCl_2$ 25mM	2µL
dNTPs Set 2mM	2µL
Forward Primer (10 pmol)	1µL
Reverse Primer (10 pmol)	1µL
cDNA (depending upon concentration)	1µL

Taq Polymerase (2.5U)	0.5µL
Nuclease Free Water	10.5µL
Total reaction volume	20µL

The amplification conditions used for optimization were initial denaturation at 95°C for 5 minutes with temperature profile: denaturation at 95°C for 45 seconds, annealing at 55-60°C for 45 seconds and extension at 72°C for 45 seconds, final extension was carried out for 10 minutes at 72°C. Amplification was completed after 35 cycles.

PCR products were mixed with 6X loading dye prior to running on 2% agarose gel stained with ethidium bromide along with 50 bp DNA ladder (GeneRuler™ ready-to-use by Fermentas) and visualized by Wealtech gel documentation system. Sharp band of desired product size indicated successful amplification.

3.11 Optimization of Amplification Conditions on Real Time PCR

After optimizing the primers and reaction conditions on the conventional PCR, the amplification conditions were tested on Applied Biosystems 7300 Real-Time PCR System by using Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Catalog No: K0221, Fermentas).

The reaction mixture for real time PCR had following composition.

Maxima® SYBR green Green/ROX qPCR Master Mix (2x)	12.5µL
Forward Primer (0.3µM)	0.75µL
Reverse Primer (0.3µM)	0.75µL
Template cDNA	1µg
Water, Nuclease-free	up to 25 µL
Total volume	25 µL

The temperature profile used for real time PCR was initial denaturation at 95°C for 10 minutes, denaturation at 95°C for 15 seconds and annealing / extension at 60°C for 1 min. Amplification was completed using 40 cycles. Statistical analysis of the data was performed by using Graph Pad Prism version 6.

3.12 Flow cytometry-Annexin V

To investigate the percentage of cell death among lymphocyte population of PBMCs, Annexin V staining was performed using BD Flow cytometer (BD). Flow cytometry is done for quantitative and qualitative analyses of cells via intracellular or intercellular cell staining. After harvesting and centrifugation, approximately 5 × 105 cells were washed with FACS-PBS (PBS supplemented with 0.1% BSA, 0.03% sodiumazide, pH 7.2) and fluorescently labelled with Anti-CD3 FITC for 30 min at 4°C. Cells were than washed with FACS-PBS and fixed in 1.5 ml of formaldehyde (7% in PBS) in dark at room temperature. Next, the cell pellet was resuspended in 100 µl of Annexin Binding Buffer (1X) and stained with Annexin PE antibody (ExBio). Samples were kept in dark for 20 min followed by immediate analysis by Cellquest BD FACS software (BD Biosciences).

Chapter 4

RESULTS

4.1. Clinicopathological Data of Collected Samples

Peripheral blood samples of chronic HCV patients were collected from Armed Forces Institute of Pathology (AFIP). Only non-responder category patients (HCV RNA detectable even after completion of therapy up to 6 months) were included in the study. Patients who were co-infected with other viruses i.e HBV and HIV or had any co morbidities were not included in the study. Samples were screened for possible risk of other infections before processing them. The details of sample collected are given in following table.

Table 4.1. Chronic HCV Patients

Sr No.	Age	Gender	ALT 4-42 U/L	Alkaline Phosphatase 35-105 U/L	Viral Titer IU/mL	HCV Genotype
1	28	F	71	107	36587	3a
2	36	M	241	426	475696	3a
3	34	F	41	72	4248147	3a
4	33	F	75	73	134356	3a
5	46	M	99	457	2.5×10^5	3a
6	41	F	87	141	130045	3a
7	32	M	36	97	1560377	3a

8	58	F	93	181	502671	3a
9	48	M	66	131	5.9×10^4	3a
10	45	F	42	94	26001	3a
11	31	M	58	217	475625	3a
12	43	F	88	155	2226677	3a
13	41	M	68	106	1845206	3a
14	47	M	55	97	7.8×10^6	3a
15	46	F	73	209	2265998	3a

4.2. Cell Culture

4.2.1. Isolation of PBMCs from Peripheral Blood Samples

Peripheral Blood Mononuclear Cells were separated from whole blood by density gradient centrifugation using Histopaque R-1077. The white buffy layer containing PBMCs was collected at the interface of plasma and Histopaque layer (Figure 4.1a). Cells were then washed with PBS and pellet was cryopreserved and stored for further experiments (Figure 4.1b)

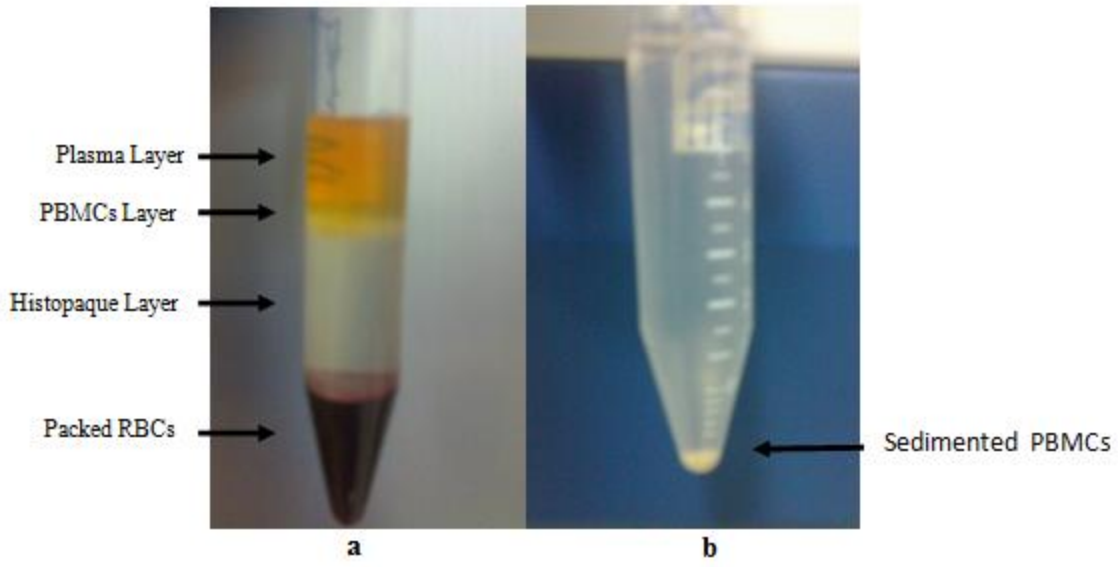


Figure 4.1

(a) PBMCs isolated from peripheral blood. Arrows show fractionation of blood into different layers upon centrifugation. White buffy layer at the interface show PBMCs population.

(b) PBMCs pellet sedimented after washing with PBS.

4.2.2 Cell Counting

The cells were counted, using a hemocytometer, based on trypan blue exclusion method. Cell suspension was prepared by adding 10µl of cells and 10µl of trypan blue [dilution factor=total volume/dilute volume (20/10=2)]. The mixture was loaded onto the hemocytometer grid and was focused by 100X objective of the microscope. The hand tally counter was used to count the cells in four 16 corner squares (eliminating the dead cells, stained by trypan blue). Average of the cell count was taken:

$$X = \frac{\text{No. of cells in each 16 square chamber (A+B+C+D)}}{4}$$

$$= \frac{111+122+100+96}{4} = 107.25$$

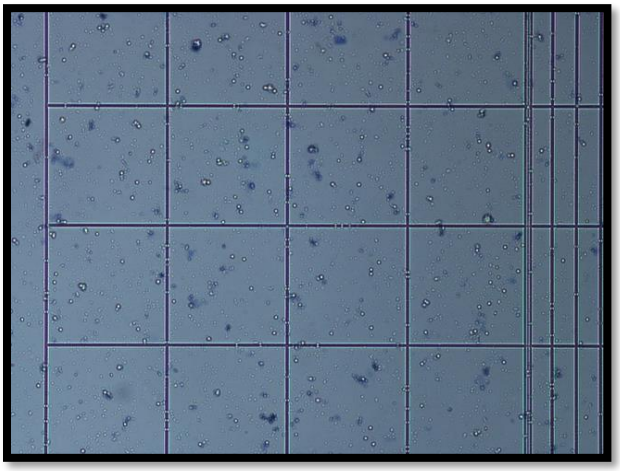


Figure 4.2. Figure showing single block of hemocytometer comprising 16 squares used for counting of PBMCs by trypan blue exclusion method. Microscope resolution was set at 20X.

4.2.3 PBMCs Culturing and Interferon Stimulation

The extracted PBMCs were cultured in RPMI 1640 media (10% FCS, 1% pen/strep). The cells 4x10^6 were plated in 2ml media per well in 6-well plate and stimulated with Interleukin-6 (5ng, 10ng, 20ng/ml). The cells were incubated for 3 hours in 5% C02 and at 37℃ in humid incubator. Antibiotics in the media prevented any bacterial contamination and the media supported the growth and cultivation of the cells. PBMCs are grown in suspension and thus are not attached to the surface. Cells are harvested after 3 hours for RNA extraction and FACS analysis.

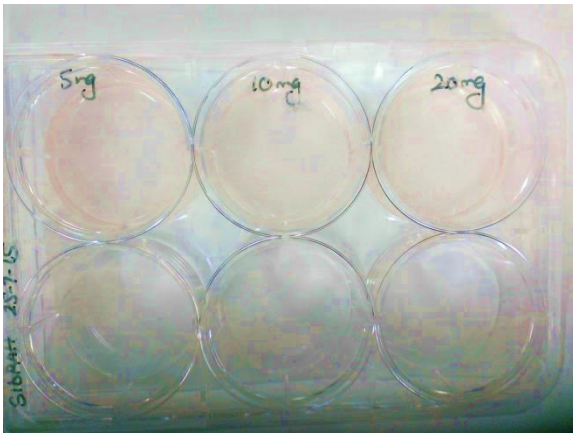


Figure 4.3. Culturing of PBMCs at different doses 5ng, 10ng, 20ng for dosage optimization at different time points in 6 well plate.

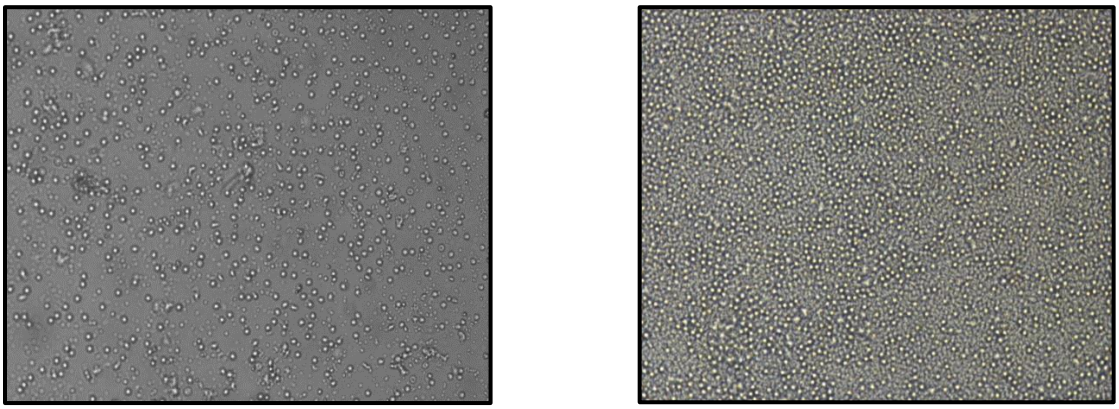


Figure 4.4. (a) unstimulated PBMCs observed after 3hrs w/o IL-6 in RPMI media.

(b) PBMCs observed after stimulation with IL-6 in and cultured in RPMI media

4.3. Analysis of Extracted cellular RNA and cDNA synthesis

RNA was extracted from PBMCs via TRIzol® method and its quantification was determined by Nanodrop. Quality of RNA was also determined by running samples on 1% agarose gel stained with ethidium bromide. Distinct three sharp bands of 28S, 18S and 5S indicated good quality of extracted RNA (Figure 4.5).

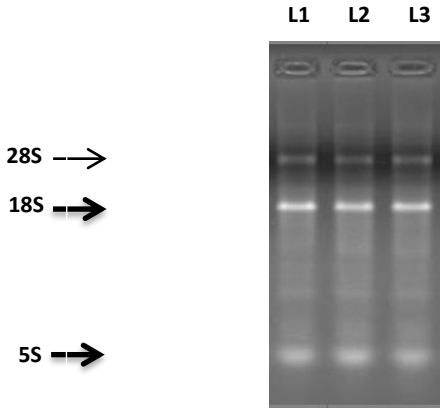


Figure 4.5. Quality of extracted RNA analysed on 1% gel. Arrows indicate distinct bands of 28S, 18S and 5S in all samples from Lane 1-3.

The extracted RNA was then reverse transcribed to cDNA. Synthesized cDNA was validated by PCR amplification of housekeeping gene (GAPDH) which is constitutively expressed in all cells.

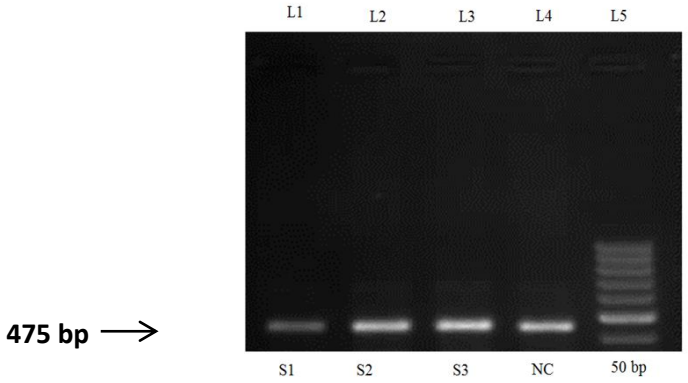
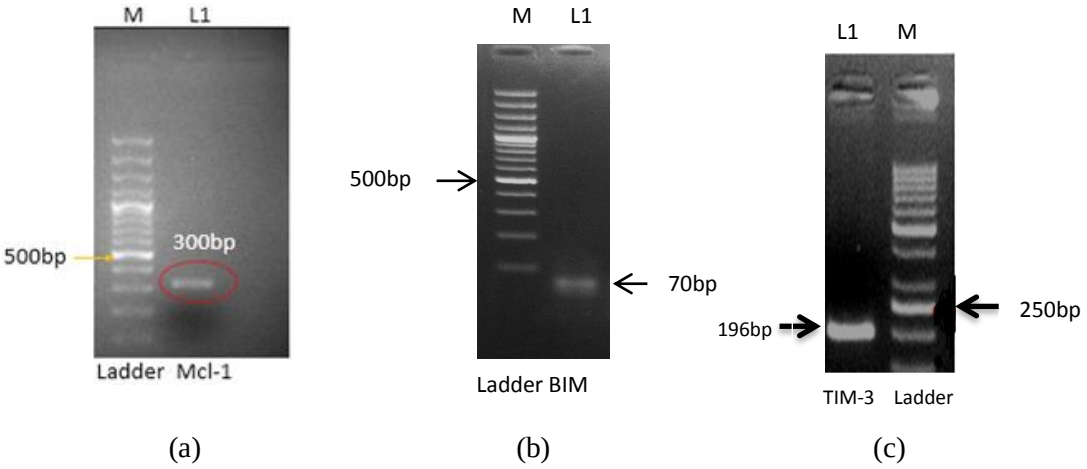


Figure 4.6. Confirmation of integrity of cDNA by PCR amplification of housekeeping gene (GAPDH) gene. Clear bands of 475bp observed in all samples from Lane 1-4 along with 50bp DNA ladder on extreme right (L5).

4.4 Optimization of Amplification Conditions on Conventional PCR

Primer specificity, their product size and melting temperatures were validated using polymerase chain reaction. The primers were optimized at different melting temperature by running gradient PCR and the best Tm was determined. The reactions were carried out in a thermocycler and the PCR products analysed on 2% agarose gel (Figure. 4.7)



(a) (b) (c)

Figure 4.7 Gel electrogram of amplified set of primers at optimum temperatures. (a) depicts the MCL-1 amplicon of 300bp, (b) amplification of 70 bp BIM gene in Lane 1 (c) amplification of TIM-3 product of 196bp by using cDNA as template.

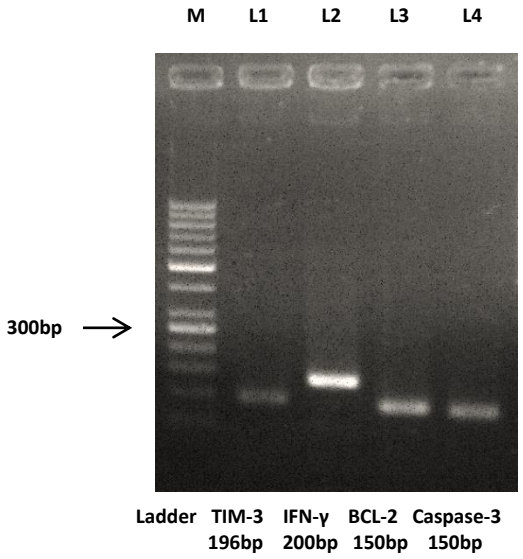
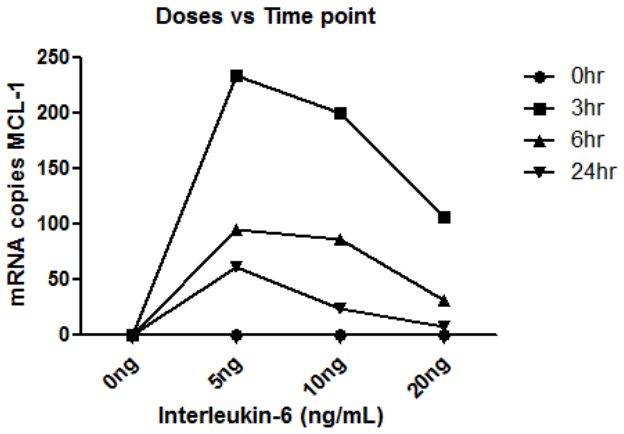


Figure 4.8. Gel electrogram of optimised set of primers using cDNA as template. Lane 1-4 depicts amplified products of TIM-3 (196bp), IFN-γ (200bp), BCL-2 (150bp) and Caspase-3 (150bp) respectively. Lane M comprise of 100bp DNA ladder.

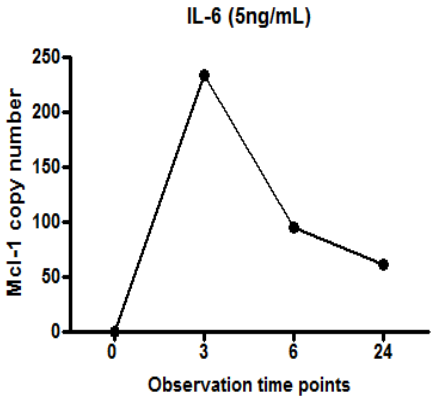
4.5. Gene Expression Studies using Quantitative Real Time PCR

4.5.1 Cytokine dose and time optimization

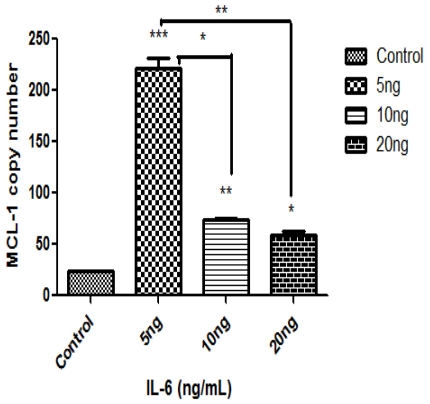
To determine the ideal dose and time point for the expression of our target gene MCL-1, we stimulated PBMCs derived from healthy individuals with three different doses (5ng, 10ng, 20ng) at three time points 3hrs, 6hrs, 24hrs. Cells were harvested at each time point and further processed for RNA extraction and cDNA synthesis. cDNA was used for quantitative analysis of MCL-1 expression at mRNA level via qRT-PCR. Experiment was performed in triplicates and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference. Results were analysed by Graphpad Prism 6 and copy number of MCL-1 were plotted against respective doses and time points (Figure 4.9). Mcl-1 expression was found to be more robust at IL-6 dose (5ng/mL) and at 3hrs with maximum number of mRNA copies generated. The expression reduced with increase in IL-6 dose and prolonged time of stimulation experiment.



(a)



(b)



(c)

Figure 4.9. Variation in copy number of MCl-1 with respect to dose and time. (a) The difference in mRNA levels of Mcl-1 was more prominent at 3hrs and 5ng dose of IL-6. The trend shows a reduction in copy number with respect to increasing dose and stimulation time. (b) Graph depicts the trend of Mcl-1 gene expression for a single dose of IL-6(5ng), striking difference in gene expression was observed after 3 hrs of stimulation. (c) significant difference was observed in case of all experimental groups and data was analysed by applying one way Anova. *P value < 0.05, **P <0.005, ***P <0.001 showed different levels of significance. All experiments were performed in triplicates.

4.5.2 Effect of Interleukin-6 on Mitochondrial Apoptosis Pathway

To investigate how IL-6 regulates other set of genes involved in mitochondrial apoptosis, quantitative expression of mRNA levels was assessed in control as well as experimental groups 3 hours' post stimulation of IL-6 (5ng). Since the optimum dose of IL-6 was already determined based on reference gene Mcl-1, rest of the experiments conducted on chronic HCV patients were performed with 5ng/mL of IL-6 stimulation for 3hrs. It was observed that expression levels of Mcl-1 and Bcl-2 were significantly increased. However, there was no significant difference in Caspase-3 and BIM expression. This shows that the overall expression of anti-apoptotic proteins is increased and no significant variation was observed in pro-apoptotic member BIM.

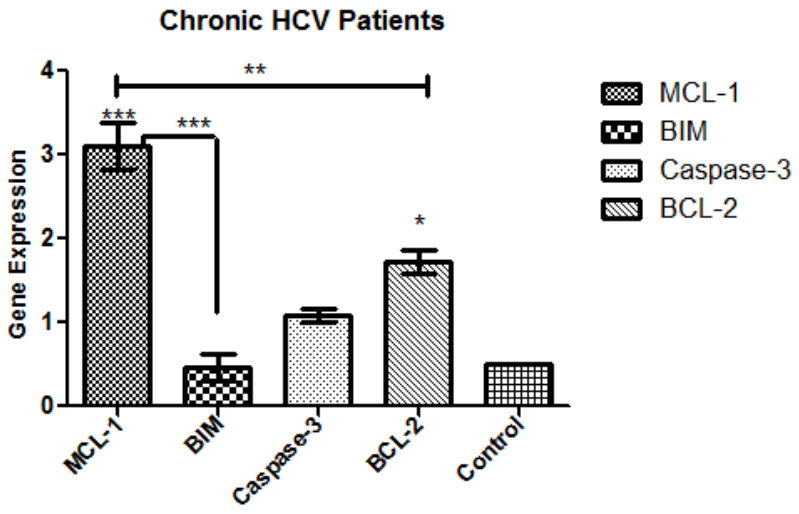


Figure.4.10 Effect of IL-6 on Bcl-2 family proteins. The transcriptional analysis showed that there was a significant increase in 3 folds and 1.5 folds in gene expression analysis of Mcl-1 and Bcl-2 respectively. However, no significant difference was observed in case of BIM and caspase-3. Data was analysed by applying one way Anova. *P value < 0.05, **P <0.005, ***P <0.001 showed different levels of significance. All experiments were performed in triplicates.

4.5.3 Effect of IL-6 on T cell inhibitory receptor

Next it was investigated whether Interleukin-6 has any effect on T cell inhibitory receptor TIM-3 whose expression is significantly increased on lymphocytes and is an important hallmark of T cell dysfunction and exhaustion. It was observed that the transcriptional expression of TIM-3 was reduced after 3hrs post culture with IL-6. However, the change was not statistically significant.

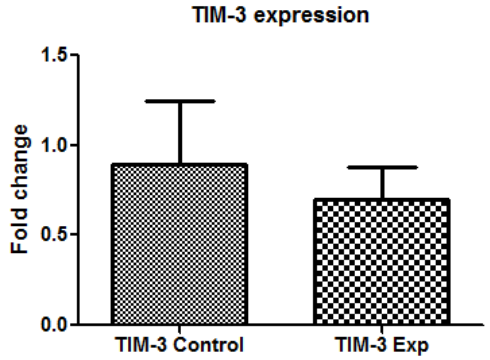


Figure.4.11 Effect of IL-6 on T cell inhibitory receptor (TIM-3). It was observed that the expression of TIM-3 was reduced in experimental groups 3 hours' post stimulation with IL-6. Data was analysed by non-parametric Mann Whitney test and $p < 0.05$ was considered significant. All experiments were performed in triplicates.

4.5.4 Effect of IL-6 on Interferon-gamma production

To investigate the capacity of PBMCs to produce IFN-γ, we determined levels of gamma interferon post stimulation. Surprisingly the mRNA levels of IFN-γ were significantly increased in PBMCs cultures treated with IL-6 as compared to those w/o IL-6.

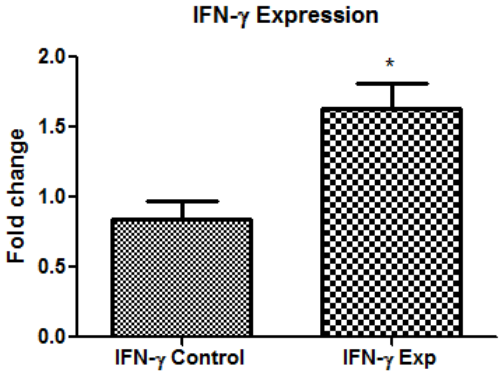
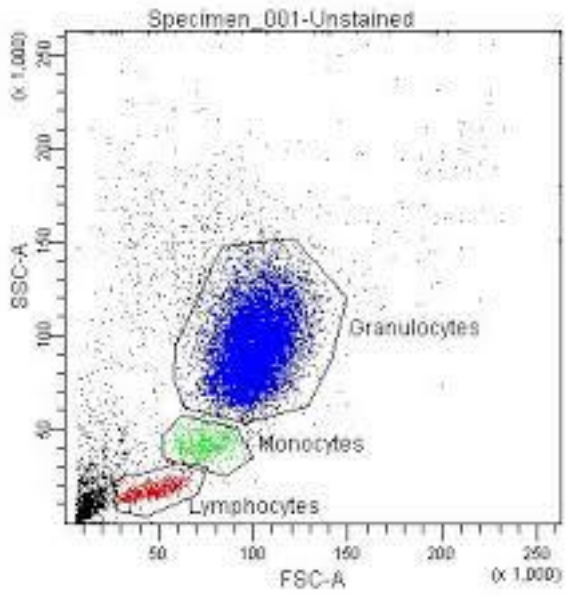


Figure.4.12 Effect of Il-6 on gamma interferon production. It was observed that the expression of IFN-γ was significantly increased in experimental groups 3 hours' post stimulation with IL-6. Data was analysed by non-parametric Mann Whitney test and $p < 0.05$ was considered significant.

Effect of Interleukin-6 on viability of PBMCs post stimulation

To evaluate the effect of Interleukin-6 treatment on viability of patient derived PBMCs, we stained PBMCs with Annexin-V in control and experimental groups after 3hrs. Analysis of cell surface stained cells was performed by Flow cytometry. Flow cytometer machine works on the principle of hydrodynamic focusing, cells are detected upon exposure to one or more lasers. Light is scattered and detected in two dimension, Forward scatter (FSC, parallel to the beam of light) and Side scatter (SSC, perpendicular beam of light). Staining for apoptosis marker Annexin-V revealed that there was a shift in viability of PBMCs, with higher percentage of apoptotic cells (Annexin+ve) in control samples as compared to stimulated cells. This indicates that IL-6 may have a potential in maintaining survival of cells. Figure 4.13 represents one of the representative experiments.



a)

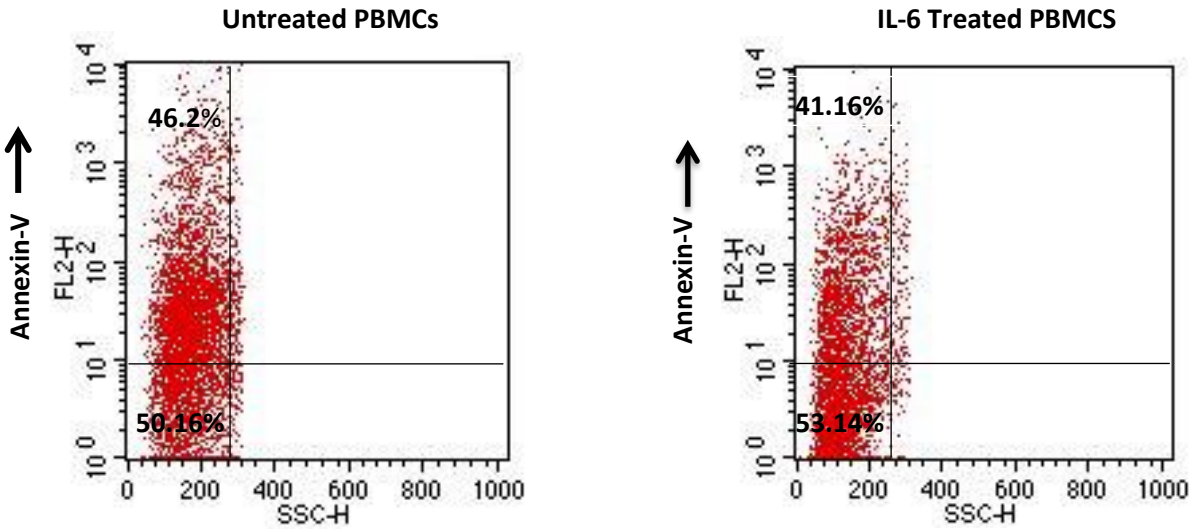


Figure.4.13 Interleukin-6 treatment increases survival of PBMCs. (a) distribution of different set of populations in PBMCS (b) Flow cytometry analysis of lymphocyte population w/o treatment with IL-6, showing greater number of Annexin +ve cells (c) Flow cytometry analysis of lymphocyte population post stimulation with IL-6, showing a reduction in Annexin +ve cells and a better cell survival.

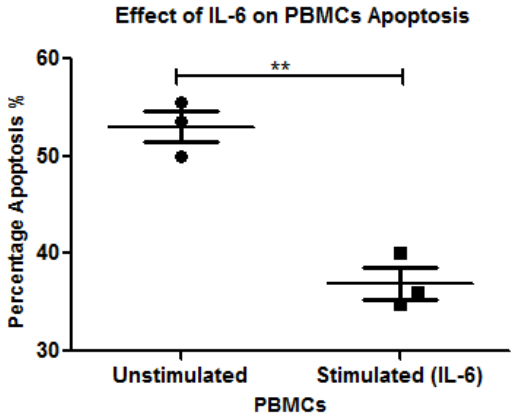


Figure 4.14 Effect of IL-6 on Percentage Apoptosis of PBMCs. Significant difference (**P<0.001) was observed in IL-6 stimulated peripheral mononuclear cells vs unstimulated cells showing a reduction in percentage of cell death.

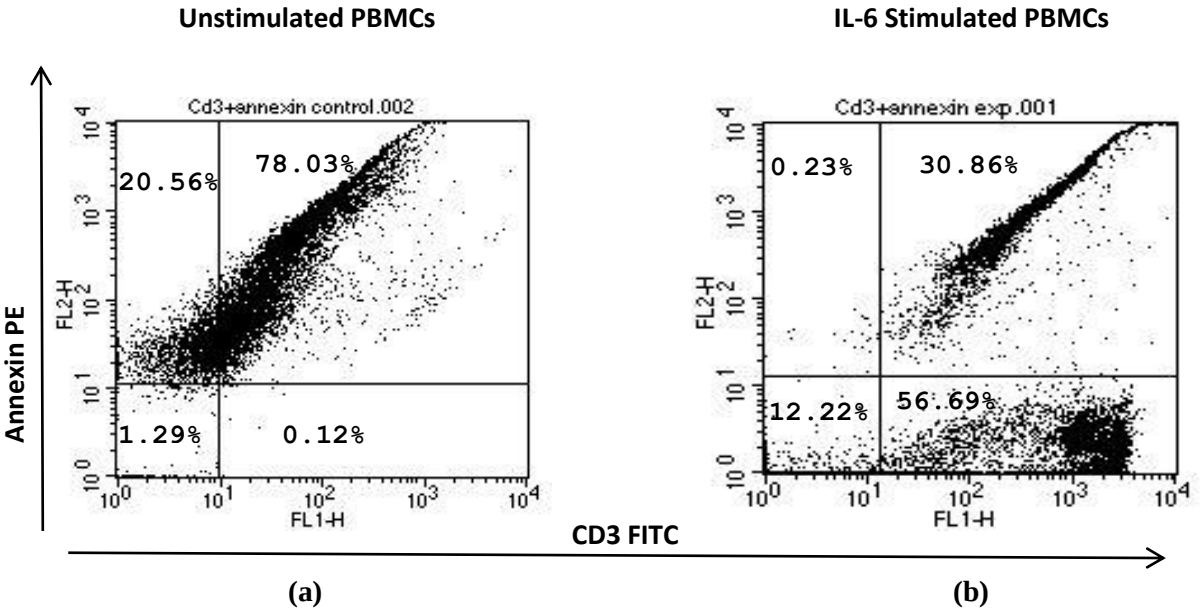


Figure. 4.15 Effect of IL-6 on survival of lymphocyte population (a) Flow cytometry analysis of lymphocyte population (CD3+ve cells) w/o treatment with IL-6, showing greater number of Annexin +ve cells (c) Flow cytometry analysis of lymphocyte population (CD3+ve cells) post stimulation with IL-6, showing a reduction in Annexin +ve cells and a better cell survival.

Chapter 5

DISCUSSION

Hepatitis C virus (HCV) has become a global health concern with an estimated 185 million people suffering from hepatitis C infection worldwide. The disease is an emerging burden on social, economic and healthcare sectors. Although the incidence of HCV has greatly reduced in United States, Australia, Japan, Western and Northern Europe, the disease is still a menace for underdeveloped and low income countries (Hajarizadeh, Grebely, & Dore, 2013). Hepatitis C virus (HCV) is highly endemic in Pakistan, with around 6.8% of the population currently infected with HCV (Umer & Iqbal, 2016).

Chronic HCV infection appears to instigate impairment and exhaustion of immune cells, to potentially assist viral persistence and disease progression. In individuals where HCV infection is not resolved, the virus promotes functional exhaustion of HCV specific T cells, anergy and deletion due to lack of positive stimulation, upregulation of inhibitory receptors and death markers, and rapid expansion of regulatory T cells (Tregs). Continuous evidence has suggested that the spontaneous apoptosis of peripheral blood mononuclear cells (PBMCs) could be one of the mechanisms responsible for diminished immune response in chronic viral infection (Barathan et al., 2015).

Interleukin 6 (IL-6) is a pleiotropic cytokine that plays an essential role in immune responses against infection. It acts both as a pro-inflammatory and anti-inflammatory cytokine. IL-6 is a prominent regulator of transition from non-specific innate immunity to a more specialized and highly specific adaptive immunity against infection. T cell homeostasis and functionality is critically monitored by IL-6 which ensures an efficient and sustained adaptive immunity (Rath et al., 2015). The present study investigated protective effect of Interleukin-6 on rescuing PBMCs from apoptosis and exhaustion induced by chronic HCV infection.

Present study explores the pro-inflammatory cytokine IL-6 as a potential homeostatic cytokine that expands T cell space by inducing expression of anti-apoptotic genes, such as Mcl-1, Bcl-2 and by downregulating expression of T cell inhibitory receptor TIM-3. Similarly, other genes such as BIM, Caspase-3 and IFN-γ were investigated to decipher the effect of IL-6 on their respective mRNA expressions. Since IL-6 expression is highly upregulated during immune activation and inflammation, IL-6 mediated T cell survival could represent a mechanism to maintain the T cell pool when signaling by other homeostatic cytokines becomes limiting. Interestingly in present study, IL-6 increased T cell number post stimulation with IL-6 independent of T cell differentiation status. Previously, a set of cytokines have been identified to promote T cell survival, which includes the γc cytokines IL-2, IL-4, IL-7, IL-15, and the gp130 family cytokine, IL-6. These cytokines not only prevented apoptosis but also provided trophic effects to maintain cell size and induce cell metabolism. Notably, all these pro-survival cytokines share the common signaling pathways of JAK-STAT and PI3-Kinase/Akt activation. This observation suggests that pro-survival cytokines could be potentially interchangeable and redundant in their effects to provide T cell survival and homeostasis.

First aim of the study was to investigate anti-apoptotic potential of Interleukin-6 in regulating genes involved in mitochondrial apoptosis. It has been previously established that IL-6 has anti-apoptotic potential and plays a major role in developing carcinogenesis by disturbing the normal balance of Bcl-2 family proteins in inflammatory conditions. However, the important role of IL-6 in regulating apoptosis could be beneficial in rescuing PBMCs and lymphocyte subsets from spontaneous cell death and exhaustion induced due to viral persistence. Therefore, present study was designed to explore the opportunities of using IL-6 for ex-vivo modification and enhancement of PBMCs derived from chronic HCV patients. Interestingly the expression of pro-apoptotic members, Mcl-1 and Bcl-2, were greatly increased after stimulating PBMC cultures with IL-6 (5ng/mL). Moreover, no significant change was observed in case of BIM, an important member of Bcl-2 family, which sequesters Mcl-1 and initiates mitochondrial apoptosis pathway. Resistance to apoptosis by upregulating anti-apoptotic proteins can allow prolonged survival of T cells in the absence of nutrients and growth factors (Michalek & Rathmell, 2010). T cell death due to exhaustion or deprivation of cytokine and growth signal is controlled by Bcl-2

family members such Bim and Mcl-1. Along with other pro-apoptotic members, BIM promotes cell death via mitochondrial/intrinsic apoptosis and its action on intrahepatic T cells is counteracted by Mcl-1(Herrant et al., 2004; Holz et al., 2010).

Another interesting aspect of current study was to investigate effect of IL-6 on inhibitory receptor TIM-3, one of the important hallmarks of T cell dysfunction and exhaustion. The expression of TIM-3 was reduced in experimental groups as compared to controls w/o IL-6 showing a promising nature of IL-6 in regulating T cell inhibitory receptors. Although the variation in expression was not significant, there is still hope that IL-6 may influence expression of TIM-3 along with other inhibitory receptors PD-1, CTLA-4, BTLA-4 and LAG-3. Exposure to chronic infection alone is sufficient to impair cytotoxic activity and differentiation of CD8+ T cells. Inflammatory pathways induced by IL-6 and IL-27 may assist in controlling viral infection and reducing the risk of CD8+ T cell exhaustion. Although a clear evidence for the regulatory effect of these cytokines on CD8+ T cells is not present, these cytokines may have a positive role in rescuing T lymphocytes. Consistently, they also have a well-known potential in modulating CD4+ T cell differentiation during chronic viral infections which further strengthens our hypothesis (Wherry & Kurachi, 2015).

IL-6 is a prominent regulator of transition from non-specific innate immunity to a more specialized and highly specific adaptive immunity against infection. T cell homeostasis and functionality is critically monitored by IL-6 which ensures an efficient and sustained adaptive immunity (Rath et al., 2015). Several lines of evidence support the notion that IL-6 markedly influences the proliferation and survival of T cells by IL-2 secretion followed by activation of STAT3 and Bcl-2 (Atreya et al., 2000; Teague et al., 1997; H. Yoshida et al., 2010). IL-6 has an immense potential in differentiating CD8 T cells to cytototoxic T lymphocyte (CTL) subsets (M. Okada et al., 1988). Surprisingly, IL-6 trans-signaling works in combination with TCR signalling to induce a rapid expression of granzyme B (GzmB) and thus contributing in development of effector T cell function (Bottcher et al., 2014). Despite its overwhelming response in cell proliferation and survival, IL-6 alone is not sufficient to sustain the adaptive immunity. However, when IL-6 is used

in combination with other cytokines like IL-7 and IL-15 it tremendously increases granzyme B and cytolytic capacity of effector cells (Gagnon et al., 2008).

To investigate the capacity of PBMCs to produce IFN-γ, we determined levels of gamma interferon post stimulation. Surprisingly the mRNA levels of IFN-γ were significantly increased in PBMCs cultures treated with IL-6 as compared to those w/o IL-6. Immunity to viral infections greatly rely on the production of gamma interferon (IFN-γ)-producing T-cell response. Previous studies have suggested a protected role of IL-6 on T lymphocyte subsets. It was demonstrated that IL-6 is necessary for generation of IFN-γ response during *M. tuberculosis* infection (Saunder *et al.*, 2000). It is now a well-established fact that IL-6 can monitor the initiation and development of both innate and adaptive immune response (Snick.J, 1990).

The data presented here strongly support the idea that IL-6 has a definitive role in maintaining immunity which cannot be compensated for by other cytokines. Annexin V staining of PBMCs populations in this study also revealed interesting findings. The number of stained cells was reduced in experimental groups showing a limited number of cells undergoing programmed cell death. This further strengthens our hypothesis that IL-6 can act as a promising candidate and a survival factor for lymphocytes.

Conclusion and Future Prospects

The findings of present study concluded that Interleukin-6 has a strong potential in regulating the mechanism of mitochondrial apoptosis in lymphocytes derived from Chronic HCV patients. Interleukin-6 has been found to upregulate anti-apoptotic proteins, reduce the expression of T cell inhibitory receptors as well as increases the production of interferon-gamma thereby proving to be a promising candidate for rescuing lymphocytes from exhaustion and apoptosis induced by persistent infections. Besides regulating the expression of candidate genes involved in apoptosis and exhaustion, IL-6 also reduces helps in maintaining integrity of PBMCs as only few cells were found to be depleted via programmed cell death. Present study could be beneficial in moving one step towards ex-vivo expansion and modification of lymphocytes for adoptive T cell therapy of chronic HCV patients. Although Interleukin-6 alone is not sufficient to produce a stable and robust response, however when used in combination with other entities could prove to be a successful candidate. Further studies need to be conducted to completely understand the nature of IL-6 in regulating survival of lymphocytes especially in persistent viral infections where they are prone to becoming exhausted and undergoing apoptosis that ultimately collapses the immune system of individual.

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APPENDIX

Cell Counting:

Cell count = X x dilution factor x 104 cell/ml

=107.25x2x104 cell/ml

=214.5x 104cell/ml

=2.14x 106cell/ml

95% cells viability was determined by dividing the live cell count (unstained by trypan blue) from the total cell count [2.14x 106cell/ml/2.26x 106cell/ml=95%).

Cytokine Calculations:

Interleukin-6=5ug

IL-6= 5000ng

5000ng diluted to 1000ng/mL

Cytokine required for stimulation experiment=5ng

1000 x X= 5ng x 2000 uL

X= 5 x 2000/1000

X= 10 uL/mL

Preparation of RPMI 1640 media:

RPMI 1640 media was supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic (Penstrep).

1X PBS

1000mL of 1 X PBS was prepared by mixing the following,

NaCl = 8g

KCl = 0.2g

Na2HPO4 = 1.44g

KH2PO4 = 0.24 g

Making the volume upto 1000mL and adjusting pH to 7.4

Annexin Binding Buffer:

10mM Hepes

140mM NaCl

2.5mM CaCl2

This will make 10X Binding Buffer. Prepare working solution of 1X before use in experiments

1% BSA in PBS (FACS-PBS):

Mix the following in PBS

1% BSA

0.1% Sodium Azide