# University of Alberta

# FUNCTION OF MYELOID DENDRITIC CELLS DURING CHRONIC HEPATITIS C VIRUS INFECTION

by

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## ABSTRACT

Approximately 70% of patients infected with hepatitis C virus (HCV) develop chronic infection, which has been reported to be due to impaired specific T cell responses. Myeloid dendritic cells (mDCs) are potent antigen-presenting cells that regulate T cell responses, however their role during chronic hepatitis C (CHC) is not fully understood.

My hypothesis was that the activity of mDCs to regulate T-cell response against HCV might be changed during CHC infection, and this change might contribute to the impaired T-cell responses that lead to the persistence of HCV infection.

The objectives of my thesis were to compare the immunogenic activity (which stimulates T-cell proliferation), tolerogenic activity (which kills T cells), and apoptosis of mDCs from CHC patients and healthy donors.

In this thesis, I found that mDCs from CHC patients expressed lower level of activating molecules, HLA-DR and CD86, compared to mDCs from healthy volunteers. When mDCs were cocultured with T cells, there were fewer T cells proliferating in the patient group than in the healthy group. This result indicated that the ability of mDCs to stimulate T cell proliferation was impaired in CHC patients.

Moreover, mDCs from CHC patients underwent spontaneous apoptosis at a higher rate than mDCs from healthy donors. Nuclear factor-kappa B (NF-κB) activity, which is critical for mDC function and prevention of apoptosis, was diminished in mDCs from CHC patients.

I further studied the tolerogenic activity of mDCs during CHC infection. mDCs from CHC patients expressed up-regulated levels of Fas ligand and the ligand 2 of PD-1 compared to mDCs from healthy volunteers. mDCs from CHC patients can kill T cells, while mDCs from healthy volunteers could not. This result indicated that the tolerogenic activity of mDCs was up-regulated in CHC patients.

In conclusion, mDCs from CHC patients demonstrated functional changes with increased apoptosis, and diminished NF-κB activity. mDCs from CHC patients have impaired immunogenic activity to stimulate T-cell proliferation, and have up-regulated tolerogenic activity to kill T cells. These changes might be additional novel mechanisms of immune evasion by HCV, and contribute to the impaired specific T-cell responses observed in CHC patients.

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| | |
|---|---|
| Ab | antibody |
| Ag | antigen |
| AICD | activation induced cell death |
| Alb-uPA | albumin-urokinase plasminogen activator |
| ALT | alanine aminotransferase |
| APC | allophycocyanin |
| APCs | antigen-presenting cells |
| ARF | an alternate reading frame protein |
| AST | aspartate aminotransferase |
| BDCA-1 | blood dendritic cell antigen-1 |
| BLyS | B lymphocyte stimulator |
| BSA | bovine serum albumin |
| CAPE | caffeic acid phenethyl ester |
| CD | cluster of differentiation |
| cDNA | complementary DNA |
| CFSE | carboxyfluorescein succinimidyl ester |
| CHC | chronic hepatitis C |
| CTL | cytotoxic T lymphocytes |

| | |
|---|---|
| DAPI | 4', 6-diamidine-2'-phenylindole dihydrochloride |
| DC | dendritic cell |
| DTT | dithiothreitol |
| E1 | envelope glycoprotein 1 |
| E2 | envelope glycoprotein 2 |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| EMSA | electrophoretic mobility shift assay |
| F protein | a frameshift protein |
| Fah | fumaryl acetoacetate hydrolase |
| FasL | Fas ligand |
| Fc | fragment crystallizable |
| FcR | Fc receptor |
| FITC | fluorescein isothiocyanate |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| HAV | hepatitis A virus |
| HBV | hepatitis B virus |
| HCV | hepatitis C virus |
| HCVcc | HCV in cell culture |

| | |
|---|---|
| HCVpp | HCV pseudoparticles |
| HIV | human immunodeficiency virus |
| HLA | human leukocyte antigen |
| HRP | horseradish peroxidase |
| HS | highly sulfated heparin sulfate |
| HVR1 | hypervariable region 1 |
| IFN | interferon |
| IκBα | inhibitory kappa B-α |
| IL | interleukin |
| IRES | internal ribosome entry site |
| IRF3 | IFN regulatory factor 3 |
| IPS-1 | IFN-β promoter stimulator protein 1 |
| ISDR | Interferon Sensitivity Determining Region |
| JFH-1 | Japanese fulminant hepatitis-1 |
| LDs | Lipid droplets |
| LDLr | low-density lipoprotein receptor |
| LPS | lipopolysaccharide |
| MACS | magnetic-activated cell sorting |
| mBLyS | membrane-bound BLyS |

| | |
|---|---|
| mDC | myeloid dendritic cell |
| MFI | mean fluorescence intensity |
| MHC | major histocompatibility complex |
| MHC-I | MHC class I |
| MLR | mixed lymphocyte reaction |
| moDC | monocyte-derived myeloid dendritic cell |
| mTRAIL | membrane-bound TRAIL |
| MV | measles virus |
| NANBH | non-A, non-B hepatitis |
| $NaVO_3$ | sodium metavanadate |
| NCR | noncoding region |
| NK | natural killer |
| NS | nonstructural protein |
| NtAbs | neutralizing antibodies |
| NF-κB | nuclear factor-kappa B |
| PAGE | polyacrylamide gel |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate-buffered saline |
| PE | phycoerythrin |
| PerCP | peridinin Chlorophyll Protein Complex |
| pDC | plasmacytoid dendritic cell |

| | |
|---|---|
| PD-1 | programmed death-1 |
| PD-L1 | ligand 1 of programmed death-1 |
| PD-L2 | ligand 2 of programmed death-1 |
| PE | phycoerythrin |
| pegIFN-alpha | pegylated interferon alpha |
| PIB | phosphatase inhibitor buffer |
| pIκBα | phosphorylated inhibitory-κBα |
| PNPP | para-nitrophenyl phosphate |
| PPD | tuberculin-purified protein derivative |
| PS | phosphatidylserine |
| | |
| rs | reference sequence |
| Rag2 | recombination activating gene 2 |
| RdRp | RNA-dependent RNA polymerase |
| RIG-I | retinoic acid-inducible gene I |
| | |
| sBLyS | soluble BLyS |
| SCID | severe combined immunodeficiency disease |
| siRNA | small interfering RNA |
| SRBI | scavenger receptor class B type I |
| STAT-C | specifically targeted antiviral therapy for HCV |
| sTRAIL | soluble TRAIL |
| SVR | sustained virological response |

| | |
|---|---|
| TBS | Tris-buffered saline |
| TBS-T | Tween-20 in TBS |
| TCR | T cell receptor |
| TLR3 | toll like receptor 3 |
| TRAIL | tumor necrosis factor-related apoptosis inducing ligand |
| Treg | regulatory T cells |
| TRIF | Toll–IL-1 receptor (TIR) domain-containing adaptor inducing IFN-β |
| TNF | tumor necrosis factor |
| TUNEL | terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling |

# CHAPTER 1

# Introduction

### *1.1 General background of Hepatitis C virus (HCV)*

The history of HCV research began with the search for causative agents of blood transfusion-associated hepatitis known as non-A, non-B hepatitis (NANBH).

The modern era of blood transfusion started the same time as World War II (1939-1945), which resulted in the need for blood replacement. After that, transfusion-associated hepatitis became one of the major hazards of blood transfusion [1]. After the identification of hepatitis B virus (HBV) as an agent causing hepatitis, an all-volunteer donor system was adopted and multiple screening assays for HBV antigen were introduced in the 1970's. These interventions dramatically decreased rates of transfusion-associated hepatitis. A retrospective study showed that only 25% of transfusion-associated hepatitis was HBV-related, leaving 75% of cases tentatively classified as non-B hepatitis [1, 2]. The only other known hepatitis virus at that time was hepatitis A virus (HAV). Surprisingly, there was not a single case due to HAV. Hence, the "NANBH" was designated to describe the new form of hepatitis caused by the unknown infectious agent [3].

Despite the description of NANBH, international research efforts to identify the causative agent failed for more than a decade.

In 1989, Choo and colleagues under the direction of Dr. M. Houghton, collaborating with Dr. D. W. Bradley, utilized a novel molecular cloning approach to identify the unknown infectious agent. A random-primed

complementary DNA (cDNA) expression library was constructed from RNA extracted from the plasma of a chimpanzee freshly infected with the NANBH agent. The cDNA library was screened with antibodies in serum from a patient diagnosed with NANBH. This resulted in the isolation of a cDNA clone that encoded an antigen specifically associated with NANBH infections [4]. The virus was confirmed to be present in a panel of NANBH specimens by Dr. H. J. Alter and coworkers, and the virus was re-named HCV [5]. The discovery of HCV has led to significant improvements in the diagnosis of HCV, improved blood safety by screening assays, and a rapid expansion in epidemiology, pathogenesis and antiviral research.

### *1.2 The HCV particle*

HCV is a small (50 nm in size), enveloped, positive-strand RNA virus, the only known member of the genus *Hepacivirus* of the *Flaviviridae* family. The *Flaviviridae* family also includes Yellow Fever virus and West Nile virus [6]. The HCV particle consists of a RNA genome in an icosahedral nucleocapsid surrounded by a lipid bilayer. The genome consists of a single open reading frame that is approximately 9600 nucleotide bases long. The lipid bilayer is of cellular origin, and two viral envelope glycoproteins, E1 and E2, are embedded in lipid bilayer on the envelope [6]. The HCV genome contains a 5' noncoding region (NCR), followed by an open reading frame that codes for structural and nonstructural (NS) proteins and a 3' NCR that is required for replication. The translation product is a polyprotein approximately 3000 amino acids long, which

is cleaved by viral and cellular enzymes into individual proteins including three structural proteins (core, E1, E2) and six NS proteins (P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) [6]. The three HCV structural proteins include core, envelope glycoproteins E1 and E2 of the viral particle. The E1 and E2 glycoproteins play crucial roles in mediating HCV binding to and entry into target cells. E2 contains two hypervariable regions (HVRs), called HVR1 and HVR2. They are considered as hotspots for extreme sequence variability. Epitopes localized to these HVRs evolve rapidly, and are targets of neutralizing antibodies [7, 8].

The six NS proteins include the p7 ion channel, the NS2 protease, the NS3 serine protease and RNA helicase, the NS4A polypeptide, the NS4B and the NS5A proteins, and the NS5B RNA-dependent RNA polymerase (RdRp). The p7 is a small hydrophobic protein with 63 amino acids. Following its release from the polyprotein, p7 oligomerizes on the endoplasmic reticulum (ER) membrane to form calcium ion-conductive pores [9]. While deletion of p7 does not appear to affect HCV entry and replication, it significantly abrogates infectious virion assembly and release [10]. The NS2-3 cysteine protease consists of the C-terminal domain of NS2 in conjunction with the N-terminal region of NS3. It is responsible for the autocatalytic cleavage of the NS2-NS3 junction [11]. The NS3 is a multifunctional protein with serine protease activity provided by its N-terminal domain, RNA helicase and nucleotide triphosphatase (NTPase) activity produced by its C-terminus domain. The NS3 serine protease domain,

which engages the NS4A polypeptide as a cofactor, is responsible for the cleavage of all of the remaining junctions in the NS portions of the polyprotein [12]. The NS4A is required for efficient polyprotein processing by functioning as viral protease cofactor and provides stability to NS3 [13]. The NS4B is an integral membrane protein that for many years was characterized mainly as a protein of unknown function. Recently, however, NS4B has been found to be responsible for the formation of a novel intracellular membrane structure, termed the membranous web, which appears to be the platform upon which viral replication occurs. Furthermore, NS4B has been implicated in modulation of NS5B's RdRp activity [14, 15]. The NS5A phosphoprotein has generated wide interest in HCV research because its ability to modulate the IFN response of host cells. The NS5A harbors a potential Interferon Sensitivity Determining Region (ISDR), in which sequence variations within this cluster may contribute to IFN-alpha therapy resistance that is often observed with HCV [16]. While no known enzymatic function has been ascribed to NS5A, it is an essential component of the HCV replication. Furthermore, NS5A has been reported to be an RNA-binding protein [17]. Being the RdRp for HCV, NS5B is critical for RNA replication in the cells and is membrane-associated [18]. These NS proteins are important targets of specific antiviral agents for HCV.

In addition to the polyprotein, an alternate reading frame (ARF) protein or frameshift protein (F) is also produced [19]. The F protein was identified in the HCV core-coding region that has the potential to encode a protein of up to 160

amino acids. Specific antibodies to and T cells actively against the F protein have been detected in patients with chronic HCV infection, which suggests that the F protein is expressed during HCV infection. The precise function of the F protein is unclear, although the F protein may play a role in HCV replication since mutations in this protein delayed RNA replication and reduced virus titers [20, 21].

### 1.3 HCV genotypes

HCV isolates are classified into six (or seven) major genotypes and more than 100 subtypes. HCV main genotypes are identified as genotypes 1 through 6 (or 7), and have greater than 30% sequence divergence between them. Each genotype is further divided into subtypes designated by lowercase letters, with each subtype differing from one another by 10-30%. Subtypes are further clustered into quasispecies based on their genetic diversity, which are generated by the low-fidelity rate of viral RdRp [22].

The distribution of HCV genotypes varies globally. In North America, genotype 1a predominates and is followed by 1b, 2a, 2b and 3a [23]. Genotypes are clinically important in that it is one of the principal determinants of potential response to interferon (IFN)-based therapy and the required duration of such therapy. For example, HCV-infected patients with genotype 1 and 4 are less responsive to standard IFN-based therapy than other genotypes [23]. Duration of IFN-based therapy to genotypes 1 and 4 is 48 weeks, while that for genotypes 2 and 3 is 24 weeks.

### 1.4. HCV lifecycle

The HCV lifecycle comprises viral entry, uncoating and release of the viral genome into the cytoplasm followed by the translation and replication of the RNA, cleavage of the polyprotein, assembly into new particles, and egress (Fig. 1-1). The major host cell supporting HCV infection and replication *in vivo* is the human hepatocytes.

HCV entry is the first step of interaction between virus and the target cell. Being a complex multi-step process, HCV entry occurs in a pH- and clathrin-dependent manner with several host receptors being involved in. The current model of HCV entry includes (1) the initial attachment of HCV to glycosaminoglycans (GAG) and/or low-density lipoprotein receptor (LDL-R), (2) binding of HCV to CD81 and /or scavenger receptor class B type I (SR-BI) and (3) re-location to tight junctions via binding of HCV to members of the claudin family (CLDN1, 6 and 9) and occludin [6, 24].

In patient serum, HCV is mostly (more than 85%) associated with lipoproteins and thus has been termed as lipo-viro-particles (LVP) [25]. Density gradient analysis of serum reveals that HCV-RNA is found in both low (1.06-1.15 g/ml) and high (1.17-1.25 g/ml) density fractions. The HCV in the low-density fractions has a higher infectivity than the HCV in the high-density fractions [26, 27]. Observed under electron microscope, the HCV LVP with the unusual low-density are spherical with up to 100 nm in diameter [25]. After delipidation

with detergents, the LVP are converted to smaller particles resembling nucleocapsids with a diameter of 30-40 nm [25]. Characterization of the unusual low-density LVP from patient serum demonstrated that they contain apolipoprotein B and apolipoprotein E proteins, triglycerides, and cholesterol [25], which are components of low density protein (LDL) and very low density lipoprotein (VLDL) particles. Moreover, blocking LDL-R with antibody can block HCV entry [28], indicating that the LDL-R is a co-receptor for HCV entry.

The initial attachment is probably mediated by the lipoproteins (apolipoprotein E, apolipoprotein B) on HCV particles with LDL-R expressed on cells [28, 29]. Pretreatment of HCV LVP with heparin or cells with heparinase decreased HCV attachment, indicating that the involvement of GAGs [29]. After the initial attachment of HCV to LDL-R and GAGs, CD81[30] and SR-BI [31] are involved in the uptake of HCV. The E2 protein of HCV interacts with the large extracellular loop of CD81, a post-attachment entry co-receptor [30]. E2 can also interact with SR-BI [31]. The role of CD81and SR-BI in HCV entry is post-binding, as antibodies against either CD81 [30] or SR-BI [31] inhibit infection after HCV attachment. Following the interaction of HCV with CD81 and SR-BI, HCV re-locates to tight junctions via binding to members of the claudin family (CLDN1, 6 and 9) and occludin [6, 24]. Claudin-1, a tight junction component that is highly expressed in the liver, is essential for HCV entry at a late step. Antibody against claudin-1 could block HCV infection [32]. Another tight junction protein, occludin, was subsequently identified as a novel

receptor required for HCV entry. This further highlights the importance of tight junction complex in HCV entry [33].

After HCV comes into contact with claudin-1 and occludin, HCV undergoes clathrin-dependent endocytosis [34]. Clathrin is protein that is involved in the formation of coated vesicles. Clathrin is one of the coat-proteins that are used to build small vesicles in order to safely transport molecules between cells. Small interfering RNA-mediated clathrin depletion inhibited HCV entry and infection [34]. Following internalization via endocytosis, HCV genome is delivered from the endosome to the cytoplasm by a pH-dependent fusion process. Being a positive sense RNA, HCV genome RNA acts as mRNA and is therefore directly translated. Upon release of the viral genome into the host cell, the HCV polyprotein is produced using an internal ribosome entry site (IRES)-dependent translation mechanism in the infected cell cytoplasm [35]. Translation of HCV genome RNA occurs at rough endoplasmic reticulum. Translation of the HCV open reading frame yields a polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases into mature structural and NS proteins. The structural proteins and the p7 polypeptide are processed by the endoplasmic reticulum (ER) signal peptidase, while the NS proteins are processed by viral proteases, the NS2-3 protease and the NS3-4A serine protease. NS2-3 protease is also known as autoprotease. The auto-catalytic activity of the NS2-3 autoprotease resides in the C-terminal half of NS2 and the N-terminal one-third of NS3 protein [6]. With the NS4A

polypeptide functions as a cofactor for the NS3 serine protease, the NS3-4A serine protease complex is responsible for the process of all other NS proteins [6, 24]. During or shortly after the polyprotein processing, the replication complex is formed, presumably involving viral proteins, viral RNA, and host cell factors. The NS5B RdRp protein begins synthesis of the complementary negative-strand RNA immediately after infection, which then serves as a template to generate genomic positive-strand RNA. The newly synthesized positive-stand RNAs are used for translation, further RNA production, or the positive-strand RNA becomes the genetic material for virion packaging. Replication of HCV induces membranous web synthesis, which concentrates lipid-rich structures that facilitate HCV replication and assembly [6].

Although little is known regarding the late steps of HCV lifecycle, HCV may use the assembly and secretion of VLDL to exit the infected cells. Inhibition of apolipoprotein B by short hairpin RNA inhibits the production of infectious HCV [36]. Furthermore, inhibition of VLDL secretion leads to the accumulation of intracellular of HCV-RNA and inhibited secretion of HCV [37]. Therefore, the association of HCV nucleocapsid with lipid droplets has been proposed to mediate the exit of HCV [24, 37].

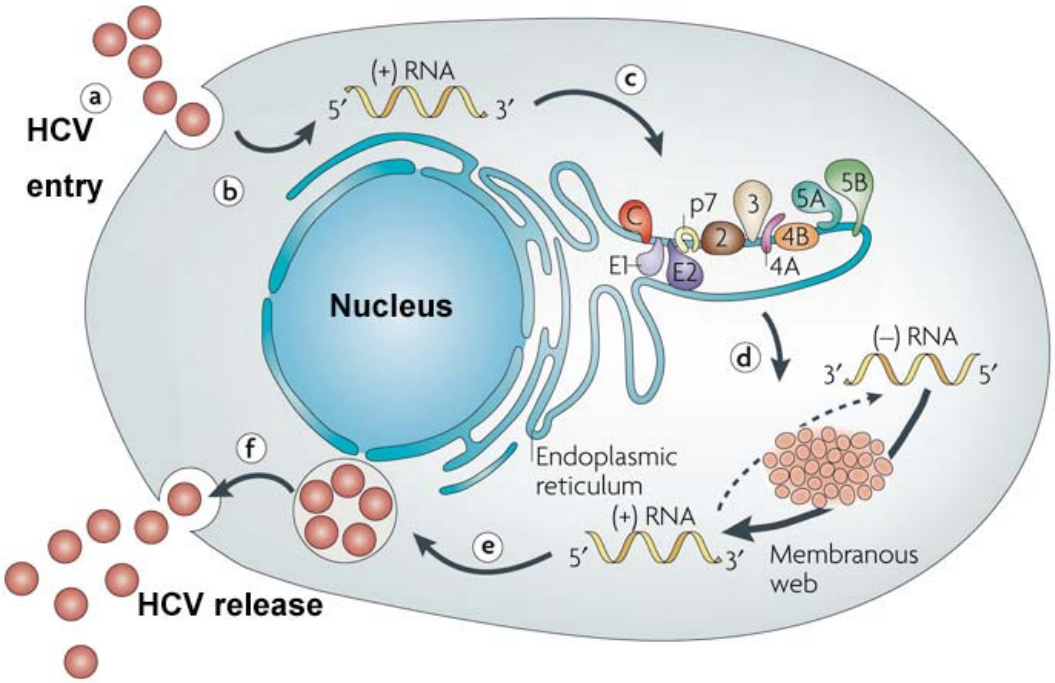


Fig. 1-1 HCV lifecyle [(modified from reference [6])].

The HCV lifecycle primarily includes (a) HCV binding and internalization; (b) cytoplasmic release and uncoating; (c) IRES-mediated translation and polyprotein processing; (d) RNA replication; (e) packaging and assembly; and (f) HCV maturation and release.

As HCV encodes only a few viral proteins, HCV depends heavily on host factors to propagate. There are a large number of host factors required for HCV translation, replication and production. For example, autophagy proteins Beclin-1, Atg4B, Atg5 and Atg12 are suggested as proviral factors required for the translation of incoming HCV RNA and thereby the initiation of HCV replication [38]. Lipid droplets (LDs) in the vicinity of active replication structures are essential for HCV assembly in liver-derived cells. The capsid protein core and NS5A interact with the LDs, and this allows HCV assembly [39]. The use of small interfering RNA (siRNA) screens has further identified dozens of host factors required for HCV replication [40]. The discovery of these host factors has advanced our understanding of virus-host interaction, and has identified potential new targets for preventive and therapeutic interventions.

### 1.5 HCV epidemiology

With approximately 170 million infected individuals worldwide, or approximately 3% of the world's population, HCV is the major cause of chronic hepatitis. HCV is primarily transmitted by blood-to-blood contact. Any practice or activity that involves blood-to-blood exposure can potentially be a source of HCV infection. Most patients with chronic HCV infection were infected through transfusion with unscreened blood or blood products, via injection drug use (IDU), or through sexual exposure, even though transmission by sexual contact is low [41]. Being at a particular high risk of HCV exposure and infection,

injection drug users (IDUs) constitute one half to two thirds of HCV-infected patients in Canada [42].

Vertical transmission of HCV from an infected mother to her child during the birth process is known to occur. However, transmission occurs only among women who are HCV RNA positive at the time of delivery, and the risk of transmission in this setting is relatively low as less than 5% of infants born to HCV positive mothers become infected [43].

Occupational exposure leads to HCV transmission in health care workers, with the highest proportion of occupational transmission being from percutaneous injury via hollow-bore needles used in vascular access [44] .

**1.6 Signs and symptoms of HCV infection**

Acute HCV refers to infection during the first 6 months after infection. Symptoms of acute HCV infection may include decreased appetite, fatigue, abdominal pain, jaundice, and flu-like symptoms. However, more than 70% of patients do not develop symptoms during the acute phase, which makes acute HCV infection difficult to be diagnosed. Acute hepatitis C is marked by appearance of HCV RNA in serum within 1 to 2 weeks after infection followed by elevated serum level of liver-associated enzymes, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [45]. The

appearance of antibodies to HCV tends to arise late, ranging from 3 to 15 weeks after infection [46].

In the natural course of hepatitis C infection, approximately 75-85% of the infected patients become chronic carriers of HCV [46]. Chronic HCV infection is defined as infection with HCV persisting for more than six months. It is often asymptomatic clinically and discovered accidentally, e.g. by routine examination, blood donations or insurance screening. Most patients have evidence of inflammation on liver biopsy, while the rate of liver fibrosis varies significantly between individuals [45].

Whereas HCV viremia may reach a stable level and remains fairly constant over years after infection, chronic HCV infection is usually associated with persistently elevated and frequently fluctuating elevations in ALT and AST [46].

The most common severe sequelae of chronic HCV infection include progressive hepatic fibrosis, cirrhosis, and hepatocellular carcinoma. Once chronic HCV infection has progressed to cirrhosis, signs may appear indicating decreased liver function and/or portal hypertension (increased pressure in the liver circulation). These signs include ascites (accumulation of fluid in the abdomen), varices (enlarged veins, especially in the stomach and esophagus), and hepatic encephalopathy (a syndrome of cognitive impairment due to the accumulation of ammonia and other substances normally cleared by a healthy liver) [41, 47].

Extrahepatic manifestations of chronic HCV infection include mixed cryoglobulinemia, Sjogren's syndrome, B cell non-Hodgkin's lymphoma, presence of auto-antibodies in sera, and type II diabetes [45]. There is a 2-3-fold increase in type II diabetes in individuals with chronic hepatitis C infection [48-51]. The mechanisms underlying these extrahepatic manifestations are not fully understood.

### 1.7 Management of patient with chronic HCV infection

The management of HCV infection includes serological and virological tests, liver biopsy and ultrasound-based tests [52, 53].

Anti-HCV antibody indicates that a patient has been infected with HCV [52, 53]. However, specific virological testing for HCV RNA differentiates the patients who have been infected and then cleared the virus from those that become chronic carriers. Molecular determinations of the HCV genotype are important tests that help in the managements of chronic carriers of HCV. The presence of HCV RNA in the absence of anti-HCV antibodies strongly indicates acute HCV infection [52, 53].

Liver biopsy and ultrasound-based tests are often used to evaluate the severity of HCV-related liver disease. Serum enzymatic assay, such as elevated ALT level, indicate the indirect effects of HCV on infected hepatocytes, which reflect

immune injury and altered liver function since infected hepatocytes are targets of host cellular and humoral immunity. Liver biopsy and ultrasound-based tests have been used to assess HCV-associated liver disease, including inflammation, steatosis, fibrosis and hepatocellular carcinoma [52, 53].

## 1.8 Factors that promote progression of chronic hepatitis C

Multiple factors, including host factors, transmission route of HCV and viral factors, promote progression of chronic hepatitis C. Host factors include race, age, gender, coinfection with HIV or HBV, obesity, alcohol, HLA and interleukin-28 (IL28) genotyping. HCV clearance is less common in blacks than whites [50]. The average time from infection to development of cirrhosis and hepatocellular carcinoma in older patients (average age, 58 years) was more than twice as long as in the younger patients (average age, 29 years) [54, 55]. Women tend to eliminate HCV more rapidly, have a lower rate of disease progression than men, and a lower mortality rate from HCV-related liver disease [56]. Viral factors include genotype and viral mutations.

### *1.8.1 Human genetic variation in IL28 locus*

A genetic polymorphism near the IL28B gene, encoding IFN-lambda, is associated with the variable outcome of HCV infection. For example, minor G allele at the reference sequence (rs) 8099917 in IL28B locus was associated with chronicity [57]. The association of the IL-28B locus with HCV control indicates the importance of innate immunity and IFN-lambda in the pathogenesis of HCV

infection [58]. IFN-lambda proteins are encoded by the IL28A/B and IL29 genes. The human genetic polymorphism near the IL28B gene is found on chromosome 19 and is 3 kilobases (kb) upstream of the IL28B gene [59]

Furthermore, genotype at the rs12979860 polymorphism affects the progress of steatosis in treatment of naïve patients with chronic HCV infection. The CC genotype is associated with a lower rate of steatosis, which indicated less disturbance of lipid metabolism [60]. Furthermore, the CC genotype is associated with spontaneous clearance of acute HCV infection [61] as well as response to IFN-based treatment [62, 63].

### *1.8.2 Human leukocyte antigen (HLA) haplotypes*

Both HLA-I and HLA-II haplotypes affect the clinical course of HCV-infected patients. For example, HLA-I B54 is closely associated with the progression of liver injury and HLA-C*04 is associated with HCV persistence [64]. This may be attributed to the observation that certain HLA-I molecules may not be capable of presenting HCV epitopes to cytotoxic T cells, which are required for clearance of HCV infection. The association of HLA-C*04 with viral persistence suggests that natural kill (NK) cells may play a role in HCV clearance, since HLA-C*04 is a ligand for receptors on NK cells [65].

### *1.8.3 HIV co-infection*

HIV co-infection accelerates the liver disease progression in HIV/HCV co-infected patients. HCV reactivation and persistence have been reported in patients coinfected with HCV/HIV even though some of these patients had previously cleared HCV infection when they were HIV-negative. Cirrhosis progresses more quickly in co-infected patients than in HCV mono-infected patients [66].

Studies on the possible mechanism yielded controversial results. Daar *et al.* suggested that it is due to the deteriorating immune status in HIV co-infected patients, based the fact that as $CD4^+$ lymphocyte count decreases there was an increase in HCV RNA level [67]. However, Strasfeld *et al*. demonstrated that this effect is independent of severity of immunosuppression, since they found that there was not a significant correlation of $CD4^+$ lymphocyte count with HCV RNA level [68].

### *1.8.4 Lifestyle risk factors*

The incidence of cirrhosis is higher in HCV-infected subjects who consume alcohol daily [50]. The mechanism of liver injury in these patients is multi-factorial, which includes a combination of diminished immune clearance of HCV, oxidative stress, hepatic steatosis, increased iron stores, and increased apoptosis of hepatocyte [69]. Tobacco and obesity are also lifestyle risk factors associated with hepatocellular carcinoma [70].

### *1.8.5 HCV genotype*

In comparison to other genotypes, the HCV genotype 1 is related to a higher rate of HCV persistence and a lower successful treatment rate with IFN-based therapy. Genotype 1 patients also have a higher rate of insulin resistance [71, 72]. The HCV genotype 3 is associated with steatosis and fibrosis development. This could result from genotype-3a core protein, which is stronger than the genotype-1b core protein in up-regulating the fatty acid synthase promoter [73].

### 1.9 Occult HCV Persistence

Recently, several papers have revealed that patients continue to harbor HCV in their plasma at very low levels and the replicative intermediates of HCV were detected in peripheral blood mononuclear cells (PBMCs), monocyte-derived dendritic cells (moDCs), and hepatic tissues as long as 8 years after resolution of hepatitis C [74-76]. Such persistent replication of residual HCV after spontaneous or therapy–induced recovery from hepatitis C is referred to as occult HCV persistence.

The correlation of occult HCV infection with disease progression has not been documented. The significance of occult HCV remains to be determined.

### 1.10 Therapy of HCV infection

#### *1.10.1 Combination therapy and its efficiency*

Until recently, the standard therapy for HCV was a combination of pegylated interferon alpha (pegIFN-alpha) and ribavirin for 6 to 12 months depending on the viral genotype [77]. Antiviral effect is monitored by measuring viral loads in patient plasma before and during treatment, with at least a 2-$\log_{10}$ decrease from

baseline in HCV RNA by 12 weeks required if therapy is to continue for the full course of 48 weeks [77, 78].

Only patients with detectable HCV RNA should be considered for pegIFN-alpha and ribavirin combination therapy. HCV genotypes must be determined before treatment since it dictates the duration of treatment, the dose of ribavirin and the virological monitoring procedure. For HCV genotype 1, patients require 48 weeks of treatment, with HCV RNA quantification performed at weeks 4, 12, 24 and 48 to monitor treatment. For genotypes 2 and 3, therapy is monitored at 4 and 12 weeks and a normal course of therapy requires 24 weeks of treatment. Genotype 4 requires therapy for 48 weeks, similar to genotype 1 [77].

Sustained virological response (SVR) is defined as the absence of detectable HCV RNA in the serum 6 months after therapy was complete. Most sustained virological responders remain HCV-RNA negative for the rest of their lives. HCV genotype is an important prediction of the outcome of therapy. For genotype 1, therapy for 48 weeks produces a SVR in 40-50%. For genotype 2, this increases to near 90% with 6 months of standard therapy. Patients with genotype 3 have a SVR of approximately 70% with 4 months of therapy [77].

As discussed under Section 1.9.1, a human genetic polymorphism near the IL28B gene on chromosome 19 is highly predictive of virus clearance with pegIFN-alpha plus ribavirin treatment in adults with the genotype 1 virus [58]. The study

concentrated on SVR in chronic HCV patients involving a 48-week course of pegIFN-alpha-2b or pegIFN-alpha-2a combined with ribavirin. Because the polymorphism that is associated with better treatment response occurs in substantially greater frequency in European than in African populations, this genetic polymorphism helps to explain the differences in response rates between African-Americans and patients of European ancestry [59]. The frequencies of genotypes TT, GT and GG for the rs8099917 were 0.42, 0.51, and 0.07 among patients with treatment failure versus 0.68, 0.29, and 0.03 among those with SVR, respectively. Minor G allele carriers had a higher risk of treatment failure than the patients with the TT allele ($P = 3.11\times10^{-8}$) and thus the G allele was defined as the risk allele. Besides rs8099917, other alleles in IL-28B locus have been reported to be associated with the outcome of HCV infection, including rs8105790, rs11881122, rs7248668 [57] and rs12979860 [61].

### *1.10.2 Evaluation of therapy adherence for predicting SVR*

Combination therapy with pegIFN and ribavirin is recommended as the first-line treatment approach for chronic hepatitis C patients. However, IFN therapy is associated with adverse effects including psychological disturbances, poor appetite, skin rash, anemia and leukopenia [79]. These adverse effects are more common in patients receiving combination therapy than in those receiving IFN monotherapy [80].

The severe adverse effects of therapy frequently lead to dose reduction and/or treatment discontinuation. Treatment discontinuation owing to severe adverse effects has been reported in 6-13% of HCV-infected patients who receive combination therapy [81]. Furthermore, dose reduction of pegIFN and/or ribavirin owing to adverse effects has been reported in 25-40% of patients who receive such therapy [80, 81].

Good adherence to treatment enhances SVR in chronic HCV patients treated with combination therapy [82, 83]. Discontinuing ribavirin as part of hepatitis C therapy reduced SVR [82], and reducing the ribavirin dose within the first 12-20 weeks of treatment in patients with genotype 1 was associated with a significant decline in SVR [83]. These studies indicate that adherence to full dose combination therapy is important for obtaining a SVR.

**1.11 Specifically targeted anti-viral therapy for HCV (STAT-C)**

New HCV therapies have demonstrated increased efficient antiviral activity. HCV is a RNA virus that is highly mutable and can easily develop mutant strains to resist therapies. Viral enzyme inhibitors targeting polymerase, protease and helicase are emerging as new therapies. Many of the STAT-C antivirals are in preclinical and clinical development. The protease inhibitor, BILN 2061, targets NS3. It was the first protease inhibitor shown to have high efficacy against HCV both *in vitro* and *in vivo* [84]. However, due to cardiac toxicity its clinical development was stopped. Another new HCV protease inhibitor, VX-950

(Telaprevir), was developed into the first STAT-C therapy to be licensed in 2011. However, as monotherapy, some patients quickly developed viral breakthrough, which was related to the selection of viral variants with decreased sensitivity to Telaprevir [85]. Boceprevir is the second protease inhibitor of NS3 to be licensed. It reduces viral load when administered as a monotherapy, and in combination with IFNα and ribavirin further reduces viral load [86]. Telaprevir along with Bocepravir have been licensed as the protease inhibitors to be used with pegIFNα and ribavirin. The combination therapy can prevent the emergence of mutant HCV strains [87]. The addition of either of these protease inhibitors to pegIFN and ribavirin increases the SVR to near 75% from 45-50% for genotype 1 virus. Both of these protease inhibitors are genotype 1 specific [88, 89].

The new antiviral therapies mentioned above have been shown to be highly specific with tolerable adverse effects. Adverse effects of the standard HCV therapies have restricted their use. While these new STAT-C antivirals are being used with pegIFN and ribavirin, the ultimate goal would be to develop combination of antivirals that are highly efficient, but free of pegIFN [88, 89].

**1. 12 HCV quasispecies and host selective pressure**

In persistently infected hosts, the RNA virus genome is described as a dynamic population of heterogeneous closely related variants in host. The fundamental cause of the generation of quasispecies in HCV is due to their replication enzymes.

RNA viruses, including HCV, influenza virus and HIV, replicate by viral polymerases. Their enzymes are error-prone and lack 3' exonuclease proof-reading activity [6].

Host selective pressure plays a dominant role in driving HCV evolution. It has been reported that HCV does not undergo high rates of mutation without selective pressure from host immune responses [90]. The selective pressures on mutants include specific anti-HCV antibodies, HCV-specific helper T lymphocytes and HCV-specific cytotoxic T lymphocytes. These specific immune responses neutralize HCV infection of target cells, mediate the killing of HCV-infected cells and prevent HCV dissemination. However, since HCV is highly mutable, the HCV genome can persist as quasispecies and evade these host immune responses. If the host immune responses are strong and sustained during the first few months of acute HCV infection, HCV infection will be cleared. Otherwise, HCV infection will persist, and the inadequate host immune responses provide an environment that drives HCV evolution [90].

Hypervariable region 1 (HVR1) of E2 is an example of a highly mutable site subject to immune pressure. HCV genes encoding the envelope glycoproteins (E1 and E2) are the most heterogeneous, especially the 81 nucleotides encoding HVR1. HVR1 is a 27-amino-acid sequence located in the N-terminal portion of the HCV envelope protein from positions 384 to 410. HVR1 can be used both to identify individual HCV strains and to study HCV quasispecies. It contains linear B-cell epitopes and is thought to be the major immunogenic domain of E2.

HVR1 is mutated during the natural course of HCV infection. HVR1 amino acid substitutions, which occur rapidly during the acute phase of infection, help the virus escape from the host immune response [7].

The HVR1 is under selective pressure since it is important for virus infection and it is a target for host immune response.

**1. 13 HCV quasispecies and viral fitness cost**

The balance between immune evasion and virus fitness cost determines the outcome of immune escape mutations [91, 92].

One of the mechanisms used by HCV to actively evade the host response is the highly genetic variability and accumulated mutations within CTL epitopes that impair CTL recognition. Although HCV is highly mutable, variability is not unlimited. For example, the NS3 protease does not mutate extensively. This might be an indication that the NS3 gene is indispensable for HCV fitness, and mutations within it may be lethal for HCV. A recent study examined whether the variability of the NS3 protease was limited by viral fitness [91]. In this study, the conserved nature of NS3 was confirmed by sequence analysis of blood samples from HCV-infected patients. Artificial mutations were introduced at five positions in the epitope between residues 1073-1081 of the NS3 protease by site-directed mutagenesis. Introduction of the five mutations prevented CTL recognition, implying immune evasion. However, three of the five mutations reduced NS3 protease activity and RNA replication, suggesting there was a

significant viral fitness cost for the immune evasion. This may explain why some of these mutations are not likely to appear in nature, since they reduce viral fitness. In another study, polymerase inhibitors were used to obtain resistant HCV replicon variants that contained mutations in the NS5B polymerase gene [92]. It was found that certain mutations in the HCV replicons showed reduced replication capacity, which again suggests a balance between viral mutation to evade immune response and a viral fitness cost. These two studies of HCV support the concept that the balance between immune evasion and virus fitness cost determines the outcome of immune escape mutations [91, 92].

In conclusion, these studies indicate that the balance between immune evasion and virus fitness indeed determines the outcome of immune escape mutations. Although a single mutation may impair HCV fitness, virus escape mutations may bring about improved viral fitness. When immune evasion does not seriously reduce HCV fitness, the mutants are likely to exist. However, with immune evasion at the cost of HCV fitness, the mutations are not likely to occur naturally, or will not persist [91, 92].

### 1.14 Experimental animal models for HCV

Currently, the two most important animal models used for HCV research are chimpanzees and albumin-urokinase plasminogen activator/severe combined immunodeficiency disease (Alb-uPA/SCID) mice transplanted with human hepatocytes.

Chimpanzees are the only natural host of HCV infection other than humans. Importantly, the clinical courses of HCV infection observed in chimpanzees and humans are similar, which is an important prerequisite for the use of chimpanzees as a model for HCV infection in humans**.** For example, a study performed in chimpanzees has contributed to the understanding of the specific cellular immune responses that are essential for the resolution of HCV infection [93]. HCV-infected chimpanzees demonstrated genomic response to IFN-alpha therapy [94]. Further studies in chimpanzees have shown that HCV evades host immune responses partly by altering the population of regulatory T cells (Treg) [95]. Although the chimpanzees will continue to be an indispensable model for HCV research, their utilization has been severely restricted due to the limited availability, the cost for these animals and their maintenance and ethical concerns related to their use [96, 97].

As an alternative animal model to chimpanzees for HCV infection, the Alb-uPA/SCID mice can be used as a platform to support engraftment and proliferation of transplanted human hepatocytes. The human hepatocyte population rapidly expands and replaces much of the diseased mouse parenchyma. The mice with chimeric human livers can then be inoculated with human serum or cell culture supernatant containing infectious HCV particles and support durable HCV replication at levels comparable to those levels seen in HCV-infected humans [98, 99]. Recently, a second mouse model has been reported based on

immunodeficient mice lacking fumaryl acetoacetate hydrolase (Fah), recombination activating gene 2 (Rag2) and the gamma chain of the receptor for IL-2 (IL-2rgamma). This mouse model allows the transplantation of human hepatocytes at any age of the mouse, which might improve the robustness of engraftment and mortality [19, 100].

The mouse models are becoming more readily produced and easier to be handled than chimpanzees. They allow the study of the HCV infection of human hepatocytes *in vivo*, and the assessment of antivirals targeting the viral or host factors present in transplanted human hepatocytes. For example, the gene expression profiles from HCV-infected chimeric mice have demonstrated a story of the innate immune response during HCV infection [101]. However, the mouse models are limited by significant mortality due to bleeding issues associated with high expression of urokinase in liver cells and the variable robustness of infection. Being deficient in adaptive immune responses, the use of mouse models is limited in the study of the pathogenesis of liver disease, immune-based therapies and vaccines.

In brief, the chimpanzee model has improved our understanding of HCV pathogenesis, the interaction of HCV with host immune system, and the potential to develop new vaccines. The Alb-uPA SCID mouse transplanted with human hepatocytes has been useful for the testing of new antiviral agents *in vivo* [19], the

study of innate immune response [101] and can be used to demonstrate protective immunity to HCV.

## 1. 15 HCV in cell culture

### *1. 15.1 HCV replicons*

The HCV life cycle and host-virus interactions that determine the outcome of infection had been difficult to study for many years, because the cell culture model of HCV infection was not available. Since 1999, HCV replicons have been used to study HCV in cell culture [19, 102].

A HCV replicon is an RNA construct that contains necessary viral elements for virus replication and a selection marker. It can replicate efficiently in certain cultured cells. It contains a selection marker, neomycin resistance gene, which facilitates identification of cell clones with replicon replication. The electroporation of Huh7 hepatoma cells with HCV replicon and culture with neomycin permits the selection of clones in which HCV replicon replication occurs [19, 102].

Since HCV replicons contain NS3-NS5 genes or the full-length coding region that is enough to drive HCV replication, they have also been used to develop drugs targeting HCV protease, helicase or polymerase in cell culture. HCV replicons do not replicate efficiently without adaptive mutations, nor do they release infectious HCV particles into culture medium [19, 102].

Studies based on HCV replicons have revealed some of the host-virus interactions that regulate the processes of HCV translation and RNA replication, and have been used to discover and develop drugs for the treatment of HCV infection.

### 1.15.2 Retroviral or lentiviral HCV pseudoparticles (HCVpp)

HCVpp consist of HCV envelope glycoproteins assembled onto retroviral or lentiviral core particles. The presence of green fluorescence protein or luciferase marker genes packaged within these HCVpp facilitates the reliable and fast determination of entry as well as antibody-mediated neutralization of infection [19, 103, 104].

Although differences in the export pathway of HCVpp and native HCV could affect the properties of the viral envelope, the HCVpp system is widely used because it is highly robust and amenable to high-throughput assays for quantification of virus neutralization studies [19].

### 1.15.3 JFH-1 strain of HCV

In 2005, three laboratories reported the first cell culture system for HCV [26, 105, 106]. The newly isolated virus was from a Japanese patient with fulminant hepatitis C (JFH-1) and infected Huh7-derived cells produced the infectious virus. JFH-1 can infect human hepatoma-derived Huh7 cells, human non-hepatic cells (Hela and 293 cells), chimpanzees, and the chimeric mouse model (Alb-uPA

SCID mouse) [26, 105, 106]. This system has facilitated both *in vitro* and *in vivo* studies of HCV.

The JFH-1 strain of HCV contains the full-length HCV genome, can replicate efficiently in Huh7 cells, releases infectious HCV particles, and can be passed effectively in cell cultures [105]. It provides a tool for studying detailed aspects of the HCV life cycle, including entry, trafficking, viral assembly, and egress, that had not been previously approachable using HCV replicons or HCVpp [19].

The JFH-1 strain is HCV genotype 2a and can be used for the creation of chimeric HCV strains of different genotypes. The chimeric viruses can be produced using the structural proteins of other isolates, but robust infection requires the presence of the NS proteins from the JFH-1 isolate [107]. This limitation precludes the analysis of full-length patient-derived strains. Another limitation is the dependency of transformed hepatoma cell lines to grow the virus, which does not reflect the cell physiology of the primary human hepatocytes, the natural target of HCV infection [19]. Moreover, JFH-1 does not infect immune cells, a characteristics different from HCV in patient serum [108]. However, the JFH-1 strain has laid a foundation to examine new aspects of the HCV life cycle and to develop new drugs both *in vitro* and *in vivo* for combating HCV.

### *1.16 Hepatitis C vaccine*

HCV is able to establish persistent infections in up to 85% of infected individuals with severe clinical consequences. Although it is a heavy burden on public health, there is currently no vaccine available for hepatitis C. Since vaccination is one of the most cost-effective approaches for the management of infectious diseases, development of an effective HCV vaccine is an important objective for the prevention of HCV infection. Evaluating different vaccination strategies that can induce HCV-specific immunity is critical for the development of an effective vaccine to prevent HCV infection and reduce HCV-related mortality and morbidity [45].

Induction of HCV-specific antibodies as well as multi-specific, functional CD4 and CD8 T cells is the immunological hallmark of a successful HCV vaccine in humans [109-112]. However, HCV quasispecies and multiple genotypes continue to challenge the development of an efficient vaccine.

## 1.17 Innate immune control and escape of HCV

### *1.17.1 HCV attenuates innate immune responses in hepatocytes*

The first response to HCV infection is believed to be IFN-β production by infected hepatocytes. This response is initiated by two types of receptors, toll like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I) [113]. HCV dsRNA in endosomes activates TLR3, which recruits the adapter molecule Toll–IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF). In addition, RIG-I recognizes the polyuridine motif of the HCV 3’ UTR in

cytoplasm, and then recruits the adapter molecule IFN-β promoter stimulator protein 1 (IPS-1). Both of these processes result in downstream signaling, nuclear translocation of nuclear factor-kappa B (NF-κB) and IFN regulatory factor 3 (IRF3). NF-κB and IRF3 work in synergy and lead to the synthesis of IFN-β, which induces an antiviral state [113, 114].

However, HCV may attenuate the IFN response. For example, HCV NS3/4A protein in cell culture is able to cleave the adapter molecules TRIF [115] and IPS-1 [116], and thus blocks TLR3 and RIG-I signaling. This phenomenon still needs to be demonstrated as being important *in vivo* in the HCV-infected liver, however, it is likely a significant strategy of viral immune evasion [113].

### *1.17.2 HCV interferes with natural killer (NK) cell activity*

NK cells are an essential part of the innate antiviral response. The cells were named "natural killers" because of the initial notion that they do not require activation to kill cells that are missing "self" markers of major histocompatibility complext class I (MHC-I). NK cells kill virus-infected cells by releasing perforin and granzyme that cause the target cells to die by apoptosis [117].

NK cells are polarized toward cytotoxicity in chronic hepatitis C in an IFN-α-dependent manner [118]. In addition to releasing perforin and granzyme, NK cells can also express tumor necrosis factor-related apoptosis inducing ligand (TRAIL) to kill HCV-infected cells. As reported recently, IFN-α can induce the

expression of TRAIL on NK cells, which is associated with the control of HCV infection [119].

HCV infection may interfere with the cytotoxic activity of NK cells. NK cells recognize virus-infected cells that are missing MHC-I. However, the increased expression of MHC-I on the surface of HCV-infected hepatocytes suppresses the cytotoxic activity of NK cells, and might contribute to HCV persistence [120]. An inhibitory effect of the HCV envelope protein E2 on NK cell functions has also been observed [121].

Dendritic cells (DCs) are also an important component of the innate immune system. Their roles during HCV infection will be described later in Section 1.19.

## 1.18 Specific immune responses against HCV infection

During HCV infection, both humoral immune response (antibody-mediated) and cellular immune response (CD4 and CD8 T cell-mediated) specific for HCV are activated. The two types of responses interact with each other in response to infection. Their roles during HCV infection are different.

### *1.18.1 Specific humoral immune responses against HCV*

Chronic HCV infection is associated with B cell activation. However, chimpanzees that cleared the acute infection have strong CD4 and CD8 T-cell

responses but had poor antibody response. This suggested that T cell responses are better correlated with protection against HCV infection than antibodies [122]. The neutralizing effect of neutralizing antibodies (NtAbs) on different strains of HCV is variable. Cross-reactive NtAbs against HCV in chimpanzees vaccinated with genotype 1a E1/E2 envelope glycoproteins were detected against 1a, 4a, 5a, and 6a, but had limited reactivity against 2a and 3a [123]. Similarly, cross-genotype NtAbs are induced in chronic HCV infection (with a single genotype) and strongly cross-neutralized HCVpp from genotypes 1a, 4a, 5a, or 6a but showed only a limited neutralizing effect against genotypes 2a or 3a [124].

Even in the presence of NtAbs, chronic HCV infection in adults is characterized by high levels of viremia [125]. NtAbs of maternal origin did not prevent transmission of HCV or progression to chronicity in children [124]. These studies suggest that the appearance and persistence of NtAbs do not correlate with control of viremia or with the clearance of HCV. Anti-HCV NtAbs with a neutralizing capacity hardly induce protective immunity [90], probably because of the extreme genetic variability of HCV.

Extrahepatic manifestations of chronic HCV infection occur in as many as 74% of HCV-infected individuals and are thought to, in part, directly relate to B-cell activation. Consequences of B-cell activation include hypergammaglobulinemia, cryoglobulinemia, lymphoproliferative disorders, and autoantibodies [121].

A number of viruses, including Epstein-Barr virus (EBV), human T-cell lymphotropic virus 1 (HTLV1) and HIV, have been associated with lymphoma. HCV has recently been recognized as a potential cause of B-cell lymphoma [126]. The lymphoplasmacytoid lymphoma, which is an overt B-cell lymphoma, is most frequently associated with HCV. In a study from Italy where the prevalence of HCV is particularly high (up to 12.6% in parts of the south), up to 32% of cases with lymphoplasmacytoid lymphoma were associated with HCV [127]. Treatment of HCV with antiviral therapy could lead to regression of some lymphomas [126]. Of note, there are studies showing that B-cell lymphoma is not associated with HCV. For example, a study from France reported that the prevalence of HCV infection was low (1.83%) in patients with B-cell non-Hodgkin lymphoma, and thus HCV did not play a major role in the pathogenesis of B-cell lymphoma in this patient cohort in France [128]. The discrepancy between the studies might be due to geographical variations in viral genotype and population genetics [126].

Patients with vasculitis caused by cryoglobulinemia are often treated with the B-cell depleting anti-CD20 antibody rituximab. Rituximab depletes B cells in the circulation by antibody-dependent lysis, and reduces the cryoglobulins that cause vasculitis [129].

### *1.18.2 Specific cellular immune responses against HCV*

The consensus is that T cell responses determine the outcome of acute HCV infection [90, 130-134]. Strong T cell responses are essential for HCV clearance. Acute HCV-infected patients with strong HCV-specific T cell responses spontaneously clear HCV infection, while those with the impaired T cell responses develop chronic hepatitis C (CHC) [90, 130-134].

During acute HCV infection, the presence of a vigorous, multispecific and sustained HCV-specific CD4 and CD8 T cell response is associated with a self-limited course of infection. Both CD4 and CD8 T cells are present in the liver and blood during HCV infection. CD8 cytotoxic T cells serve an effector function by killing HCV-infected cells to control infection, whereas CD4 helper T cells provide a helper function to maintain the CD8 T cell response [133]. Resolution of acute HCV infection is probably T-cell–mediated because it coincides temporally with expansion of HCV-specific CD8 T cells [122] and their acquisition of an activated phenotype (CD38+ and HLA class II+) [135].

Curiously, acute HCV infection can sometimes be resolved without an increase in the ALT level, as reported by a study on chimpanzees [130]. This could reflect non-cytolytic control of some infections by T-cell–derived cytokines such as IFN-gamma, which was detectable only in the livers of the chimpanzees that cleared or controlled the virus [130]. However it is also possible that relatively few hepatocytes are infected in these patients, which would limit CD8 T-cell–mediated liver damage.

The capacity of CD8 T cells alone to terminate HCV infection is limited and CD4 T cells are essential for immune protection to HCV. In a study in chimpanzees, high frequencies of HCV-reactive T cells targeting all HCV proteins (core-NS5) were detectable in the blood 6 months after spontaneous resolution of acute HCV infection. The chimpanzees did not have detectable HCV envelope glycoprotein-specific antibody responses [136]. This indicated that resolution of acute infection correlate specifically with strong and sustained T cell responses. Grakoui *et al.* temporarily depleted CD4 T cells with a monoclonal antibody immediately before rechallenging the chimpanzees with HCV. The monoclonal antibody was administered intravenously before reinfection with the same HCV dose and strain as used in the original infection which had been cleared. A 99% reduction in the absolute number of circulating CD4 T cells was observed in chimpanzees treated with the monoclonal antibody against CD4 T cells, and intrahepatic CD4 T cells were also efficiently depleted. Reinfection of the chimpanzees after CD4 T-cell depletion resulted in persistence of HCV RNA for more than 300 days. The magnitude and breadth of HCV-specific CD8 T cell responses in blood were also substantially reduced after the CD4 T cell depletion. This result suggested that immune control in the absence of CD4 T cells was inadequate for the resolution of HCV infection. Without sustained CD4 T cell help, CD8 T cells may not be able to terminate HCV infection [136].

The failure of the CD4 T-cell response to HCV is a defining feature of CHC [90, 130, 137, 138]. Antigen-driven proliferation of CD4 T cells is observed in patients who cleared HCV infection, but is inconsistently detected in patients who developed CHC. CHC patients fall into two groups depending on HCV replication patterns during the first few months of infection. Patients in the first group are unable to mount an HCV-specific CD4 T-cell response and these patients develop chronic HCV infection. The second group of patients have strong HCV-specific CD4 T-cell activity and clear HCV RNA transiently from their serum, however, the CD4 T-cell activity weakens just before a rebound in viremia that results in chronic infection [90, 139].

## 1.19 Role of DCs during HCV infection

### *1.19.1 Function of DCs*

DCs are essential for the development and regulation of specific immune responses. DCs initiate specific immunity by serving as professional antigen presenting cells (APCs) that take up antigens in their local microenvironment, which are then processed and presented to naïve T cells. After the initiation of specific immune responses, DCs can also modulate T-cell responses that they generate. Compared to other professional APCs (macrophages and B cells) which can only activate memory T cells, DCs can activate both naïve and memory T cells. DCs are the most potent of all the APCs. Being the link between the innate and specific immunity, DCs play key roles in immunity against infection, tumor immunity and modulating immune homeostasis [140, 141].

There are two distinct subpopulations of DCs: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). The primary function of mDCs is the uptake, processing and presentation of antigens, while pDCs are the main producers of type I interferons [142]. The mDCs are referred to as conventional DCs due to their important role in antigen presentation (Figure 1-2).

It has been well accepted that DCs have immunogenic function. DCs express major histocompatibility complex (MHC, signal 1) and co-stimulatory molecules (signal 2, e.g. CD80, CD86) that facilitate DCs to present antigens to T cells. Besides the expression of these molecules, DCs can produce inflammatory cytokines (signal 3, e.g., IFN-$\alpha/\beta$ and IL-12) to further help T cell activation and proliferation [142].

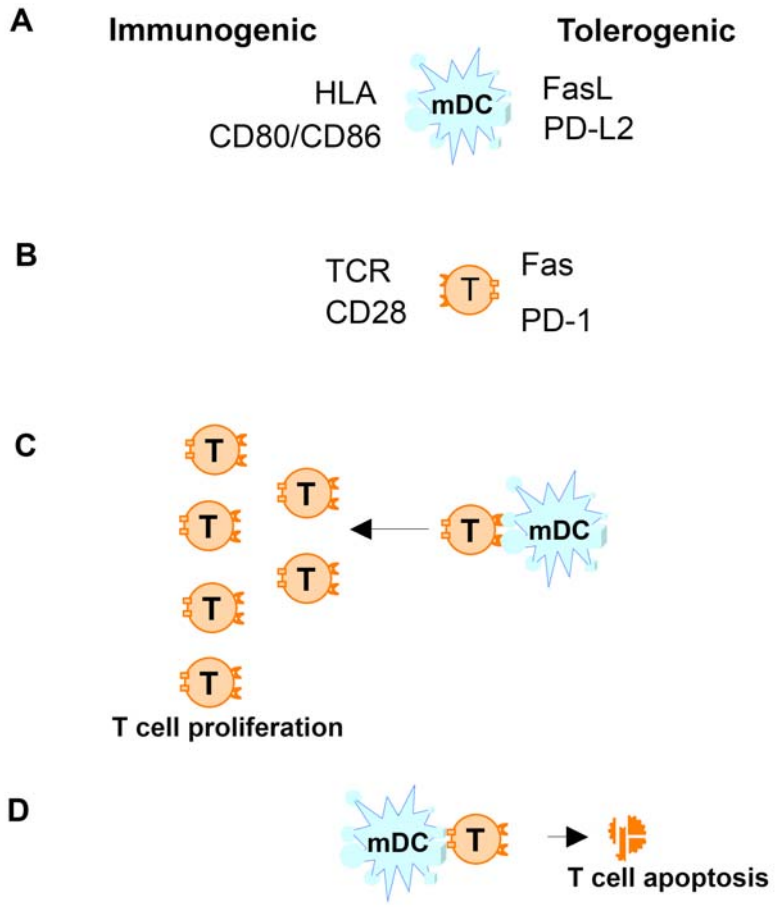


**Fig. 1-2. Current model of mDC function.**
mDCs can be immunogenic by expressing activating molecules, HLA and CD80/CD86 (**A**), which interact with TCR and CD28 expressed on T cells (**B**) and lead to T-cell proliferation (**C**).
mDCs may also be tolerogenic by expressing inhibitory molecules, Fas ligand (FasL) and the ligand 2 of programmed death-1 (PD-L2) (**A**), which interact with Fas and programmed death-1 (PD-1) expressed on T cells (**B**) and lead to T-cell apoptosis (**D**).

Recently, the tolerogenic function of DCs has been reported and its clinical implications have been indicated for cancer [143], microbial infections [144] and autoimmune diseases [145]. Mice with DC deficiency develop fatal autoimmune diseases [146]. DCs can express inhibitory molecules on cell surface and these molecules can lead to T-cell apoptosis. The ability of DCs to directly mediate cellular cytotoxicity was first reported by a study published in 1996 showing that, in mice, DCs induced the apoptotic death of CD4 T cells via Fas ligand (FasL)/Fas -dependent mechanism [147]. DCs can limit T-cell response by limiting their production of interleukin (IL)-2, a cytokine essential for T-cell survival and function [148]. It was suggested that the induction of T-cell apoptosis by DCs may represent a mechanism for maintaining peripheral T-cell tolerance [146, 147]. Soon after that, the presence of killer DCs was reported in association with tumors [143], microbial infections [144], and autoimmune disease [145]. DCs can produce regulatory/suppressive cytokines (e.g. transforming growth factor-beta and IL-10) to induce T-cell hyporesponsiveness and induce regulatory T cells (Treg) to dampen T-cell immune responses [145]. Thus the development of autologous DCs with tolerogenic function is one of the therapeutic strategies for autoimmune diseases, e.g., rheumatoid arthritis [145].

During virus infections, the DC-induced immune tolerance could be classified into three mechanisms.

(1) DCs express or release inhibitory molecules, e.g. TNF-related apoptosis inducing ligand (TRAIL) and FasL, which take effect through receptor-ligand

interactions. The increased expression of membrane-bound TRAIL on DCs was reported during human immunodeficiency virus (HIV) infection [149], and the increased secretion of soluble TRAIL by DCs was reported during measles virus infection [150]. The direct killing activity is also referred to as cytotoxicity of killer DCs. In Chapter 3 of my thesis, I studied whether or not the direct killing activity (cytotoxic activity) of mDCs is altered during HCV infection.

(2) DCs produce immunosuppressive cytokines and other inflammatory mediators to dampen T-cell response. For example, during respiratory syncytial virus infection, DCs are induced to up-regulate their production of IL-10, IL-11 and prostaglandin E2 [151]. These mediators are immunosuppressive and are believed to be responsible to the delayed protective immune response against respiratory syncytial virus. IL-10 is also produced by DCs during many other chronic virus infections, such as HBV [152], HCV [153] and HIV [154, 155]. The increased expression of IL-10 and transforming growth factor-beta by immune cells was found in Epstein-Barr virus infection [156].

(3) DCs induce an increase in population of Treg, which are capable of suppressing immune response directly and indirectly. For example, DCs induce the proliferation of Treg during chronic HCV infection [157]. Treg function by producing IL-35, a cytokine inhibiting T-cell proliferation [158]. Granzyme B is another key component of cell contact-mediated suppression by Treg [159]. Treg can also indirectly suppress effector T-cell response by consuming local IL-2, which is critical for the survival of actively dividing effector T cells [160].

In conclusion, DCs can have immunosuppressive effect and induce immune tolerance by multiple mechanisms, and this strategy may be used by viruses to evade host immune response and thus facilitates virus persistence.

### *1.19.2 DC deficiency*

With the crucial role of DCs to prime naïve T cells and subsequently shape T cell responses by their recognition, processing and presentation antigens, the deficiency of DCs may lead to severe immune-related diseases. Indeed, mice with DC deficiency have fatal diseases [146].

Cell ablation has been instrumental in determining the role of DCs in mice. Diphtheria toxin (DTx) was specifically expressed in the DCs of the transgenic mice. DTx can terminate the protein synthesis in DCs. The DCs of the transgenic mice specifically undergo apoptotic death and are efficiently ablated. A major fraction of the transgenic mice with DC deficiency die by the age of 2 months. Although the exact reason for the death of the mice remains unclear, researchers found that the mice had auto-antibodies with tissue-reactivity, hyper-immunoglobulinemia and generalized lymphoproliferative syndrome. Researchers concluded that these mice might have developed fatal autoimmunity [146]. DCs play a critical role in the protection against many pathogens, including influenza [161] and herpes simplex viruses (HSV) [162, 163]. Furthermore, the mice with DC deficiency exhibit a significant reduction in Treg cells in their blood circulation, which suggests a link between the number of the DC and peripheral Treg cell compartment [163].

DC deficiency in humans has been identified recently. Among the three genetically defined syndromes of DC deficiency in humans, mutation of the GATA-binding factor 2 (GATA2) has been identified as the cause of the largest group of cases that are called DC, monocyte, B and NK lymphoid (DCML) deficiency. GATA2 encodes a transcription factor involved in the homeostasis of haematopoietic stem cells and it has recently been identified as a new hereditary leukemia gene. Patients with DCML deficiency have very low numbers of circulating monocytes and no detectable mDCs or pDCs in their peripheral blood. Patients with DC deficiency are susceptible to infection. For example, human papilloma virus (HPV) infection is a prominent feature of DCML deficiency [164, 165]. DCML deficiency is also associated with myeloproliferation and loss of Treg cells. Besides DCML deficiency, interferon regulatory factor 8 (IRF8) mutation and leukopenic states that affect DCs also lead to DC deficiency in humans, and these patients demonstrate similar syndromes [164].

DCs in the mice with the DC deficiency are ablated specifically, while the humans with the DC deficiency have deficiencies in other immune cells, (e.g., DCML is deficient in monocytes, B and NK cells). Nevertheless, DC deficient individuals (both mice and humans) demonstrate three major characteristics: (1) susceptibility to microbial infections, (2) autoimmune diseases, and (3) loss of Treg cells. The first two characteristics can be related to the immunogenic and

tolerogenic function of DCs. However, the mechanism underlying the association of DC deficiency with the loss of Treg cells is unclear.

### *1.19.3 Activity of DCs during HCV infection*

The role of DCs during HCV infection is not fully understood. Whether the mDC function is altered or not during CHC remains controversial [166-174]. In human blood, about 0.6% of the peripheral blood mononuclear cells (PBMCs) are mDCs [175]. Due to the low numbers of circulating mDCs in peripheral blood, several studies have used interleukin-4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) to obtain *in vitro* generated monocyte-derived myeloid DCs (moDCs) [166, 173, 174], which demonstrate similar phenotype and functions as mDCs *ex vivo* isolated from peripheral blood. Previous studies have examined the functions of moDCs, including mixed lymphocyte reaction (MLR), expression of co-stimulatory molecules and cytokine secretion. These studies have yielded conflicting results. Some studies report that the function of mDCs in CHC patients [172-174] and chimpanzees [171] is comparable to the function of mDCs in healthy controls, while other studies indicate that mDC function is significantly inhibited in CHC patients [166-170].

MLR is *an in vitro* assay that directly determines the overall ability of DCs to induce T cell proliferation. It reflects the strength of all the signals T-cell require to activate, including the expression of HLA (signal 1, which can be detected by flow cytometry), the expression of co-stimulatory molecules (signal 2,

which can be detected by flow cytometry) and cytokine secretion (signal 3, which can be detected by ELISA) by DCs. Analyzing the methods to generate results of MLR in different studies may help to understand the discrepant results.

Averill L. *et al.* demonstrated that mDCs from CHC patients showed a diminished capacity to induce a MLR in response to polyinosinic: polycytidylic acid (poly I:C, which is structurally similar to double-stranded RNA) stimulation [170]. The mDCs from CHC patients or mDCs from healthy donors were cocultured with allogeneic healthy $CD3^+$ T cells from a healthy subject (third party). On day 6, the cells were pulsed with $^3$H-thymidine and 16 hours later, the thymidine incorporation (proliferation) was quantified. Averill L. *et al.* found that mDCs from CHC patients demonstrated a lower allostimulatory capacity to induce T cell proliferation compared to mDCs from healthy donors [170]. Of note, in the studies demonstrating mDC function is inhibited in CHC patients [166-170], the mechanism of the inhibition of mDC function in CHC patients has yet to be determined. NF-κB activity is crucial for effective antigen presentation by DCs [176], however, its activity in DCs during HCV infection has not been reported.

However, contrary to the studies demonstrating mDC function is inhibited in CHC patients [166-170], Barnes E. *et al*. found that mDCs retain their functional capacity in patients following infection with HCV [174]. Poly I:C was used to stimulate the maturation of mDCs, and allogeneic PBMCs from a third party were used as responder cells. After 5 days of coculture, the proliferative response of

the responder cells was evaluated by the addition of $^{3}$H-thymidine for 18 hours. Barnes E. *et al.* demonstrated that there is no significant difference in the stimulatory capacity of mDCs from HCV-infected patients and healthy controls [174].

The first obvious difference between the studies from Averill L. *et al*. [170] and Barnes E. *et al*. [174] is the responder cell (or, target T cell) used in MLR. Averill L. *et al.* [170] worked on T cells isolated using a $CD3^{+}$ T cell isolation kit and declared that the kit yielded > 98% purity, while Barnes E. *et al.* [174] used PBMCs as responder cells. PBMCs are blood cells having a round nucleus, including lymphocytes (T cells, B cells and NK cells), monocytes, basophils and DCs. Since the components of PBMCs are not pure T cells, using PBMCs as target T cells may not have been the best choice for these studies.

The second difference lies in the culture condition of mDCs. Averill L. *et al.* [170] used RPMI-1640 media supplemented with 5% heat-inactivated Human AB serum, while Barnes E. *et al*. [174] used RPMI-1640 media supplemented with 10% fetal calf serum (FCS). Although serum is required for mDC culture, heterologous serum has immunosuppressive activity on mDC function [177] and may affect results of the experiment. In mice with lymphocytic choriomeningitis virus (LCMV) infection and LCMV-induced autoimmunity, fetal bovine serum (FBS) or normal mouse serum were used to culture DCs, respectively, and their effects on DC function were compared. FBS-exposed DCs demonstrated a

reduced capacity to induce virus-specific T cells and an enhanced production of IL-4, IL-5 and IL-10 (which are all T helper 2 cytokines that favor the production of antibodies but do not stimulate T-cell immunity against infections), and delayed viral clearance. This study showed the importance of using autologous serum for DC culture to accurately determine the function of DCs. FBS is not appropriate for culture of heterologous DCs [177]. Human AB serum used by Averill L. *et al.* [170] or FCS used by Barnes E. *et al*. [174] are both heterologous serum. It is unknown whether or not these heterologous sera (either from same species or different species) have comparable immunosuppressive effect on DCs, and whether or not CHC patient DCs and healthy donor DCs demonstrate similar sensitivity to the immunosuppressive effect of heterologous serum. Because of these unknown factors that may affect DC function, the result obtained from these studies using heterologous sera may not be as reliable as studies using autologous sera. Autologous sera are expected to be used for DC culture in my studies to avoid possible interference that could arise from the use of heterologous sera.

In brief, the conflicting results in the research area of mDC function during HCV infection could possibly be due to these differences in the design of the studies. Further study with novel experiment design is expected to address the conflicts in this area. As a result of the conflicting results reported for the function of mDCs from CHC patients compared to mDC function from healthy control patients, I have focused my research on studying mDC from CHC patients and healthy uninfected individuals.

## 1.20 Hypotheses

Myeloid DCs, the most potent APCs, have impaired function during chronic HCV infection, which leads to the impaired T cell responses against HCV in CHC patients.

NF-κB activity is crucial for effective antigen presentation by DCs [176]. If mDC function is changed during chronic HCV infection, then NF-κB activity in mDCs should be determined.

## 1.21 Study objective

The aim of this study was to determine whether or not mDC function is impaired during chronic HCV infection. Cell samples were obtained from chronic hepatitis C patients and healthy volunteers. Assays were performed to detect the expression of activation molecules on mDCs and the ability of mDCs to stimulate T cell proliferation. The expression of inhibitory molecules on mDCs was also determined and the cytotoxic activity of mDCs on T cells was examined. The result of this study may assist us to further understand how immune cells are altered by chronic HCV infection.

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# CHAPTER 2

# Functional Changes, Increased Apoptosis and Diminished NF-kappaB Activity of Myeloid Dendritic Cells during Chronic Hepatitis C Infection

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## 2.1 Introduction

Efficient activation of an immune response depends on the interaction of APCs and T cells. DCs are the most potent professional APCs [1].

In 1973, DCs were identified as a novel cell type by Ralph Steinman and Zanvil Cohn at Rockefeller University. By observing cells from mouse spleen that adhere to glass and plastic surfaces, they found that there was a cell population with distinct microscopic properties from mononuclear phagocytes, granulocytes and lymphocytes. The cell nucleus was large, refractile, contorted in shape, and contains small nucleoli. The abundant cytoplasm was arranged in varying length and width, and contained many large spherical mitochondria. These cells comprised about 1.0-1.5% of the total nucleated cell population. The cell population was subsequently named DCs [2]. For the discovery of the DCs and their key role in controlling specific immunity, Ralph Steinman was awarded the 2011 Nobel Prize in Medicine [3].

DCs play a central role in regulating immune responses. This is because of their optimal positioning as sentinels in the periphery, their rapid migration to the draining lymph nodes, their ability to acquire and present antigen in HLA molecules, and their high expression of the costimulatory molecules CD80 and CD86 [1, 4, 5]. In the draining lymph nodes, antigen-bearing DCs interact with CD4 T cells. The T cell receptor (TCR) is triggered by HLA class II and antigen peptide (“signal 1”) and CD28 is engaged by CD80/CD86 (“signal 2”) expressed

on DCs. These interactions result in clonal expansion of antigen-specific T cells [6].

HCV is the major cause of chronic hepatitis with approximately 170 million infected individuals worldwide. An important characteristic of HCV infection is that 70% to 80% of HCV-infected patients develop chronic hepatitis C (CHC) [7-10]. T cell responses determine the outcome of acute HCV infection, and strong T cell responses are essential for HCV clearance. Acute HCV-infected patients with strong HCV-specific T cell responses spontaneously clear HCV infection, while those with the impaired T cell responses develop CHC [11-16].

The major function of mDCs is the uptake, processing and presentation of antigens [17]. As described in Chapter 1 (page 40-44), the role of mDCs during HCV infection is not fully understood. Whether mDC function is altered or not during CHC remains controversial [18-26]. Previous studies have examined the functions of mDCs. Some studies report that the function of mDCs in CHC patients [24-26] and HCV-infected chimpanzees [23] is comparable to the function of mDCs from healthy human or chimpanzee controls, while other studies indicate that mDC function is significantly inhibited in CHC patients [18-22]. However, in the studies that report inhibition of the function of mDCs in CHC patients, the mechanism of the inhibition has not been determined.

In this study, the function and apoptosis of *in vitro* generated moDCs from CHC patients and healthy donors were examined. To study the function of moDCs, three assays were performed. MLR was used to indicate overall moDC ability to stimulate T cell proliferation, followed by two detailed functional assays that examined endocytosis and expression of stimulatory molecules. To study the apoptosis of moDCs, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) was used. These assays found differences in the function and apoptosis of moDCs between CHC patients and healthy donor groups. This led to studies of the possible mechanisms for these differences. The activity of NF-κB, a transcription factor that regulates DC function and prevents apoptosis, was examined in moDCs. Furthermore, mDCs were *ex vivo* isolated from peripheral blood of CHC patients and healthy donor groups. The function, apoptosis and NF-κB activity assays were performed on *ex vivo* isolated mDCs to determine if the *ex vivo* isolated mDCs from patients demonstrate similar characteristics to the *in vitro* generated moDCs from patients.

## 2.2 Materials and Methods

### *2.2.1. Study patients*

CHC patients with detectable HCV antibodies and who had been HCV-RNA positive for more than 3 years were included in this study. These patients were either treatment-naïve or had failed therapy and had not been on treatment for at least 6 months prior to blood samples being collected. Control samples were collected from healthy, HCV-negative individuals (age- and gender-matched).

Ethics approval for human blood collection was obtained from the University of Alberta, Faculty of Medicine and Dentistry Research Ethics Board, and informed consent was obtained from all donors. Patient characteristics are shown in Table 2-1.

**Table 2-1. Clinical characteristics of CHC patients**

| Characteristics | CHC patients |
|---|---|
| Age (year); mean (range) | 53.32 (29-64) |
| Sex (M/F) | 21/3 |
| ALT (IU/L); mean (range) | 70.32 (13-384) |
| HCV viral load (IU/Ml); mean (range) | $3.29 \times 10^6$ ($8.5 \times 10^5$ -$1.5 \times 10^7$) |
| HCV RNA genotype | genotype1/genotype 2/genotype3 =18/3/3 |

### *2.2.2 Culture and identification of in vitro generated moDCs*

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and plated to a density of $3.0\times10^7$/well in 6-well plates. The cells were incubated for 2 hours in RPMI-1640 medium, and the non-adherent cells were discarded. The adherent cells were cultured in RPMI-1640 containing 1% autologous human serum, 50 ng/mL human GM-CSF (PeproTech Inc., Rocky Hill, New Jersey) and 10 ng/mL human IL-4 (PeproTech Inc.). Cells harvested on the $5^{th}$ day of culture were identified as immature moDCs and were used for endocytosis assay. On the 6th day of culture, 0.1 μg/mL of lipopolysaccharide (LPS, E.coli, Sigma-Aldrich, St. Louis, MO) was added to stimulate the moDCs. After 6 hours, cells were harvested [27, 28] and >99% were identified as mature moDCs based on cell surface expression of HLA-DR and CD11c. All assays in this study except the endocytosis assay used mature moDCs.

### *2.2.3 Ex vivo isolation of mDCs from peripheral blood*

After PBMCs were obtained from peripheral blood as described above, mDCs were isolated from PBMCs with >90% purity using blood dendritic cell antigen-1 (BDCA-1) positive isolation kit (Miltenyi Biotech, Auburn, CA) by magnetic-activated cell sorting (MACS) according to the manufacturer's instructions as described below. BDCA-1 is a specific marker for mDCs. The isolation procedures of mDCs include (1) removal of cell clumps, (2) Fc receptor

(FcR) blocking, (3) depletion of $CD19^+$ B cells and labeling of PBMCs with BDCA-1–biotin antibody, (4) incubation of the PBMCs with anti-biotin microbeads which can bind to BDCA-1–biotin antibody, and (5) positive selection of $BCDA\text{-}1^+$ mDCs.

The detailed isolation procedures include: (1) Buffer (pH 7.2) for MACS was prepared by adding 0.5% bovine serum albumin (BSA) and 2 mM EDTA into phosphate buffered saline (PBS). PBMCs were suspended in MACS buffer and were then passed through 30 μm nylon mesh using pre-separation filters (Miltenyi Biotech) to remove cell clumps that may clog the column. (2) PBMCs ($10^8$ cells) were resuspended in 200 μL of buffer. FcR blocking reagent (Miltenyi Biotech, Auburn, CA) was supplied in buffer containing stabilizer and 0.05% sodium azide. It was used to block the binding of MACS MicroBeads or antibodies to the FcR of human FcR-expressing cells. FcR blocking reagent was added (100 μL per $10^8$ cells) to block nonspecific antibody binding. (3) CD19 microbeads (provided in BDCA-1 positive selection kit, 100 μL per $10^8$ cells) and BDCA-1–biotin antibody (provided in BDCA-1 positive selection kit, 100 μL per $10^8$ cells) were added. Cells were mixed well and incubated for 15 minutes at 4°C. Cells were washed by adding 20× labeling volume (approximately 40 mL) of buffer and were centrifuged at 300× g for 10 minutes at 4°C. Supernatant was pipetted off completely. After cells were resuspended in 500 μL of buffer per $10^8$ cells, $CD19^+$ B cells were depleted by negative selection. Firstly, columns (Miltenyi Biotech) were placed in the magnetic field of a MACS separator

(Miltenyi Biotech) and prepared by rinsing with 2 mL buffer. Secondly, cell suspension was applied onto the columns. Unlabeled cells that passed through were collected and the columns were washed with 1 mL buffer twice. Finally, the flow-through of cells was collected as the B cell-depleted fraction. The remaining PBMCs had also been labeled with BDCA-1, which is a specific marker for mDCs. (4) Cells were centrifuged at 300× g for 10 minutes and supernatants were pipetted off completely. Cells were resuspended in 400 μL of buffer per $10^8$ cells. Anti-biotin microbeads (provided in BDCA-1 positive selection kit, 100 μL per $10^8$ cells) were added. Cells were mixed well and incubated for 15 minutes at 4°C. Cells were washed by adding 20× volume of labeling buffer and centrifuged for 10 minutes. Supernatant was pipetted off completely and cells were resuspended in buffer (500 μL per $10^8$ cells). (5) Positive selection of BDCA-$1^+$ mDCs was performed. Firstly, columns were placed in the magnetic field of a MACS separator and prepared by rinsing with 2mL buffer. Secondly, the cell suspension was applied onto the columns. After unlabeled cells passed through, columns were washed with 500 μL of buffer three times. Columns were removed from the separator and placed on a suitable collection tube. After 1 mL of buffer was pipetted onto the column, the fraction with magnetically labeled BDCA-$1^+$ mDCs was immediately flushed out by firmly applying the plunger supplied with the column. Finally, the labeled BDCA-$1^+$ cells were collected as mDCs. The mDCs were cultured in RPMI-1640 containing 1% autologous human serum. On the 3rd day of culture,

0.1 μg/mL of LPS was added to stimulate the *ex vivo* isolated mDCs. 24 hours later, the cells were harvested as mature mDCs [22].

### *2.2.4 Allogeneic MLR of mature moDCs*

#### *2.2.4.1. MLR quantification by micro-beta detector*

The stimulatory capacity of irradiated mature moDCs (3000 rad; stimulator cells) was tested in MLR with healthy, allogeneic CD4 T cells ($2\times10^5$ cells in 100 μL of AIM-V medium/well, responder cells) in 96-well plates.

Third-party CD4 T cells ($2\times10^7$ cells) were isolated from PBMCs of a healthy donor using CD4 positive MACS (Miltenyi Biotech). (Step 1) Before MACS, PBMCs were incubated with FcR blocking reagent to block the binding of Microbeads to the Fc receptor of human Fc receptor-expressing cells. PBMCs ($1\times10^7$ cells) were centrifuged at 300×g for 10 minutes and supernatant was aspirated completely. Cell pellet was resuspended in 60 μl of MACS buffer (containing PBS, pH 7.2, 0.5% BSA, and 2 mM EDTA). FcR blocking reagent (20 μl) was added to cell suspension. (Step 2) Cells were mixed well with PE-conjugated anti-human CD4-antibody (BD Biosciences, 20 μl antibody/$10^6$ cells) and were incubated for 5 minutes in the dark in the refrigerator (2-8°C). Cells were washed by adding 2 mL of MACS buffer per $10^7$ cells and centrifuged at 300×g for 10 minutes. After supernatant was aspirated completely, cells (up to $10^8$) were resuspended in MACS buffer (2 mL). Cells were mixed well and were incubated with anti-PE magnetic microbeads for 15 minutes in the

refrigerator (2-8°C). Cells were washed by adding 2 mL of MACS buffer per $10^7$ cells and were centrifuged at 300×g for 10 minutes. After supernatant was aspirated completely, cells (up to $10^8$) were resuspended in MACS buffer (2 mL). (Step 3) To do magnetic separation, cells were passed through MACS pre-separation filters (Miltenyi) to get rid of cell clumps that might clog the magnetic columns. (Step 4) Magnetic columns were placed in the magnetic field of a suitable MACS separator (Miltenyi). Columns were prepared by rinsing with 500 μl of MACS buffer. Cell suspension was applied onto the column. (Step 5) Cells passing through the column were unlabeled and thus were collected as CD4-negative cells. The columns were washed three times by adding 3 ml of buffer three times, each time when the column reservoir was empty. (Step 6) Columns were removed from the separator and placed on a suitable collection tube away from the magnetic field. MACS buffer (5 ml) was added onto the column. The plunger (supplied with the column) was firmly applied to immediately flush out fraction with the magnetically labeled cells (CD4-positive cells). To increase the purity of the magnetically labeled fraction, cells were passed over a second, freshly prepared column (repeating Step 4-6). After being washed twice with 10 ml of PBS, the CD4-positive cells isolated on magnetic columns were suspended in AIM-V medium (Invitrogen, Carlsbad, CA). Primary CD4 T cells were isolated from PBMCs with >95% purity by MACS.

Third-party CD4 T cells ($2\times10^5$ cells/well) isolated from a healthy donor using MACS (Miltenyi Biotech) were suspended in 100 μL of AIM-V medium. moDCs from the same donors (either CHC patients or healthy donors) were added in triplicates at $1\times10^4$/well, $2\times10^3$/well, $1\times10^3$/well, $2\times10^2$/well and $1\times10^2$/well, respectively. Five days post-seeding, 0.5 μCi/mL of $^3$H-thymidine (Amersham Biosciences, Chalfont St. Giles, United Kingdom) was added to the culture medium. After 18 hours, the $^3$H-thymidine uptake was measured with a micro-beta detector (BMG FLUOstar OPTIMA, Waltham, MA) [28, 29].

#### *2.2.4.2. MLR observation using confocal microscopy*

Mature moDCs ($1\times10^5$ cells/well) and allogeneic CD4 T cells ($1\times10^5$ cells/well) were plated on coverslips. Five days post-seeding, cells were fixed with 4% paraformaldehyde, washed three times with PBS, stained with mouse anti-human CD11c (Becton Dickinson Biosciences), washed, and stained with Alexa 568 conjugated goat anti-mouse IgG (Invitrogen Molecular Probes, Carlsbad, CA). After permeabilization in 0.1% Triton X-100 PBS solution for 10 minutes, cells were mounted onto microscopic slides using fluorescence mounting medium (DakoCytomation) containing 1 μg/mL of 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich). Cells were observed using confocal microscopy (LSM510, Carl Zeiss, Jena, Germany). DAPI (blue) identified nucleus (both T cells and DCs) and the cells stained with anti-human CD11c (red) were DCs as seen in the confocal microscopy images.

#### *2.2.4.3. MLR quantification by flow cytometry*

Mature moDCs were incubated with tuberculin-purified protein derivative (PPD) for 3 hours [30]. Excess peptide was removed and moDCs were irradiated (3000 rad). Healthy allogeneic CD4 T cells ($10^7$ cells/ml) were labeled with 10 μM CFSE (Molecular Probes) for 10 min at room temperature. After washing twice with 40 mL PBS to get rid of the remaining CFSE in solution, the CFSE-labeled T cells ($1\times10^6$ cells/well) were cultured with moDCs ($1\times10^6$ cells/well) in AIM-V medium in 6-well plates for 5 days. Samples were washed twice with 4 mL PBS and fixed with 300 μL of 4% paraformaldehyde. Samples were analyzed by flow cytometry (FACSCalibur, Becton Dickinson Biosciences) using CellQuest software [31].

### *2.2.5 Endocytosis function of immature moDCs by confocal microscopy and flow cytometry quantification*

For the confocal microscopy of endocytosis assay, immature moDCs were plated on coverslips in 6-well plates ($10^6$ cells/well) and harvested on the $5^{th}$ day of culture. FITC-conjugated ovalbumin (Molecular Probes, Eugene, OR) was added to the culture medium to a final concentration of 2.5 μg/mL. After 60 minutes, the cells were washed twice with 4 mL PBS and fixed with 300 μL of 4% paraformaldehyde. To facilitate DAPI staining, permeabilization buffer was prepared by adding 0.1% Triton X-100 into PBS. Cells were permeabilized with permeabilization buffer for 10 minutes and washed with PBS. Samples

were put on mounting medium containing DAPI (1 μg/mL) and observed using confocal microscopy as previously described.

For endocytosis quantification, immature moDCs were incubated with FITC-conjugated ovalbumin and were examined by flow cytometry. On the 5$^{th}$ day of moDC culture, cells were incubated with FITC-conjugated ovalbumin (2.5 μg/mL) for 60 minutes. Samples were washed twice with 4 mL PBS and fixed with 300 μL of 4% paraformaldehyde. Samples were analyzed by flow cytometry using CellQuest software.

**2.2.*6 Expression of HLA-DR, CD80, CD86 and CD40 on mature moDCs and mDCs isolated from peripheral blood***

The cells were incubated with Fc receptor FcR blocking reagent to block non-specific binding of antibodies. As recommended by the manufacturer, cells were centrifuged at 300× g at 4°C for 10 minutes. Supernatants were aspirated completely. Up to $10^7$ cells were resuspended in 90 μL of blocking buffer (PBS containing 0.5% bovine serum albumin and 2 mM EDTA, pH 7.2). FcR blocking reagent (10 μl per $1\times10^7$ cells) was added and incubated with cells for 10 minutes at 4°C.

After FcR blocking reagent was used as recommended, cell samples were divided into aliquots and each aliquot was incubated with peridinin-chlorophyll protein complex (PerCP)-conjugated mouse anti-HLA-DR antibody (1.25 μg/mL),

phycoerythrin (PE)-conjugated mouse anti-human CD80 antibody (0.32 μg/mL), PE-conjugated mouse anti-human CD86 antibody (0.25 μg/mL), or PE-conjugated mouse anti-human CD40 antibody (0.075 μg/mL) (Becton Dickinson Biosciences) for 30 minutes at 4°C. Cells were washed twice with 2% FCS in PBS and then fixed with 4% paraformaldehyde in PBS. Cells were analyzed by flow cytometry. CellQuest software was used to analyze the result.

### *2.2.7 Detection of cell apoptosis by TUNEL*

During apoptosis, genomic DNA is cleaved which yields double-stranded DNA fragments and single-stranded breaks in DNA. These DNA strand breaks can be observed by labeling their free 3'-OH termini with fluorescence-labeled nucleotides in an enzymatic reaction. To detect cell apoptosis, cells were stained with TUNEL as described in the *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany), which identify and quantify apoptotic cell death at single cell level. Cells ($2\times10^6$) were collected for each sample. Flow cytometry was used to quantify cell apoptosis, and confocal microscopy was used to assess cell morphology and observe TUNEL signal in nucleus.

#### 2.2.7.1 TUNEL staining observed with FACS

Mature DCs ($2\times10^6$) were fixed with 4% fresh paraformaldehyde in PBS (300 μl) for 30 minutes. Cells were washed with 2% FCS in PBS (4 mL). After permeabilization with permeabilization solution (0.1% sodium citrate and 0.1% Triton X-100, 100 μl) for 2 minutes on ice, cells were washed with 2% FCS in

PBS (4 mL). Cells were incubated with 45 μl of label solution and 5 μl of enzyme solution for 60 minutes at 37°C in a humidified atmosphere in the dark. Cells were washed with 2% FCS in PBS (4 mL). Cells were analyzed with FACSCalibur (BD Biosciences) flow cytometer. CellQuest software (BD Biosciences) was used to analyze the result.

**2.2.7.2 TUNEL staining observed with confocal microscopy**

Cells ($2\times10^6$) cultured on coverslips were labeled as described above, and were mounted onto microscopic slides using mounting medium containing DAPI to indicate the nuclei of fixed cells. In brief, mature moDC ($2\times10^6$) on slides were fixed with 4% fresh paraformaldehyde in PBS. Cells were permeabilized in permeabilization solution (0.1% sodium citrate and 0.1% Triton X-100, 100 μl) for 10 minutes. After washing with 2% FCS in PBS, cells were incubated with 45 μl of TUNEL label and 5 μl of TUNEL enzyme for 60min at 37°C. After cells were washed with 2% FCS in PBS, cells were incubated with primary antibody, mouse anti-human CD86 antibody (BD Biosciences), for 30 minutes at room temperature. After cells were washed three times with 2% FCS in PBS, cells were incubate with secondary antibody, 1:250 diluted Alexa 588 goat anti-mouse IgG (Molecular probes, Invitrogen), for 30 minutes at room temperature. Cells were washed 3 times with 2% FCS in PBS. Coverslips were put on mounting medium containing 1 μg/ml of DAPI and observed with confocal microscope as described previously.

### *2.2.8 Detection of NF-κB activity*

Since the active form of NF-κB is in the cell nucleus, confocal microscopy was used to observe the presence of active NF-κB in cell nucleus. In addition, nuclear and cytoplasmic extracts were also prepared as described in the TransAM$^{TM}$ NF-κB family kit (Active Motif, Carlsbad, CA) for EMSA and Western assays. In brief, $2\times10^6$ cells were lysed with 1 mL of hypotonic buffer, centrifuged and the supernatant was saved as a cytoplasmic extract. Each pellet was resuspended in 50 μL of complete lysis buffer, centrifuged and the supernatants were collected as nuclear extracts. Protein concentrations of the extracts were determined as described in the protein assay kit (Bio-Rad Laboratories, Hercules, CA).

#### 2.2.8.1 Preparation of cytoplasm and nuclear extract

**N**uclear extract were prepared as described in TransAM$^{TM}$ Flexi family kit (Active Motif, Carlsbad, USA).

Firstly, mature moDCs ($10^7$ cells/sample) were harvested. Phosphatase inhibitor buffer (PIB) which contains 125 mM NaF, 250 mM β-glycerophosphate, 250 mM para-nitrophenyl phosphate (PNPP) and 25 mM sodium metavanadate ($NaVO_3$) was prepared. Prior to use, PBS/PIB was prepared by adding 0.5 ml of PIB to 10 ml of PBS.

Secondly, moDCs were washed with 10 ml of ice-cold PBS/PIB. The cells were transferred into a pre-chilled tube and spun at 300×g for 5 minutes at 4ºC. The cell pellet was resuspended in ice-cold hypotonic buffer (20 mM Hepes, 5 mM NaF, 10 μM $Na_2MoO_4$, 0.1 mM EDTA) and cells were allowed to swell on ice for 15 minutes. Triton-X of (10% solution, 50 μl) was added and mixed. Cells were centrifuged for 30 seconds at 4ºC. The supernatant was saved as cytoplasm extract.

Thirdly, lysis buffer (20 mM Hepes, 400 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 10 μM $Na_2MoO_4$, 1 mM NaVO3, 20% glycerol, 10 mM PNPP, 10 mM beta-glycerophosphate, pH was adjusted to 7.5) was prepared. The complete lysis solution was prepared by adding 1 μl of dithiothreitol (DTT) and 10 μl of protease inhibitor cocktail (Sigma, which contains 104 mM AEBSF, 80 μM Aprotinin, 4 mM Bestatin, 1.4 Mm E-64, 2 mM Leupeptin, and 1.5 mM Pepstatin A) per ml of lysis buffer. The nuclear pellet was resuspended in 50 μl of complete lysis solution. The tube was rocked gently on ice for 30 minutes on a shaking platform. The tube was centrifuged for 10 minutes at 14,000×g for 10 minutes at 4ºC. The supernatant was aliquoted as nuclear extract and stored at -80ºC.

Finally, the protein concentrations of the cytoplasm and nuclear extracts were determined by using a *DC* Protein Assay kit (Bio-Rad) and bovine serum albumin standard (Bio-Rad). The reagent package kit included a REAGENT A (an

alkaline copper tartrate solution), REAGENT B (a dilute Folin Reagent) and REAGENT S. The assay was based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent (sodium 1,2-naphthoquinone-4-sulfonate). There were two steps that lead to color development: (1) The reaction between protein and copper in an alkaline medium, (2) the subsequent reduction of Folin reagent by the copper-treated protein. Color development was primarily due to the amino acids tyrosine and tryptophan. The reduction of Folin reagent leads to its loss of 1, 2 or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue color with maximum absorbance at 750 nm.

For each ml of reagent A, 20 μl of reagent S was added to prepare fresh working reagent A'. Protein standards containing bovine serum albumin at different concentrations were prepared (1.5 mg/ml, 0.75 mg/ml, 0.375 mg/ml, and 0.1875 mg/ml). Standards and samples (5 μl) were pipetted into a clean, dry, 96-well plate. Working reagent A' (25 μl) was added into each well. Reagent B (200 μl) was added into each well and mixed by gentle agitation for 5 seconds. After 15 minutes, absorbance was read at 750 nm.

***2.2.8.2. Confocal Microscopy of NF-κB subunit p65 activity*:**

Confocal microscopy was performed as described previously. Briefly, $2\times10^6$ cells were fixed on coverslips with fresh 4% paraformaldehyde, and permeabilized in 0.1% Triton X-100 in PBS. After being washed 3 times with

2% FCS in PBS, cells were incubated with 10 μL primary antibody, rabbit anti-human p65 antibody (0.2 μg/mL, Santa Cruz Biotec, Inc.), for 30 minutes. After being washed 3 times with 2% FCS in PBS, cells were stained with a 100 μL secondary antibody, Alexa 568-conjugated goat anti-rabbit IgG (1 μg/mL, Molecular Probes), for 30 minutes. The cells were mounted onto microscopic slides using mounting medium containing 1 μg/ml of DAPI (Sigma). Cells were observed with confocal microscope (LSM510).

***2.2.8.3 Electrophoretic mobility shift assay (EMSA)*:**

EMSA was used to determine the active form of NF-κB in cell nuclear by identifying the presence of DNA: protein complex (specific binding of NF-κB to its target DNA sequence) in binding reactions. In principle, biotin-labeled duplex DNA is incubated with a nuclear extract and electrophoresed on a gel. The DNA is then transferred to a nylon membrane, UV crosslinked, probed with streptavidin-horseradish peroxidase (HRP) conjugate with the substrate.

Firstly, NF-κB binding target oligonucleotide 5’ CTT GAC AGG GTA AGT AGG GAC GAA GTA GTT GAG GGG ACT TTC CCA GGC TGA 3’ was synthesized (Integrated DNA technologies, Coralville, IA). For competition assays, an unlabelled oligonucleotide containing mutations in the NF-κB binding site 5' CTT GAC AGG GTA AGT AGG GAC GAA GTA GTT GAT ATT ACT TTT ATA GGC TGA 3' was synthesized.

To produce double-stranded oligonucleotides, the sequences were mixed with complementary oligonucleotides at a 1:1 ratio, incubated at 95ºC for 5 minutes and cooled to room temperature for 1 hour. Annealing was confirmed with polyacrylamide gel (PAGE) and the double-stranded oligo DNA was stored in 4ºC.

Binding reactions were prepared as described in the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL). Nuclear extract was prepared as described previously (Section 2.2.10.1). Binding reaction reagent (20 μl) was prepared by adding 2 μg of nuclear extract (volume varied depending on protein concentrations), biotin-labeled oligonucleotide (10 fmol), a binding buffer containing 50 ng/μl poly (dI•dC), 10% glycerol (2 μL) and 0.05% tergitol-type nonyl phenoxypolyethoxylethanol (1μL, NP-40), with or without unlabelled wild type or mutant oligonucleotides (4 pmol). The binding reagents were incubated at room temperature for 20 minutes. Each sample (20 μl) was mixed with 5× loading buffer and the mixed sample was loaded onto a 6% PAGE. The samples were separated by electrophoresis and then electrophoretically transferred to a nylon membrane. Transferred DNA was cross-linked to membranes by using a UV-transilluminator (380 mA) for 30 minutes.

Biotin signal detection was performed as described in the Chemiluminescent Nucleic acid detection kit (Pierce, Rockford, IL). The kit used an enhanced luminal substrate for HRP with optimized blocking and wash steps that together

produce high sensitivity. The blocking buffer and the wash buffer (both provided in the kit) were gently warmed to 37ºC in water bath until all particulate is dissolved. Conjugate/blocking buffer solution was prepared by adding stabilized streptavidin-HRP conjugate (66.7 μl) to 20 mL blocking buffer (1:300 dilution). To block the membrane, 20 mL of blocking buffer was incubated with the membrane for 15 minutes with gentle shaking. The blocking buffer was decanted from the membrane and was replaced with the conjugate/blocking solution (50 μL of stabilized streptavidin-horseradish peroxidase conjugate to 16 mL blocking buffer). The membrane was incubated in the 16 mL conjugate/blocking buffer solution for 15 minutes with gentle shaking. The membrane was transferred to a new container and was rinsed briefly with 20 ml of wash solution. The membrane was washed four times for 5 minutes each in 20 ml of wash solution with gentle shaking. The membrane was transferred to a new container with 30 ml of substrate equilibration buffer (provided in the kit) and was incubated for 5 minutes with gentle shaking. Substrate working solution was prepared by adding 6 ml luminal/enhancer solution to 6 ml stable peroxide solution (provided in the kit). The membrane is removed from the substrate equilibration buffer and the edge of the membrane was carefully blotted on a paper towel to remove excess buffer. A clean sheet of plastic wrap was put on a flat surface, and a puddle of working solution (16 mL) was prepared on it. The membrane was placed DNA side down onto the puddle of the working solution so the DNA side was completely covered. The membrane was incubated for 5 minutes without shaking, removed from the working solution, and

the edge of the membrane was blotted on a paper towel for 5 seconds to remove excess buffer. The moist membrane was wrapped in a plastic wrap, avoiding bubbles and wrinkles. The membrane was exposed to X-ray film for 8-10 seconds.

***2.2.8.4 Western blotting of phosphorylated IκBα (pIκBα) in cytoplasm extract***

Cytoplasmic extract was prepared as described previously (Section 2.2.10.1). Thirty micrograms of cytoplasm extract were loaded on a SDS-PAGE along with pre-stained protein molecular weight standards. Resolving PAGE (10%) and stacking PAGE (5%) were used. Gels were blotted onto nitrocellulose membrane. Upon completion of the transfer, the blots were blocked with TBS containing 5% non-fat milk for 1 hour at room temperature. Membranes were then incubated with the 2 ml rabbit anti-human phosphor-IκB-α (Ser 32) antibody (Cell Signaling Technology, Danvers, MA) or control anti-actin antibody (Chemicon, Temecula, CA) at 1:1000 dilutions in tris-buffered saline (TBS) overnight at 4ºC. After washing three times for 5 minutes each with 0.1% Tween-20 in TBS (TBS/T), membrane was incubated with 2ml HRP-conjugated goat anti-rabbit antibody (1:2000) for 1 hour at room temperature. Blots were washed with TBS and bound antibodies were detected using Western Plus kit (Western Plus, Amersham Biosciences).

***2.2.8.5 Enzyme-linked immunosorbent assay (ELISA) quantification of NF-κB activity***

ELISA was performed as described in TransAM$^{TM}$ Flexi NF-κB Family Transcription Factor Assay Kit (Active Motif).

Nuclear extract was prepared as described previously (Section 2.2.8.1). Biotinylated probe (1 pmol) and 2 μg of nuclear extract were mixed and diluted to 50 μl in complete binding buffer in a microcentrifuge tube. The reaction tube was incubated at room temperature for 30 minutes. The reaction was transferred to individual wells on the 96-well plate and incubated for 1 hour at room temperature with mild agitation. The wells were washed for 3 times. Diluted NF-κB antibody (100 μl, 1:1000 in antibody binding buffer) was added to wells. After incubation for 1 hour at room temperature, the wells were washed for 3 times and 100 μl of diluted HRP antibody (1:1000 in antibody binding buffer) was added to wells. After incubation for 1 hour at room temperature, the wells were washed for 4 times. Room-temperature developing solution (100 μl/well) was added to all wells and incubated 4-10 minutes protected from direct light. Stop solution (100 μl) was added to all wells. Absorbance was read on a spectrophotometer (Molecular Probes) within 5 minutes at 450 nm wavelength.

### 2.2.9 ELISA of cytokine concentration in culture supernatant of mature moDCs

ELISA kits for human IL-10, IL-12 (p70) (BD Biosciences) and IFN-α (PBL Biomedical Laboratories) were used to test cytokine concentrations in cell culture supernatants. Assays were performed as described in the kits. The principals

of the tests were antibody (pre-coated on wells)-antigen (in samples, to be detected)-antibody (which is conjugated to an enzyme to convert colorless substrate to a colored product, indicating the presence of antigen-antibody binding, provided in the kits) “sandwich”.

Each kit utilizes a monoclonal antibody specific for one of the cytokines coated on a 96-well plate. Cell supernatant samples (100 μL) were added to each well and incubated at room temperature for 2 hours. Washing solution was prepared by adding and mixing deionized water to 20× Wash Concentrate (provided in the kits, which contains concentrated detergent solution). Samples were aspirated and washed 5 times using washing solution (300 μL/well). After the last wash, the plates were blotted on absorbent paper to remove any residual buffer. Within 15 minutes prior to use, working detector was prepared by adding Detection Antibody (biotinylated anti-cytokine monoclonal antibody as provided in the kits, 95 μL/well) and 20× Enzyme Concentrate (concentrated Streptavidin-HRP conjugate with BSA as provided in the kits, 5 μL/well). Working detector (100 μL/well) was added to each well and incubated for 1 hour at room temperature. The working detector was aspirated and plates were washed 7 times using washing solution (300 μL/well). Substrate solution (hydrogen peroxide and tetramethybenzidine as provided in the kits, 100 μL/well) was added to each well and incubated for 30 minutes at room temperature. Stop solution (phosphoric acid as provided in the kits, 50 μL/well) was added to each well and absorbance

was read on a spectrophotometer (Spectramax Plus, Molecular Devices, California) at 450 nm wavelength.

### 2.2.10 Inhibition assay of NF-κB activity in moDCs

Caffeic acid phenethyl ester (CAPE, Sigma-Aldrich) was used as a specific inhibitor of NF-κB [32]. moDCs ($2\times10^6$ cells/sample) from healthy donors were cultured with or without CAPE (25 μg/mL) for 12 hours before LPS was applied into the culture medium to stimulate moDC maturation [32].

### *2.2.11 Frequency and subtype distribution of circulating DC*

To quantify mDCs and pDCs in human blood, antibody staining, red blood cell lysing and four-color flow cytometric analysis were performed on fresh peripheral blood. (1) Blood samples were retained in VACUTAINER blood collection tubes with K3-EDTA as anticoagulant (Becton Dickinson Biosciences) at room temperature (20°-25°C) prior to staining and lysing, and K3-EDTA was the anticoagulant. For 100 μl of whole blood, antibodies were added including 20 μl of lineage cocktail 1 (FITC-conjugated, FL1 channel of flow cytometer), 5 μl of anti-human CD123 antibody (PE-conjugated, FL2 channel), 10 μl of anti-HLA-DR antibody (PerCP-conjugated, FL3 channel), and 5 μl of anti-human CD11c antibody (APC-conjugated FL4 channel). Lineage cocktail 1 contains several antibodies that, in combination, stain lymphocytes, monocytes, eosinophils, and neutrophils. It includes antibodies against human CD3, CD14, CD16, CD19, CD20 and CD56. Human blood was mixed gently with the

antibodies using Vortex mixer and micropipettor with tips, and incubated for 30 minutes in the dark at room temperature. (2) After antibody staining, the blood samples were incubated with FACS Lysing Solution (Becton Dickinson Biosciences) that lyses red blood cells under gentle hypotonic conditions while preserving the leucocytes. FACS Lysing Solution, 10× concentrate as provided in the kit, was diluted 1:10 with room temperature deionized water. For 100 μl of whole blood, 2 ml of 1× FACS lysing solution was added. The samples were mixed gently and incubated for 9 minutes in the dark at room temperature. The cells were centrifuged at 300 × g for 5 minutes. After the supernatant was discarded, the cells were washed with 4 ml of 1× PBS with 0.1% azide. (3) Cells were fixed with 0.3 ml of 4% paraformaldehyde in PBS and then analyzed with FACSCalibur (Becton Dickinson Biosciences) flow cytometer (FACS). CellQuest software (Becton Dickinson Biosciences) was used to analyze the result. Cells that express HLA-DR and CD11c but lack the lineage marker were identified as mDC. Cells express HLA-DR and CD123 but lack lineage marker were identified as pDC [33].

### *2.2.12 Statistical Analysis*

Statistical comparisons were performed with the unpaired *t* test using SPSS statistical software (SPSS Inc. Chicago, IL). A value of $p < 0.05$ was considered statistically significant.

## 2.3 Results

### *2.3.1 Functional changes were found in moDCs from chronic hepatitis C patients*

To evaluate the ability of moDCs to stimulate T cell proliferation, allogeneic MLR was performed. moDCs from CHC patients had significantly reduced ability to stimulate allogeneic T cell proliferation when compared to moDCs from healthy donors (Fig.2-1 Panel A). In the patient MLR group, the patient moDCs were less dendrite-like and there were fewer T cells after co-culture compared to the healthy MLR group (Fig. 2-1 Panel B). As T-cell proliferation was less active in patient MLR group compared to healthy MLR group, the percentage of moDCs was higher in patient group compared to healthy group (Fig. 2-1 Panel B). To further test if the observed proliferation in MLR was due to T cells, T cells were CFSE-labeled prior to coculture. There were fewer CD4 T cells in patient MLR (Fig.2-1 Panel C) compared to the healthy MLR (Fig. 2-1 Panel D). Furthermore, there were fewer proliferated CD4 T cells among total cells (moDCs and CD4 T cells) in patient MLR (Fig.2-1 Panel E) compared to the percentage in healthy MLR (Fig. 2-1 Panel F). These assays demonstrated that moDCs from CHC patients were impaired in their ability to stimulate T cell proliferation compared to moDCs from healthy donors.

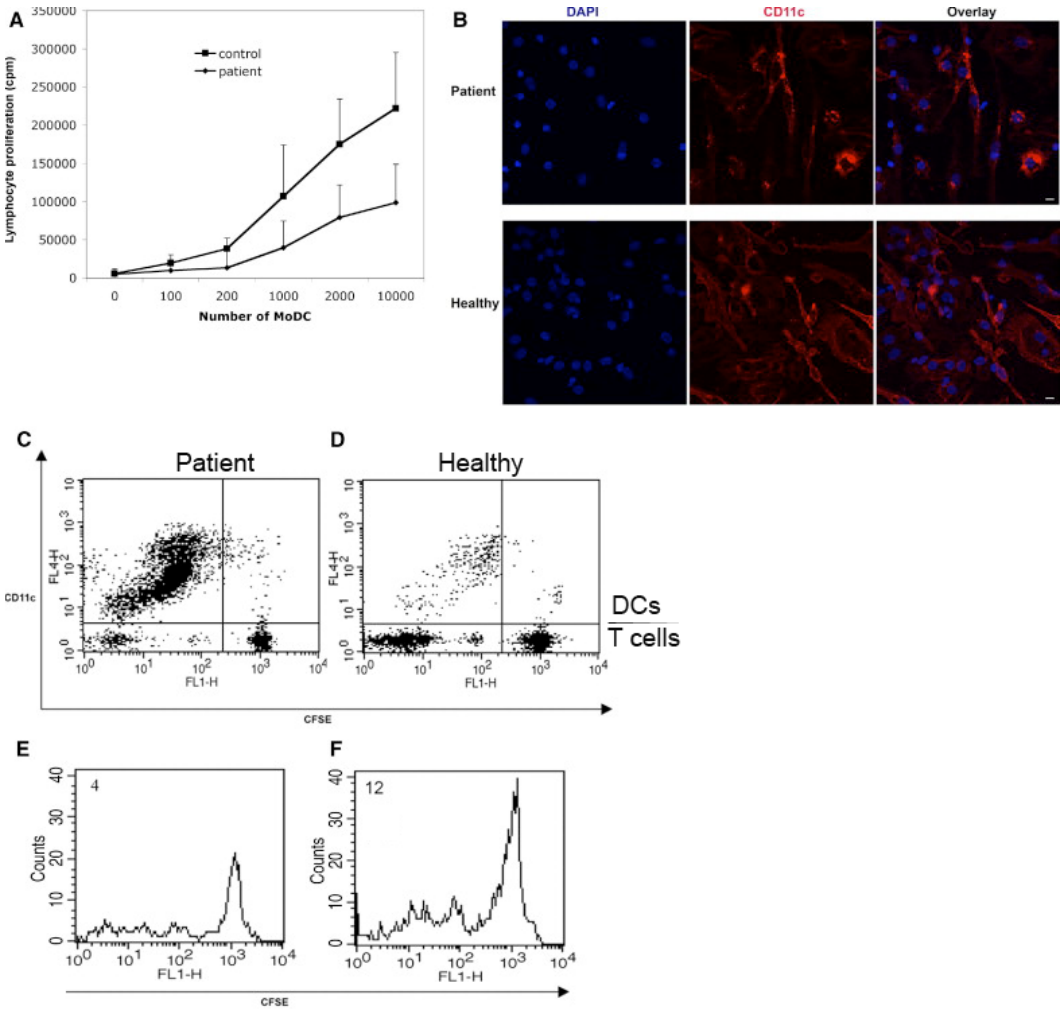


**Fig.2-1 moDCs from CHC patients are deficient in stimulating T cell proliferation [34].**

(**A**) Allogeneic CD4 T cells were plated at the concentration of $2\times10^5$ cells/well. Then mature moDCs (irradiated) from the same CHC patients and the same healthy donors were plated in triplicate at $1\times10^4$/well, $2\times10^3$/well, $1\times10^3$/well, $2\times10^2$/well and $1\times10^2$/well, respectively. Five days post-seeding, 0.5 μCi/mL of $^3$H-thymidine was added to the culture medium. After 18 hours, the $^3$H-thymidine uptake was measured with a micro-beta detector. Results were expressed as counts per minute (cpm) from experiments performed on 12 CHC patients and 12 healthy donors. Statistical results were expressed as mean ± SD ($P$ =0.032, Student's $t$ test).

(**B**) MoDCs ($1\times10^5$) were co-cultured with allogeneic CD4 T cells ($1\times10^5$) for 5 days. The cells were stained with anti-human CD11c (red, marker for DCs) and

DAPI (blue) was used to identify the nucleus. The confocal microscopy images were representative of three individual experiments. Scale bar = 10 μm.
(**C-F**) PPD (80 ng/mL)-loaded moDCs ($1\times10^6$) were co-cultured with allogeneic CFSE-labeled CD4 T cells ($1\times10^6$) for 5 days. Cells were surface stained with phycoerythrin (PE)-conjugated anti-human CD4 and allophycocyanin (APC)-conjugated anti-human CD11c. Flow cytometry plot of CD11c expression and CFSE profiles of patient MLR (**C**) and healthy MLR (**D**) were shown. CFSE profiles of CD4 T cells ($CD4^+$ $CD11C^-$) of patient MLR (**E**) and healthy MLR (**F**) were shown. Numbers indicate the percentages of T cells that proliferated among all cells (moDCs and CD4 T cells) in co-culture. The flow cytometry plots were representative of three individual experiments.

Immature MoDCs are highly endocytic. The ability of immature moDCs to endocytose FITC-conjugated ovalbumin was examined. There was no difference in the endocytosis ability of moDCs from CHC patients and healthy donors (Fig.2-2).

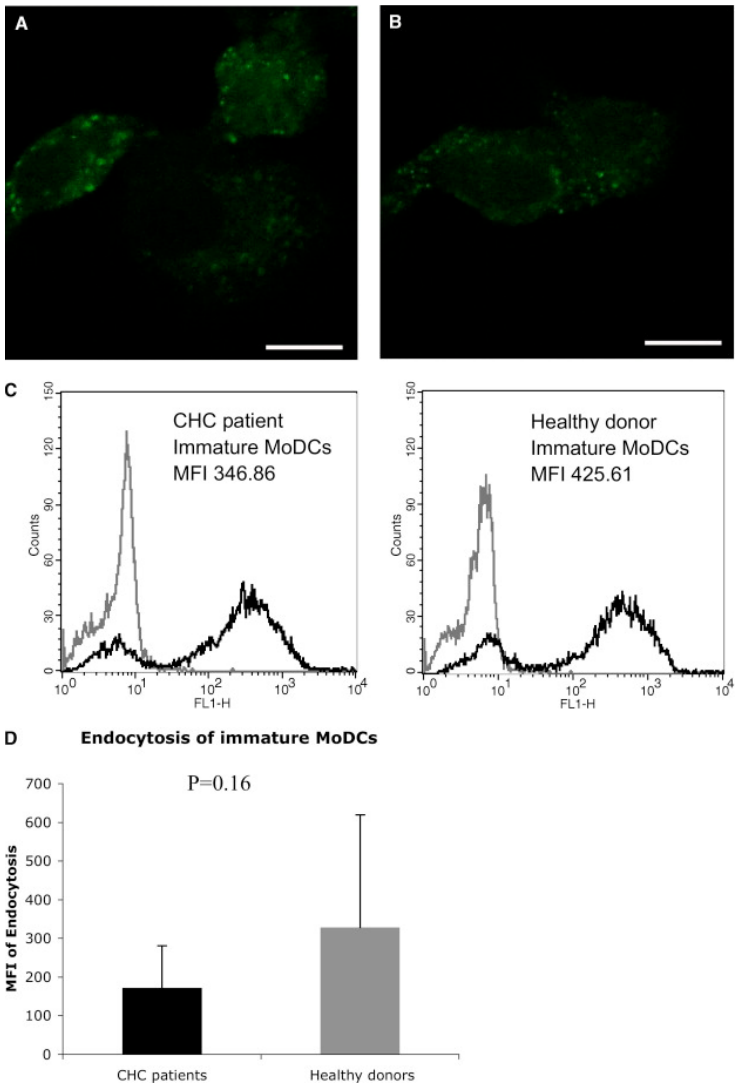


**Fig. 2-2. Comparable endocytosis function of immature moDCs from CHC patients and healthy donors [34].**

On the 5th day of moDC culture without LPS stimulation, immature moDCs were incubated with FITC-conjugated ovalbumin (2.5 μg/mL) for 60 minutes. Confocal microscopy was used to observe the fluoresceinated ovalbumin (green) in cytoplasm of moDCs from CHC patients (**A**) and healthy donors (**B**) (Representative of three individual experiments). Scale bar = 10 μm. Quantification of the endocytosis function of immature moDCs was indicated by mean fluorescence intensity (MFI) using flow cytometry. Panel **C** showed the representative flow cytometry plot of endocytosis. Grey: immature moDCs without ovalbumin incubation; Black: ovalbumin-incubated immature moDCs. Statistical results were expressed as mean ± SD from experiments performed on 20 CHC patients and 20 healthy donors ($P$ =0.16, Student's $t$ test) (Panel **D**).

Mature moDCs express high levels of HLA-DR and the co-stimulatory molecules CD80 and CD86. Expression of these molecules is a determining factor for moDCs to activate T cells [1, 4]. The expression levels of HLA-DR ($P < 0.05$, Fig. 2-3 Panel A and Panel B) and CD86 ($P < 0.05$, Fig. 2-3 Panel C and Panel D) on moDCs from CHC patients were significantly lower than the levels on moDCs from healthy donors. The expression levels of CD80 (Fig. 2-3 Panel E) and CD40 (Fig. 2-3 Panel F) on moDCs from CHC patients were comparable to those on moDCs from healthy donors. The decreased expression of HLA-DR and CD86 on moDCs from CHC patients may result in weaker activation signals sent to T cells, contributing to their diminished stimulation capacity in MLR.

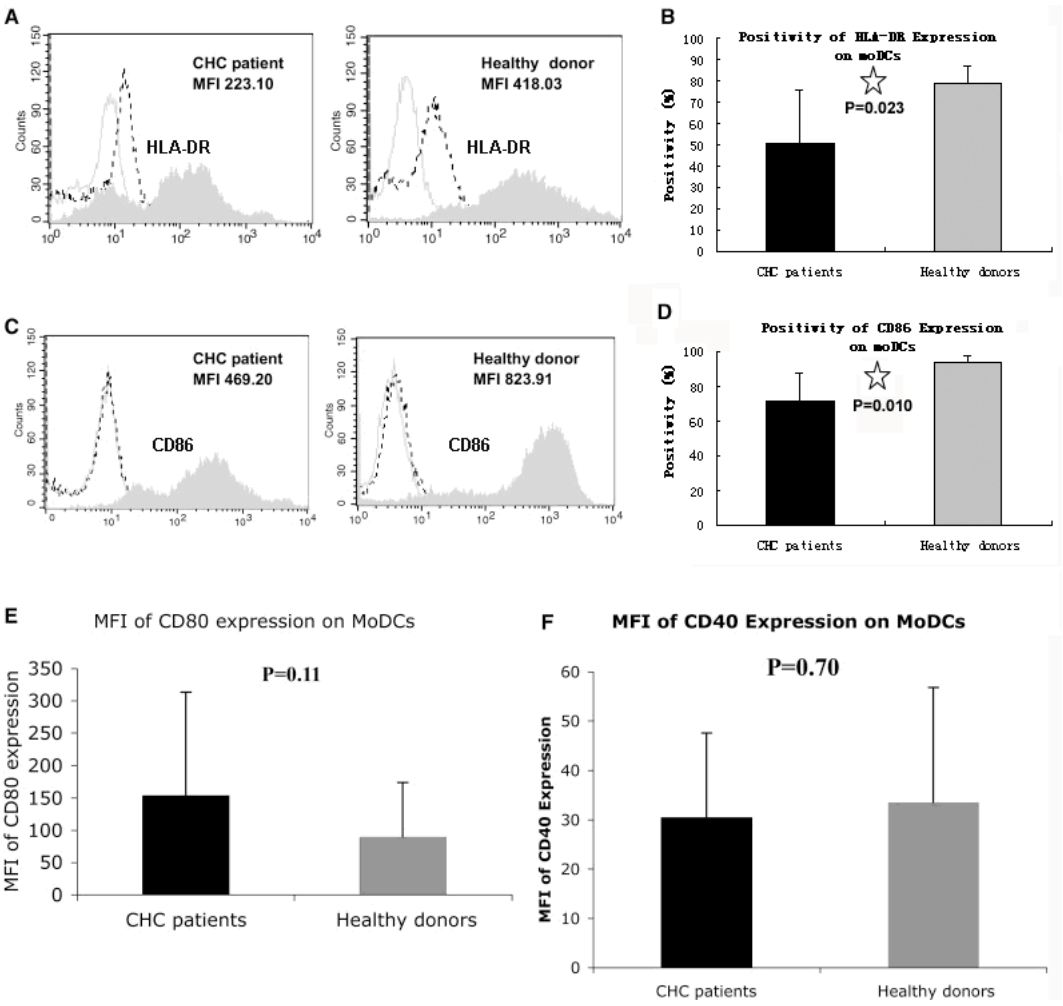


**Fig. 2-3. Decreased expression of HLA-DR and CD86 on mature moDCs from CHC patients compared to healthy donors [modified from reference 34].**

Mature moDCs from CHC patients and healthy donors were incubated with antibodies against HLA-DR (Panel. **A**, **B**), CD86 (**C**, **D**), CD80 (**E**) and CD40 (**F**), respectively. MFI and positivity were used to demonstrate the expression of these molecules on cell surface of mature moDCs as determined by flow cytometry. Grey solid line: unstained control; Black dotted line: isotype control; Filled grey histogram: HLA-DR (Panel. **A**) or CD86 (**C**). Statistical results were expressed as mean ± SD from experiments performed on 20 CHC patients and 20 healthy donors (Student's *t* test).

### *2.3.2 moDCs from CHC patients have an increased apoptosis*

It has been reported that DC apoptosis plays a central role in regulating immune tolerance [5]. It was hypothesized that if patient moDCs undergo apoptosis at a higher level than healthy moDCs, this might contribute to the poor immune responses against HCV infection in CHC patients.

To test this hypothesis, the apoptosis of patient moDCs and healthy moDCs was determined by flow cytometry and confocal microscopy. In cell culture, moDCs mature and undergo apoptosis within 24 hours after LPS stimulation. So moDCs cultured 6 hours after LPS stimulation were used in the apoptosis assays, since these moDCs are mature and functionally active but should not undergo massive apoptosis. As demonstrated by flow cytometry, the apoptosis of CHC patient moDCs was significantly higher than that of healthy moDCs (Fig.2-4 Panel A, Panel B). Confocal microscopy was used to observe the TUNEL signal in the nucleus, which indicates DNA strand breaks during apoptosis. The TUNEL signal was more frequent and stronger in CHC patient moDCs than in healthy moDCs (Fig.2-4 Panel C).

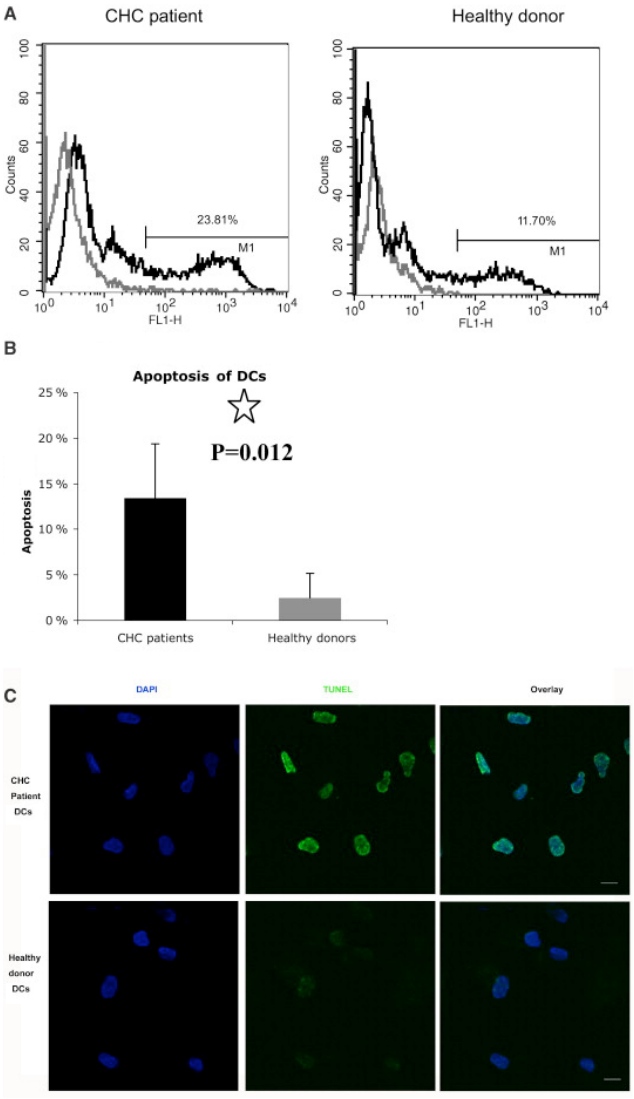


**Fig. 2-4. Increased apoptosis of mature moDCs from CHC patients as compared to mature moDCs from healthy donors [34].**

Mature moDCs from CHC patients and healthy donors were stained for apoptosis using TUNEL kit. (**A**) Representative flow cytometry plot of moDC apoptosis from patients and healthy donors. Grey: unstained control; Black: cells incubated with reaction mixture. The marker (M1) was set to make the positivity of unstained controls (background) <1%. (**B**) Flow cytometry quantification of apoptosis of moDCs. Positive percentages were expressed as mean ± SD from experiments performed on 20 CHC patients and 20 healthy donors (*P*=0.012, Student's *t* test). (**C**) Confocal microscopy of apoptosis signal in moDCs. TUNEL (green) signal indicated apoptosis. Blue signal was from

DAPI indicating the nucleus. These figures were representative of samples from three experiments (3 CHC patients, 3 healthy donors). Scale bar = 10 μm.

### 2.3.3 *NF-κB activity is diminished in moDCs from CHC patients*

With finding functional changes and increased apoptosis of moDCs from CHC patients, the NF-κB activity of patient moDCs and healthy moDCs was examined. NF-κB is a transcription factor regulating DC function and apoptosis. NF-κB is inactive in the cytoplasm since it binds to the protein inhibitor IκB. Following the phosphorylation and degradation of IκB, NF-κB translocates into the nucleus and binds to its specific target DNA sequence [35].

To localize the active form of NF-κB in the cell nucleus, confocal microscopy was used. Less NF-κB subunit p65 was observed in the nuclei of moDCs from CHC patients than in the nuclei of moDCs from healthy donors (Fig.2-5 Panel A). EMSA was used to observe the DNA-binding capacity of NF-κB in moDCs. NF-κB binding with its target DNA oligonucleotides observed in CHC patient samples was weaker than that observed in the healthy donor samples. The binding was specific since the addition of unlabelled oligonucleotides competed successfully to prevent band shift, while the addition of unlabelled mutant oligonucleotides did not (Fig.2-5 Panel B). Western blotting was used to observe the level of pIκBα in the cytoplasm, which correlates with the level of active NF-κB in the nucleus. There was less pIκBα in moDCs from CHC patients than those from healthy donors (Fig. 2-5 Panel C), suggesting that there may be less NF-κB translocating into the nuclei of moDCs from CHC patients. Finally, ELISA was used to quantify NF-κB activity in nuclear extract of moDCs. The NF-κB activity in moDCs from CHC patients is significantly

lower than that from healthy controls (P=0.008) (Fig.2-5 Panel D). These results on NF-κB activity demonstrate that NF-κB activity in DCs from CHC patients was inhibited. These results demonstrated that moDCs from CHC patients had diminished NF-κB activity, which might be an important factor in the functional changes and increased apoptosis of moDCs from patients.

To determine if the inhibition of NF-κB activity in healthy MoDCs leads to the functional changes and increased apoptosis, a specific inhibitor of NF-κB, CAPE [32], was applied into the culture medium of moDCs. CAPE-treated healthy moDCs demonstrated inhibited NF-κB activity (Fig. 2-6 Panel A), decreased expression of HLA-DR and CD86 (Fig. 2-6 Panel B) and increased apoptosis (Fig. 2-7). The changes induced in healthy moDCs by the inhibition of NF-κB activity were similar to the changes in moDCs from CHC patients. These results suggested that the changes in the patient moDCs may be due to their diminished NF-κB activity.

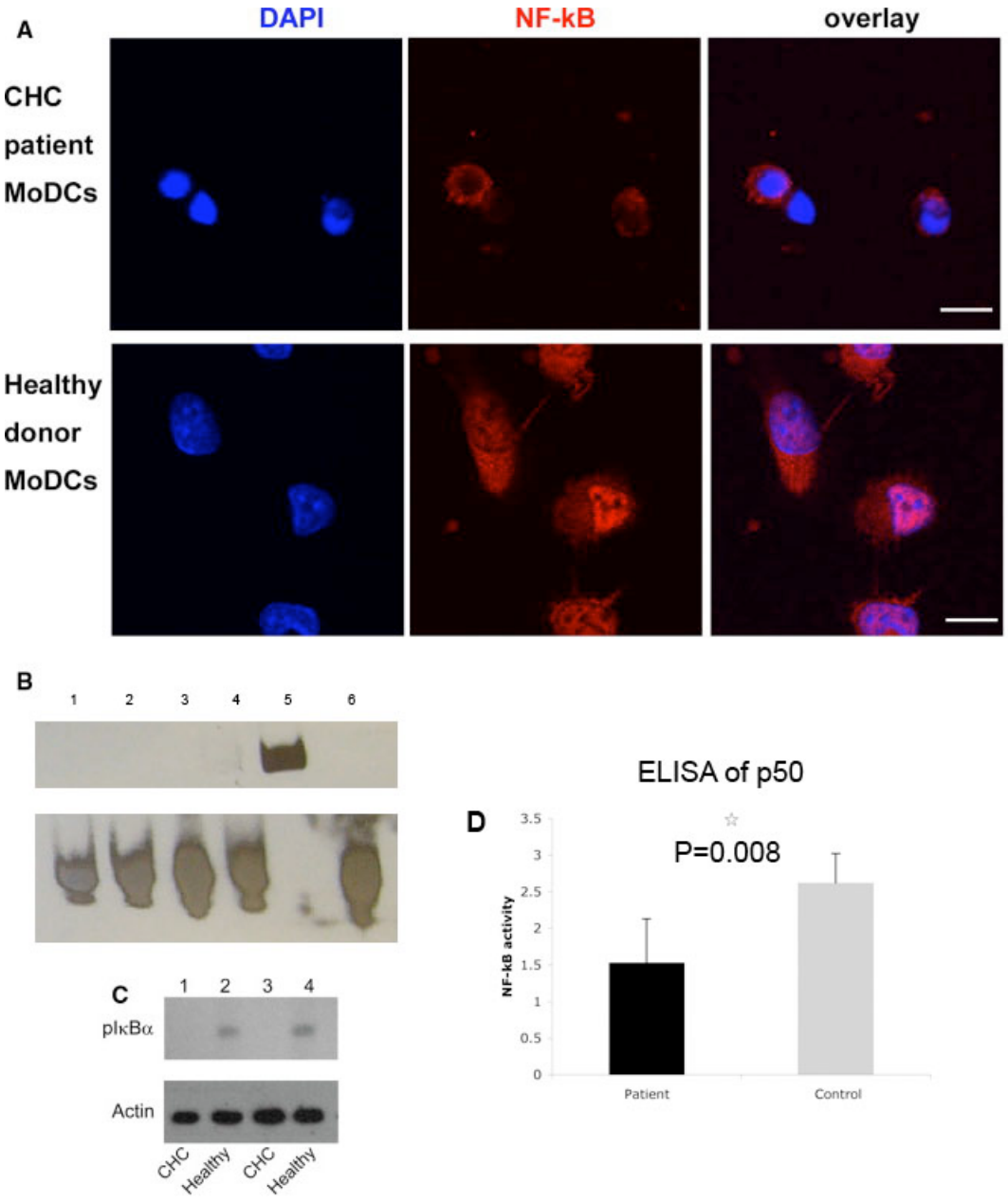


**Fig.2-5. Diminished NF-κB activity in mature moDCs from CHC patients [34].**

Mature moDCs from CHC patients and healthy donors were harvested and examined for their NF-κB activity 6 hours after LPS stimulation.
(**A**) Confocal microscopy of NF-κB subunit p65 localization. Mature moDCs were stained for p65 (red). DAPI (blue) indicates the nucleus. The active form of NF-κB was translocated into the nucleus. There was more active NF-κB in

healthy moDCs compared to moDCs from CHC patients. These figures were representatives of three experiments (3 CHC patients, 3 healthy donors). Scale bar = 10 μm.

(**B**) EMSA of NF-κB activity in nuclear extracts. Nuclear extracts of mature moDCs from a healthy donor and a CHC patient were incubated with a biotin-labeled NF-kB consensus DNA probe with or without unlabelled probe, and then separated on a 6% polyacrylamide gel. *1. Biotin-labeled DNA (20 fmol) only. 2. Nuclear extracts of moDCs from a CHC patient mixed with Biotin-labeled DNA (20 fmol). 3. Nuclear extracts of moDCs from the CHC patient mixed with Biotin-labeled DNA (20 fmol) and unlabeled DNA (4 pmol). 4. Biotin-labeled DNA (20 fmol) only. 5. Nuclear extracts of moDCs from a healthy donor as the paired control mixed with biotin-labeled DNA (20 fmol). 6. Nuclear extracts of moDCs from the healthy donor mixed with biotin-labeled DNA (20 fmol) and unlabeled DNA (4 pmol).* Binding reactions were separated by polyacrylamide gel electrophoresis (6%) and blotted onto nylon membrane. Biotin signal was identified as described in Materials and Methods. This figure was representatives of three experiments (3 healthy donors and 3 CHC patients).

(**C**) Western blotting of pIκBα in cytoplasm extracts. *Lane 1, CHC patient 1. Lane 2, healthy donor as paired control for patient 1. Lane 3, CHC patient 2. Lane 4, healthy donor as paired control for patient 2.* Cytoplasm extracts of mature moDCs from CHC patients and healthy donors were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. This figure was representatives of three experiments.

(**D**) ELISA of NF-κB subunit p50 activity in nuclear extracts. ELISA was performed as described in NF-κB Transcription Factor Assay Kit (Active Motif). 1 pmol of biotinylated probe and 2 μg of nuclear extract of moDCs were mixed to capture activated NF-κB binding. The binding reaction was transferred to individual wells on a streptavidin-coated plate provided in the ELISA kit. Anti-p50 primary antibody was added to wells. After incubation and washing, HRP-conjugated anti-IgG antibody was added to wells. After washing, developing solution and stop solution was added to all wells. Absorbance was read on a spectrophotometer. Statistical result is expressed as mean ± SD from experiments performed on 8 CHC patients and 8 healthy donors ($P$=0.008, Student's $t$ test).

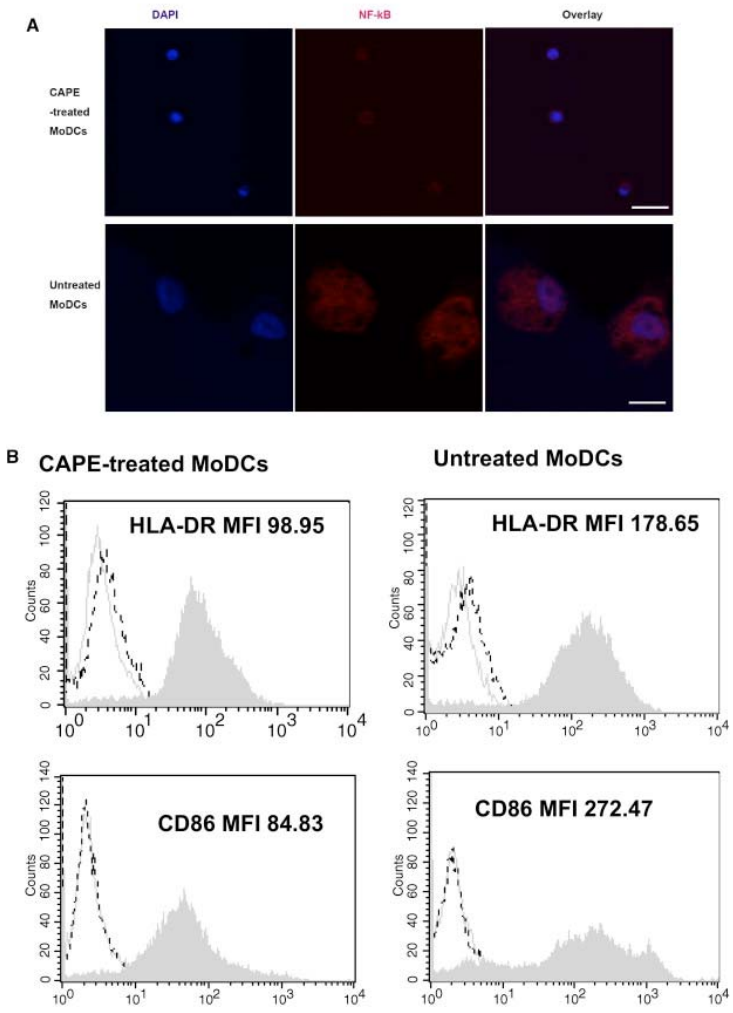


**Fig.2-6 Inhibition of NF-κB activity resulted in decreased expression of HLA-DR and CD86 on healthy moDCs [34].**

MoDCs from healthy donors were treated with or without 25 μg/mL of CAPE (a specific inhibitor of NF-κB) for 12 hours. (**A**) Confocal microscopy images about the NF-κB activity (NF-κB subunit p65, red) in CAPE-treated moDCs and untreated moDCs from a healthy donor. DAPI (blue) indicated the nucleus. The active form of NF-κB is located in the nucleus. These figures were representative of five individual experiments. Scale bar=10 μm. (**B**) The expression of HLA-DR and CD86 on CAPE-treated moDCs and untreated moDCs were determined by flow cytometry. Grey solid line: unstained control; Black dotted line: isotype control; Filled grey histogram: HLA-DR or CD86. These figures were representative of five individual experiments.

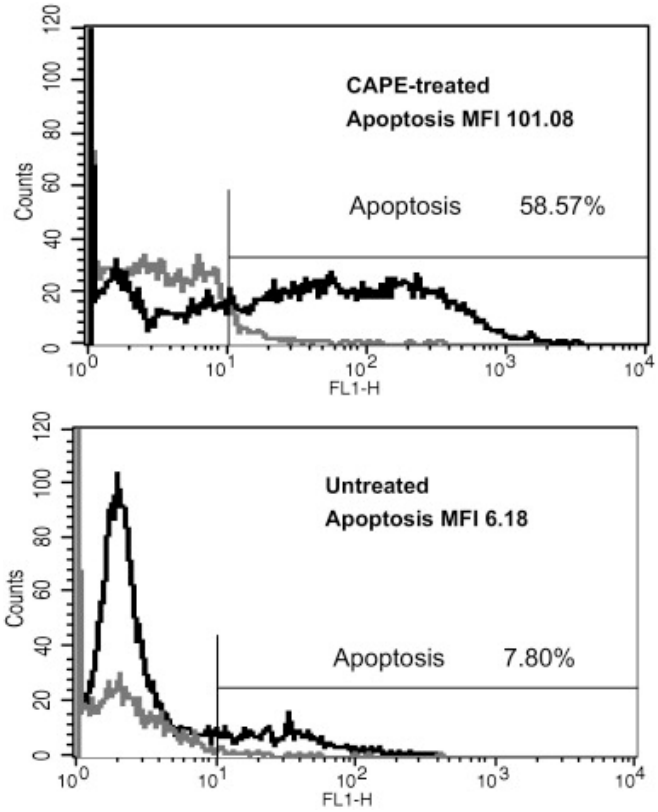


**Fig.2-7 Inhibition of NF-κB activity resulted in increased apoptosis of moDCs from healthy donors [34]**.

MoDCs from healthy donors were treated with or without 25 μg/mL of CAPE (a specific inhibitor of NF-κB) for 12 hours. The CAPE-treated moDCs and untreated moDCs were stained using TUNEL kit and their apoptosis was determined by flow cytometry. Grey: unstained control. Black: cells incubated with reaction mixture of the TUNEL kit. The marker was set to make the positivity of unstained controls (background) <1%. These figures were representative of five individual experiments.

### *2.3.4 mDCs ex vivo isolated from peripheral blood of CHC patients demonstrate decreased expression of HLA-DR and CD86, increased apoptosis and diminished NF-κB activity.*

The mDCs *ex vivo* isolated from peripheral blood of CHC patients were used to determine if their function, apoptosis and NF-κB activity correlated to those observed on *in vitro* generated moDCs from CHC patients. BDCA-1 is one of the specific markers of mDCs. The expression of BDCA-1 on the cell surface of mDCs is shown in Fig. 2-8 Panel A. The expression of HLA-DR and CD86 on mDCs isolated from peripheral blood of CHC patients was decreased compared to the expression of these molecules on mDCs from healthy donors (Fig. 2-8 Panel B). There was increased apoptosis and diminished NF-κB activity (Fig. 2-8 Panel C and Panel D) in mDCs from CHC patients compared to mDCs from healthy donors. These results obtained from *ex vivo* isolated mDCs were similar to the results obtained from *in vitro* generated moDCs.

### 2.3.5 Comparable production of cytokines by moDCs from CHC patients and healthy donors

Cytokine secretion is another important function of DC that regulates immune response. In my study, cytokine production by moDCs from CHC patients and healthy donors were generally low and highly variable. In the culture supernatant of moDCs from CHC patients, the concentrations of IL-10, IL-12 and IFNα were comparable to those from the controls (P=0.76 for IL-10, P=0.27 for IL-12, P=0.90 for IFNα) (Fig.2-9).

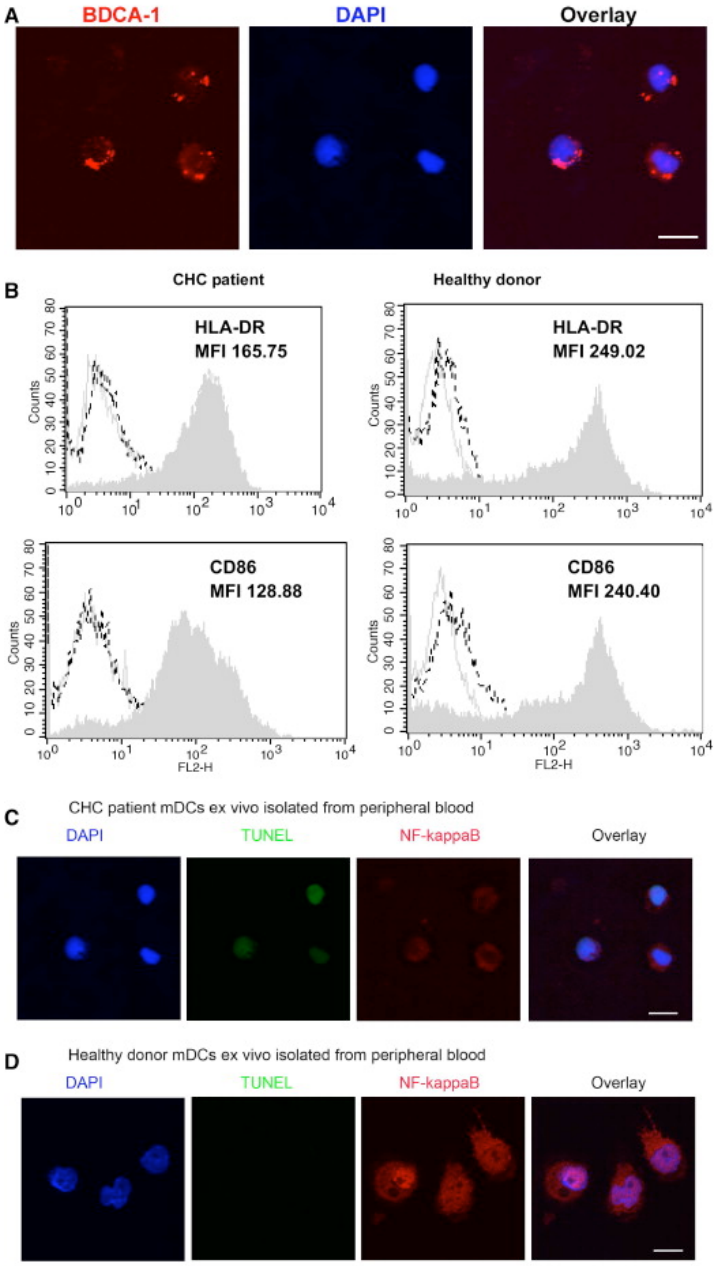


**Fig. 2-8 *Ex vivo* isolated CHC patient mDCs demonstrated functional changes, increased apoptosis and diminished NF-κB activity [34].**

(**A)** Identification of *ex vivo* isolated mDCs using confocal microscopy. The mDCs were plated on coverslips, fixed with 4% paraformaldehyde, stained with mouse anti-human BDCA-1, washed with PBS, and stained with Alexa 647-conjugated goat anti-mouse IgG antibody. The coverslips were secured on slides using mounting medium containing DAPI to indicate nucleus. Red: BDCA-1, Blue: DAPI. Scale bar =10 μm.

(**B**). Representative plot of HLA-DR and CD86 expression on cell surface of mDCs ex vivo isolated from peripheral blood of CHC patients and healthy donors. MDCs were examined by flow cytometry with gating on BDCA-$1^{+}$ $CD19^{-}$ cells. Grey solid line: unstained control; Black dotted line: isotype control; Filled grey histogram: HLA-DR or CD86. These figures were representative of five individual experiments.
(**C**) and (**D**) showed the confocal microscopy images about the apoptosis signal (TUNEL, green) and NF-κB activity (NF-κB subunit p65, red) in mDCs ex vivo isolated from peripheral blood of a CHC patient (**C**) and a healthy donor (**D**). DAPI (blue) indicated the nucleus, where the active form of NF-κB is. These figures were representative of five individual experiments. Scale bar =10 μm.

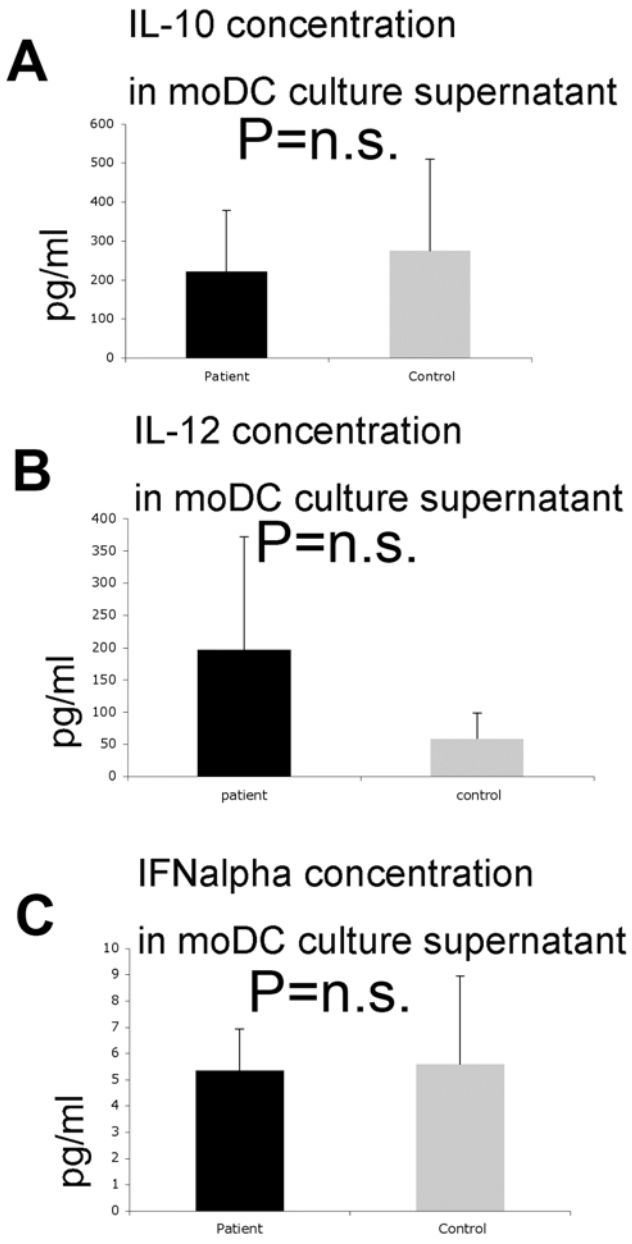


**Fig. 2-9 Comparable production of cytokines by moDCs from CHC patients and healthy donors.**

Culture supernatants of mature moDCs were collected within 12 hours of LPS stimulation. The concentrations of IL-10, IL-12 and IFN-alpha in culture supernatants were determined by ELISA. Statistical result is expressed as mean ± SD from experiments performed on moDCs from 6 CHC patients and 6 healthy donors. n.s., not significant.

### 2.3.6 Numbers of mDCs and pDCs in blood were not changed in CHC patients

The numbers of circulating mDCs and pDCs in peripheral blood of CHC patients were comparable with that of healthy controls (P=0.311 for mDCs and P=0.462 for pDCs). The ratio of circulating mDC/pDC in CHC patients was comparable with that of healthy controls (P=0.383). The data for the numbers and subtype distribution of DC in peripheral blood are presented in Table 2-2.

**Table 2-2 Subtype percentage and ratio of circulating DCs in PBMCs from CHC patients and controls**

| | HCV Patients | Controls | P value |
|---|---|---|---|
| Circulating mDC (%) | 0.498 ± 0.310 | 0.782 ± 0.512 | 0.311 |
| Circulating pDC (%) | 0.178 ± 0.080 | 0.330 ± 0.432 | 0.462 |
| Circulating mDC/pDC | 3.030 ± 1.52 | 4.490 ± 3.260 | 0.383 |

NOTE. Results are determined by flow cytometry and expressed as means ± SD from experiments performed on 20 CHC patients and 20 controls.

## 2.4 Discussion

HCV is among the most successful human viruses in establishing persistent infections. HCV persists in 70% to 80% of infected patients. Understanding immune evasion of this virus is crucial for the design of effective strategies to control HCV. The control of viral infection involves an early innate immune response and a strong adaptive immune response. The effect of HCV on the innate immune response has been extensively studied and it is well known that HCV has evolved unique mechanisms to block critical steps in the innate immune response [36]. CHC patients have impaired T-cell responses against HCV [11-16]. The decreased expression of HLA-DR and CD86 together with the increased apoptosis of mDCs from CHC patients shown in this study suggest that there may be decreased levels of "signal 1" (HLA-DR) and "signal 2" (CD86) from mDCs, the most potent professional APCs, to activate CD4 T cell responses. The decreased ability of mDCs from CHC patients to activate T-cell response correlates with previous reports that CHC patients have impaired anti-HCV T-cell responses [11-16].

Previous studies on mDC function during chronic HCV infection have yielded conflicting results [18-26]. It is important to recognize that some of the seemingly small differences in study design, such as culture media and harvesting time, could affect the results when working with DCs. Previous studies used hetorologous serum, either fetal serum [18, 19, 21, 25, 26] or heterologous human serum [22, 24], in DC cultures. However, heterologous serum has

immunosuppressive activity on DC function and autologous serum is better than heterologous serum to culture DCs [27]. To circumvent potential interference caused by heterologous serum, this study used autologous serum in DC culture. Furthermore, harvesting DCs at different time points after LPS stimulation yields different assay values. To ensure that the assays were done at the appropriate time, the same harvesting time was set for each assay and one patient sample and its control sample were worked on at the same time. My study correlates with previous reports that mDC function was inhibited in CHC patients [18-22]. Furthermore, my study indicates that there is an increased apoptosis of mDCs in CHC patients, and suggest that diminished NF-κB activity in patient mDCs is probably responsible for the function and apoptosis changes.

Given that *in vitro* generated moDCs may differ from mDCs *ex vivo* isolated from peripheral blood in certain aspects [37], assays were performed on the both types of mDCs. In this chapter, assays on moDCs and mDCs isolated from peripheral blood yielded similar results.

Clonal expansion of antigen-specific T cells requires two signals sent from APCs by their expression of HLA class II ("signal 1") and CD80/CD86 ("signal 2") [6]. The major function of HLA class II molecules is to present processed antigens, which are derived primarily from exogenous sources, to CD4 T cells. Therefore, the HLA-DR molecule is important to initiate an antigen-specific response [38]. If signal 2 from CD80/CD86 is lacking, antigen presentation leads to T cell

anergy, an important mechanism of immunologic tolerance [6]. In the functional assays, I demonstrated that moDCs from CHC patients showed diminished capacity to stimulate T lymphocyte proliferation compared to those from healthy donors. This could be attributed partly to their decreased expression of HLA-DR and CD86 compared to the moDCs from healthy donors.

There are significantly more apoptotic mDCs in CHC patient samples than those in healthy donor samples. This may contribute to the diminished capacity of mDCs from CHC patients to stimulate T cell proliferation. Mature mDCs reside in regional lymph nodes while immature mDCs circulate in peripheral blood [1, 4]. The increased apoptosis of patient mDCs suggest that there might be fewer mature mDCs in the hepatic lymph nodes of CHC patients to stimulate the appropriate T cell responses. Future studies on the hepatic lymph nodes from CHC patients and non-CHC patients obtained at the time of liver transplantation could be used to examine this possibility.

NF-κB is a transcription factor crucial for immune responses. NF-κB controls the expression of HLA-DR and CD86, and acts as an anti-apoptotic factor for mDCs [39-42]. Here, I found that NF-κB activity in moDCs from CHC patients was diminished compared to that in moDCs from healthy donors. Furthermore, the characteristics of moDCs from CHC patients were similar to those of CAPE-treated healthy moDCs in which NF-κB activity was specifically inhibited. These results suggest that the diminished NF-κB activity may be responsible for

the functional changes and increased apoptosis of mDCs from CHC patients. These results also suggest that the NF-κB activity in mDCs may regulate protective immune responses during HCV infection. While HCV primarily replicates in hepatocytes, there are reports that PBMCs can be infected by HCV, with monocytes containing the greatest HCV RNA loads [43]. It will be of interest to determine if HCV proteins [44] or DC infection by HCV [45] can suppress the NF-κB activity in mDCs.

Apoptosis is triggered by multiple stimuli through a variety of signaling pathways. Among these, three pathways seem to be important for DCs. These signaling pathways include NF-κB, TRAIL and Fas. There are some reports that viruses can induce an increased apoptosis of DCs, such as herpes simplex virus (HSV) [46] and HIV [47]. These studies indicate that DC apoptosis during virus infection may be a common mechanism of virus immune evasion. It has been reported that mature DCs are resistant to Fas-associated apoptosis for that they express C-FLIP, an inhibitor of the Fas-mediated apoptosis [48]. In the present study, I found that the diminished NF-κB activity might account, in part, for the increased apoptosis in DCs during HCV infection.

In conclusion, my study suggests that mDCs may play an important role in the induction of T cell responses that can control HCV infection. I demonstrated that mDCs from CHC patients have decreased immunogenic function and

increased apoptosis and presented evidence that the inhibition of NF-κB activity of mDCs is correlated to these changes.

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# CHAPTER 3

# Myeloid DCs Can Kill T Cells

# During Chronic Hepatitis C Virus Infection

## 3.1 Introduction

HCV infects about 3% of the world's population [1]. The failure of CD4 T-cell response to HCV is a defining feature of CHC [2-5]. Antigen-driven proliferation of CD4 T cells is observed in patients who cleared HCV infection, but is inconsistently detected in patients who developed CHC. CHC patients fall into two groups depending on HCV replication patterns during the first few months of infection. Patients in the first group are unable to mount an HCV-specific CD4 T-cell response and remain chronically infected. The second group of patients have strong HCV-specific CD4 T-cell activity and clear HCV RNA transiently from their serum, however, the CD4 T-cell activity weakens just before a rebound in viremia that results in chronic infection [2, 6].

DCs are the most potent professional APCs that regulate T-cell responses [7, 8]. Besides their well-known immunogenic function of stimulating T-cell proliferation, DCs also have tolerogenic activity of killing T cells. The killer DCs express Fas ligand (FasL: CD178) [9-15] or tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL: CD253) [16-19]. The presence of killer DCs has been reported during measles virus (MV) [16, 17] and HIV [18, 19] infections. MV induces TRAIL expression in DCs and these DCs induce the apoptosis of activated T cells [16] and can kill tumor cells [17]. During HIV infection, killer DCs produce TRAIL and induce the apoptosis of CD4 T cell lines [18, 19]. However, whether DCs regulate the apoptosis of T cells during HCV infection remains to be determined.

B lymphocyte stimulator (BLyS), a molecule also termed as B cell-activating factor belonging to the TNF family (BAFF), is a member of the tumor necrosis factor (TNF) superfamily [20-22]. Full-length BLyS is expressed on the plasma membrane as membrane-bound BLyS (mBLyS), which is capable of co-stimulating T cell activation [23]. mBLyS expressed on DCs has co-stimulatory activity on both $CD4^+$ and $CD8^+$ T cells at multiple stages, including naïve T cells, recently antigen-primed T cells, and memory T cells [23, 24]. The expression of mBLyS on DCs during HCV infection has not been reported.

In Chapter 2, my study [25] has shown that the ability of mDCs to stimulate CD4 T-cell proliferation was impaired in CHC patients. In this chapter (Chapter 3), the tolerogenic activity of mDCs from CHC patients was examined. Due to the low numbers of circulating mDCs in peripheral blood, monocytes were cultured *in vitro* with IL-4 and GM-CSF to obtain moDCs. The expression of inhibitory ligands, including FasL, programmed death-1 (PD-1) ligands 1 and 2 (PD-L1: CD274 and PD-L2: CD273), on moDCs was examined by flow cytometer. The moDCs from CHC patients and healthy donors were co-cultured with different target T cells, including healthy CD4 T cells, Jurkat T cells and autologous patient CD4 and CD8 T cells. Cell apoptosis was examined by TUNEL. These assays found that patient moDCs have up-regulated tolerogenic activity and are capable of killing CD4 and CD8 T cells isolated from human peripheral blood. Possible

mechanisms for the killing were explored by examining the expression of T cell receptor (TCR), PD-1 and Fas on target T cells. To determine whether the killing was mediated by soluble factors or cell-cell contact, moDCs and T cells were cultured either in the presence of blocking antibodies, or in two chambers separated by a polycarbonate membrane in transwells. Furthermore, mDCs were *ex vivo* isolated from the peripheral blood using MACS, and killing assays were performed to determine if these mDCs of CHC patients demonstrate up-regulated tolerogenic activity compared to those of healthy donors.

## 3.2 Patients and Methods

### *3.2.1 Blood Samples*

Male CHC patients who had been HCV-RNA positive and had detectable HCV antibodies for more than 3 years were included in this study. If patients were given a trial of therapy with pegIFN and ribavirin, they were off therapy for at least 6 months. Patient characteristics are shown in Table 3-1. Control samples were collected from HCV-negative healthy male donors (age-matched). Ethics approval for human blood collection was obtained from the University of Alberta, Faculty of Medicine Research Ethics Board. Informed consent was obtained from all donors.

**Table 3-1. Clinical characteristics of CHC patients**

| Characteristics | CHC patients |
|---|---|
| Age (year); mean | 49.5 |
| ALT (IU/L); mean | 126.5 |
| HCV viral load (IU/Ml); mean | $2.8 \times 10^6$ |
| HCV RNA genotype | genotype1/genotype 2/genotype3 =20/4/16 |

Male patients have more aggressive HCV infection in general than female patients. Women tend to eliminate HCV more rapidly, have a lower rate of disease progression than men, and a lower mortality rate from HCV-related liver disease [26]. In the pilot study, I observed more killing activity of moDCs from male CHC patients however poor killing activity of moDCs from female CHC patients. In this chapter, I concentrated on immune cells from male patients and male healthy donors.

### *3.2.2 Antibodies*

Mouse anti-human FasL, PD-L1, PD-L2, TCR, Fas, PD-1, CD11c and isotype antibodies were purchased from BD Biosciences (San Diego, CA). Alexa 568 conjugated goat anti-mouse IgG was purchased from Molecular Probes (Invitrogen, Carlsbad, CA).

### *3.2.3 Culture of in vitro generated moDCs and ex vivo mDCs*

moDCs were cultured as described before (Chapter 2.2). In brief, PBMCs were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation. The cells were plated to a density of $3.0\times10^7$ cells/well in 6-well plates, incubated for 2 hours, and the non-adherent cells were removed. The adherent cells were cultured in RPMI-1640 containing 1% autologous human serum, 50 ng/mL of human GM-CSF and 10 ng/mL of human IL-4. On the $5^{th}$ day of culture, 0.1 μg/mL of LPS was added to stimulate the moDCs. After 6

hours, the cells were harvested [27, 28] and >99% were identified as mature moDCs based on cell surface expression of HLA-DR and CD11c.

*Ex vivo* mDCs were isolated as described previously (Chapter 2.2). In brief, *ex vivo* mDCs were isolated from PBMCs with >90% purity by MACS using BDCA-1 positive isolation kit. The *ex vivo* mDCs were cultured in RPMI-1640 containing 1% autologous human serum. On the 3rd day of culture, 0.1 μg/ml of LPS was added to stimulate mDCs. After 24 hours, the cells were harvested as mature *ex vivo* mDCs [29].

### *3.2.4 Culture of primary CD4 T cells and Jurkat T cell line*

Primary CD4 T cells were isolated from PBMCs with >95% purity by MACS (similar as that described in Chapter 2. 2). Briefly, PBMCs isolated from donors were incubated with FcR blocking reagent and PE-conjugated anti-human CD4-antibody (BD Biosciences) for 30 min at 4°C, washed twice with cold MACS buffer (PBS containing 2% BSA and 2mM EDTA), and incubated with anti-PE magnetic microbeads (Miltenyi Biotec) for 15 min at 4°C. The cells were washed twice with cold MACS buffer and isolated on magnetic columns (Miltenyi Biotec) according to the manufacturer's instructions [30]. T cells were suspended in AIM-V medium (Invitrogen).

A T cell leukemia cell line, Jurkat T cells, were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich) [13].

### *3.2.5 Flow cytometry analysis of FasL, PD-L1, PD-L2, TRAIL and BLyS expression on cell surface of moDCs*

To block non-specific binding of antibodies, moDCs ($1\times10^7$ cells) were incubated with 20 μl of FcR blocking reagent (Miltenyi Biotec) for 15 minutes. After FcR blocking, moDCs ($1\times10^6$/sample) were individually incubated with PE-conjugated mouse anti-human antibodies against FasL (20 μl), PD-L1 (20 μl), PD-L2 (20 μl), TRAIL (20 μl), and BLyS (20 μl) for 30 minutes. Cells were washed twice with 2% FCS in PBS, fixed in 4% paraformaldehyde and then analyzed by flow cytometry using CellQuest software as described in detail in Section 2.2.6 [31].

### *3.2.6 ELISA of TRAIL concentration in serum and moDC culture supernatant*

ELISA of TRAIL concentration in serum and moDC culture supernatant was performed as described in TRAIL ELISA kit (Diaclone, Fleming, France). The principal of the test was antibody (a monoclonal antibody specific for TRAIL, pre-coated on wells)-antigen (to be detected, in samples of sera or culture supernatant)-antibody (which is conjugated to HRP enzyme to convert colorless substrate to a colored product, indicating the presence of antigen-antibody binding) “sandwich”.

Assays were performed as described in the kit. Each sample, including standard samples and patient samples, were assayed in duplicate. (Step 1) Preparation of TRAIL standard samples. Standard buffer diluent 10× concentrate (provided in the kit) was diluted 10 times with distilled water. Standard (freeze-dried TRAIL antigen provided in the kit) was reconstituted with standard buffer diluent to make a stock concentration of 3000 pg/mL TRAIL. Standard was allowed to stand for 5 minutes with gentle swirling prior to making dilutions. Serial dilutions of standard were made by adding standard diluent to standards. The final standards range from 3000, 1500, 750, 375, 187.5, 93.75 to 0 pg/mL. (Step 2) Add samples of sera or moDC culture supernatant into wells of the 96-well plate (provided in the kit). Particulates and aggregation of supernatant or serum samples were removed by spinning at 1000×g for 10 minutes. Samples or appropriate standard diluent (100 μL) was added into wells. (Step 3) Biotinylated anti-TRAIL antibody was prepared by dilution with the biotinylated antibody diluent (0.4 mL, provided in the kit) in a glass vial. Biotinylated anti-TRAIL antibody (50 μL) was added to each well. The wells were covered with a plate cover and incubated for 3 hours at room temperature. (Step 4) Washing solution was prepared by adding and mixing distilled water to 200× Wash Concentrate (provided in the kit, which contains concentrated detergent solution). The cover was removed and the wells were washed 3 times using washing solution (300 μL/well). After the last wash, the plates were blotted on absorbent paper to remove any residual buffer. (Step 5) Streptavidin-HRP

solution (5 μL) was prepared just before use by diluting with 0.5 mL of HRP-Diluent (provided in the kit). Streptavidin-HRP solution (100 μL/well) was added to each well and the wells were covered and incubated for 30 minutes. The wells were washed three times as previously described. (Step 6) Substrate solution (tetramethybenzidine as provided in the kits, 100 μL/well) was added to each well and incubated for 30 minutes at room temperature while wrapped in aluminum foil to avoid direct exposure to light. (Step 7) Stop solution ($H_2SO_4$ as provided in the kit) was added to each well (100 μL/well) to inactivate the enzyme. (Step 8) Absorbance was read on a spectrophotometer at 450 nm wavelength.

### 3.2.7 *Cell apoptosis assay*

DCs were washed and re-plated in AIM-V medium in 6-well plates ($1\times10^6$/well). Then primary T cells isolated from human blood or Jurkat T cells were added in 6-well plates at different effector: target (DCs: T cells) ratios ranging from 8:1, 4:1, 2:1, 1:1, 1:2 to 1:4. After 5-hour incubation, the cells were collected.

To detect apoptosis, the cells were fixed with 4% paraformaldehyde and were stained with TUNEL using the *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany), and analyzed by flow cytometry or confocal microscopy [13, 32]. TUNEL staining was used to detect nuclear DNA fragmentation, which is a marker of late stage apoptosis.

To observe whether cells in coculture were DCs and T cells, antibody staining was used in my pilot studies. However, antibody staining (CD3, CD4 and CD8 for T cells, and CD11c for DCs) was not efficient in labeling cells in the killing assays, which might because that apoptotic cells lose cell surface markers. Since CFSE fluorescence can be well retained in both healthy and apoptotic cells, CFSE (Molecular Probes), rather than antibody staining, was used to label cells in the killing assay. CFSE passively diffuses into cells. It is colorless and non-fluorescent until its acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester group of CFSE reacts with intracellular amines to form fluorescent conjugates that are well retained in cell cytoplasm. To make sure that one type of the cells in coculture were specifically labeled, DCs were pre-labeled with CFSE before being co-cultured with CD4 T cells. The reasons to label DCs with CFSE, but not T cells, were that (1) DCs do not divide while T cells do, and cell division can lead to decreased fluorescence in divided cells. (2) DCs can endocytose apoptotic T cells. If CFSE-labeled T cells undergo apoptosis, unlabeled DCs can endocytose CFSE-positive T cells in coculture and also become CFSE-positive. So in this chapter, DCs were labeled with CFSE but T cells were not labeled. DCs were suspended in PBS at a concentration of $1\times10^6$ cells/mL and incubated with 10 μM of CFSE for 10 minutes. The cells were then washed twice with 45 ml of ice-cold culture medium AIM-V.

#### *3.2.7.1 Flow cytometry of TUNEL staining*

Cells ($2\times10^6$) were fixed with 4% fresh paraformaldehyde in PBS (300 μl) for 30 minutes. Cells were washed with 2% FCS in PBS (4 ml). After permeabilization with permeabilization solution (0.1% sodium citrate and 0.1% Triton X-100, 100 μl) for 2 minutes on ice, cells were washed with 2% FCS in PBS (4 ml). Cells were incubated with 45 μl of TUNEL label solution and 5 μl of enzyme solution for 60 minutes at 37°C in a humidified atmosphere in the dark. Cells were washed with 2% FCS in PBS (4 ml). After TUNEL labeling and washing, the apoptosis of the DCs was determined by flow cytometry as described in Section 2.2.7.

#### *3.2.7.2 Confocal Microscopy of TUNEL staining*

Confocal microscopy was used to assess mDC morphology and nuclear DNA fragmentation.

After coculture, cells were fixed with 4% paraformaldehyde. After permeabilization in 0.1% Triton X-100 in PBS for 10 minutes, cells ($2\times10^6$) were incubated with 45 μl of TUNEL label solution and 5 μl of enzyme solution for 60 minutes. The coverslips were secured on slides using a mounting medium containing DAPI to indicate the nucleus. Labeling of cells was observed using a confocal microscope. The details of the assay have been described previously in Section 2.2.7.

### **3.2.8** ***Flow Cytometry Analysis for Expression of TCR, PD-1 and Fas on T cells***

Primary T cells or Jurkat T cells ($2\times10^6$ cells/sample) were individually incubated with PE-conjugated mouse anti-human antibodies against TCR (10 μl), PD-1 (20 μl) or Fas (5 μl) for 30 minutes. Cells were analyzed by flow cytometry as described above [31].

### 3.2.9 *Blocking assays*

To determine if the cytotoxic activity of moDCs requires cell-to-cell contact, transwell cell culture inserts (Corning Inc., Corning, NY) were used. T cells ($2.5\times10^5$ cells) and moDCs ($1\times10^6$ cells) were separated by a transwell insert (0.4 μm pore) [33] in 5 ml of AIM-V medium and co-cultured for 5 hours. Previous studies [13] have shown that 5 hours are the optimal coculture time for killing, and my study confirmed this observation.

To determine which molecules are involved in the cytotoxic activity of moDCs, cells were pre-incubated with individual antibodies (2 μg/mL) against FasL, Fas, PD-L2, PD-1 or TCR for 45 minutes [1, 31, 34]. moDCs ($1\times10^6$ cells) and target T cells ($2.5\times10^5$ cells) were co-incubated in 5 ml of AIM-V medium for 5 hours. After 5 hours of co-culture at a moDC/T cell ratio of 4:1, cells were fixed and stained with TUNEL. Cell apoptosis as determined by TUNEL staining was analyzed by flow cytometry as described above.

### *3.2.10 Cytotoxic effect of CHC patient moDCs on CD8 T cells*

During an infection, CD8 cytotoxic T lymphocyte (CTL) precursors proliferate and become effector cells by recognizing foreign peptides bound to MHC class I molecules expressed by APCs [35]. I have studied the interaction between DCs and CD4 T cells. Since DCs also interact directly with CD8 T cells, I extended my studies to determine whether moDCs from CHC patients have cytotoxic activity on CD8 T cells as well as on CD4 T cells.

PBMCs were isolated from CHC patients and healthy donors. Primary CD8 T cells were isolated from PBMCs with >95% purity by MACS (similar as that described in Chapter 2. 2). (Step 1) Before MACS, PBMCs were incubated with FcR blocking reagent to block the binding of Microbeads to the Fc receptor of human Fc receptor-expressing cells. PBMCs ($1\times10^7$ cells) were centrifuged at 300×g for 10 minutes and supernatant was aspirated completely. Cell pellet was resuspended in 60 μl of MACS buffer (containing PBS, pH 7.2, 0.5% BSA, and 2 mM EDTA). FcR blocking reagent (20 μl) was added to cell suspension. (Step 2) Cells were mixed well with PE-conjugated anti-human CD8-antibody (BD Biosciences, 20 μl antibody/$10^6$ cells) and were incubated for 5 minutes in the dark in the refrigerator (2-8°C). Cells were washed by adding 2 mL of MACS buffer per $10^7$ cells and were centrifuged at 300×g for 10 minutes. After supernatant was aspirated completely, cells (up to $10^8$) were resuspended in MACS buffer (2 mL). Cells were mixed well and were incubated with anti-PE magnetic microbeads for 15 minutes in the refrigerator (2-8°C). Cells were washed by adding 2 mL of MACS buffer per $10^7$ cells and centrifuged at 300×g

for 10 minutes. After supernatant was aspirated completely, cells (up to $10^8$) were resuspended in MACS buffer (2 mL). (Step 3) To do magnetic separation, cells were passed through MACS pre-separation filters (Miltenyi) to get rid of cell clumps that might clog the magnetic columns. (Step 4) Magnetic columns were placed in the magnetic field of a suitable MACS separator (Miltenyi). Columns were prepared by rinsing with 500 μl of MACS buffer. Cell suspension was applied onto the column. (Step 5) Cells passing through the column were unlabeled and thus were collected as CD8-negative cells. The columns were washed three times by adding 3 ml of buffer three times, each time when the column reservoir was empty. (Step 6) Columns were removed from the separator and placed on a suitable collection tube away from the magnetic field. MACS buffer (5 ml) was added onto the column. The plunger (supplied with the column) was firmly applied to immediately flush out fraction with the magnetically labeled cells (CD8-positive cells). To increase the purity of the magnetically labeled fraction, cells were passed over a second, freshly prepared column (repeating Step 4-6). After being washed twice with 10 ml of PBS, the CD8-positive cells isolated on magnetic columns were suspended in AIM-V medium.

MoDCs ($1\times10^6$ cells) were labeled with CFSE (as described in Section 3.2.7) before being co-cultured with CD8 T cells ($2.5\times10^5$ cells). After 5 hours of co-culture at a moDC/CD8 T cell ratio of 4:1, cells were fixed and stained with

TUNEL. The apoptosis of CD8 T cells ($CFSE^-$) were analyzed by flow cytometry.

## 3.3 Results

### 3.3.1 CHC patient moDCs demonstrate increased expression of FasL and PD-L2 compared to healthy donor moDCs

CHC patient moDCs demonstrated increased expression of FasL (Fig. 3-1) and PD-L2 (Fig. 3-2), while their expression of PD-L1 was unchanged compared to healthy donor moDCs (Fig. 3-3). The expressions of membrane-bound TRAIL and BLyS on cell surface membrane of moDCs were comparable in CHC patients and healthy donors (Table 3-2). The concentrations of soluble TRAIL (sTRAIL) in patient serum and in culture supernatant of patient moDCs were comparable to those of healthy donors (Table 3-3).

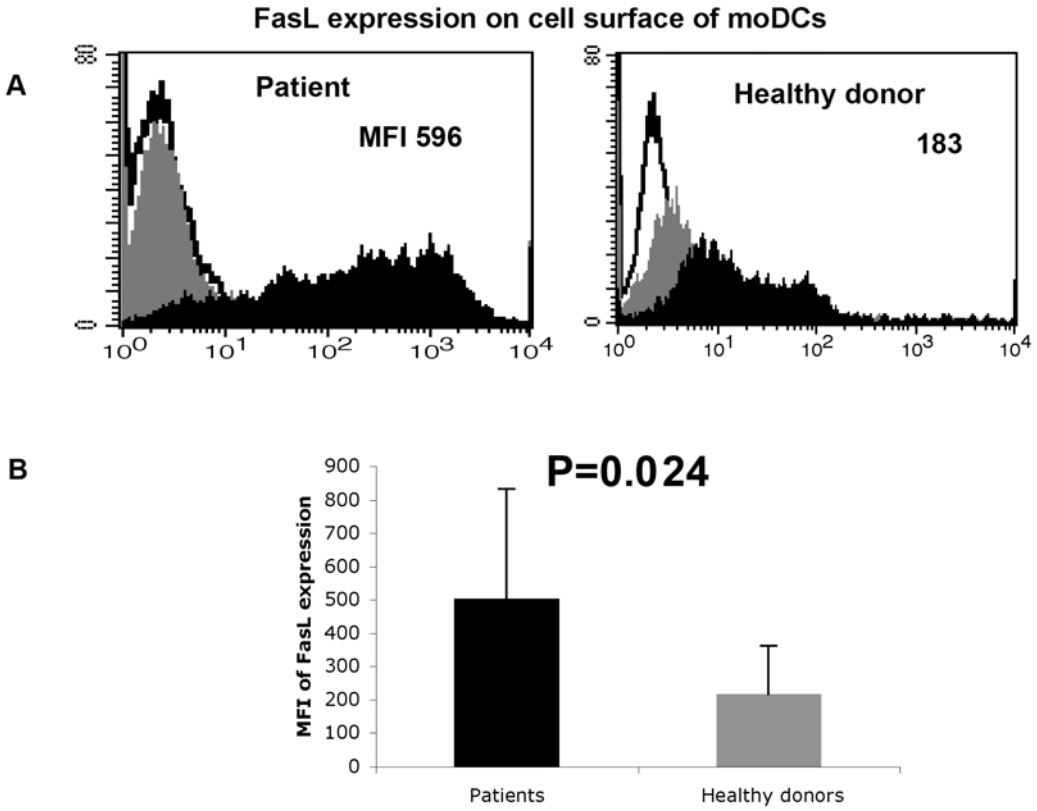



**Fig.3-1. moDCs from CHC patients express FasL at a higher level than moDCs from healthy donors.**

moDCs were prepared from adherent cells of PBMCs as described in *Materials and Methods*. The expression of FasL on the cell surface of moDCs from 20 CHC patients and 20 healthy donors was determined. moDCs were stained with specific antibodies or isotype antibodies, and analyzed by flow cytometry. White indicates unstained control, gray indicates isotype control and black indicates specific staining. Numbers indicate the mean fluorescence intensity (MFI) of FasL expression (**A)**. Unpaired *t* test is used for statistical analysis and mean ± SD is shown (**B**).

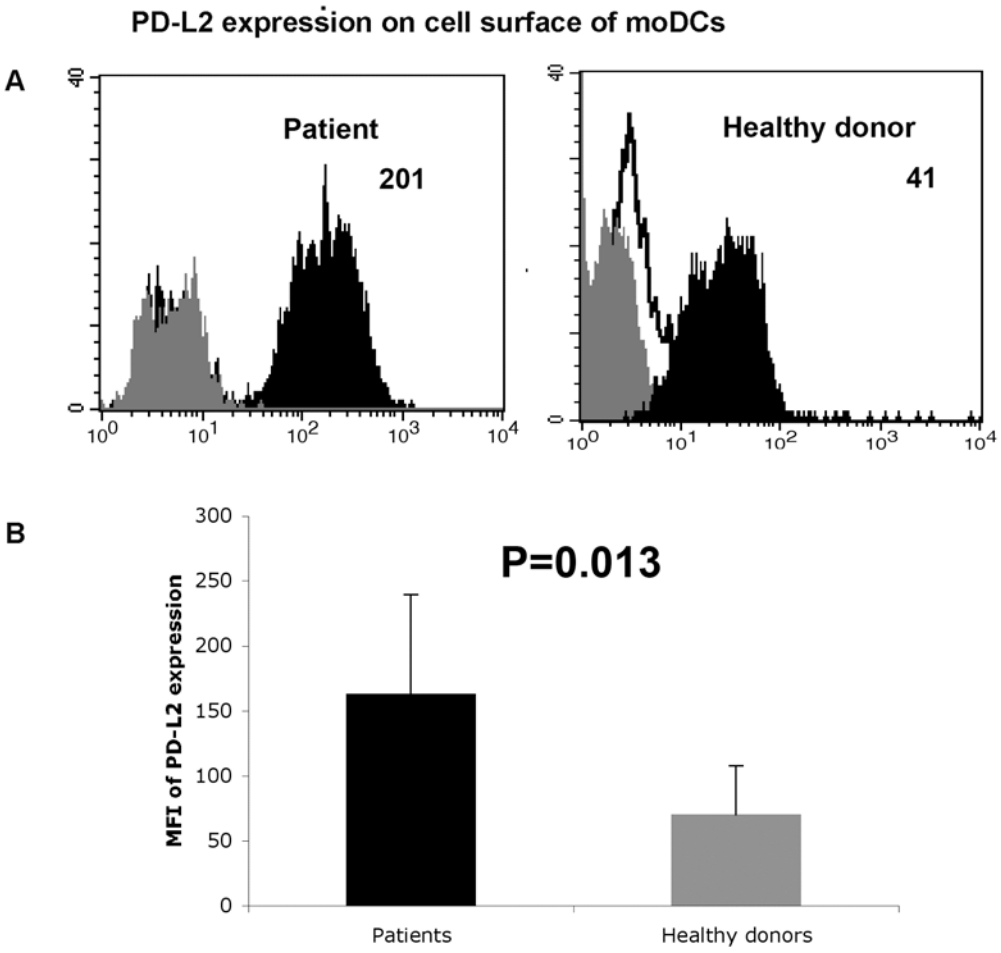



**Fig.3-2. moDCs from CHC patients express PD-L2 at a higher level than moDCs from healthy donors.**

moDCs were prepared from adherent cells of PBMCs as described in *Materials and Methods*. The expression of PD-L2 on the cell surface of moDCs from 20 CHC patients and 20 healthy donors was determined. moDCs were stained with specific antibodies or isotype antibodies, and analyzed by flow cytometry. White indicates unstained control, gray indicates isotype control and black indicates specific staining. Numbers indicate the MFI of specific expressions (**A**). Unpaired *t* test is used for statistical analysis and mean ± SD is shown (**B**).

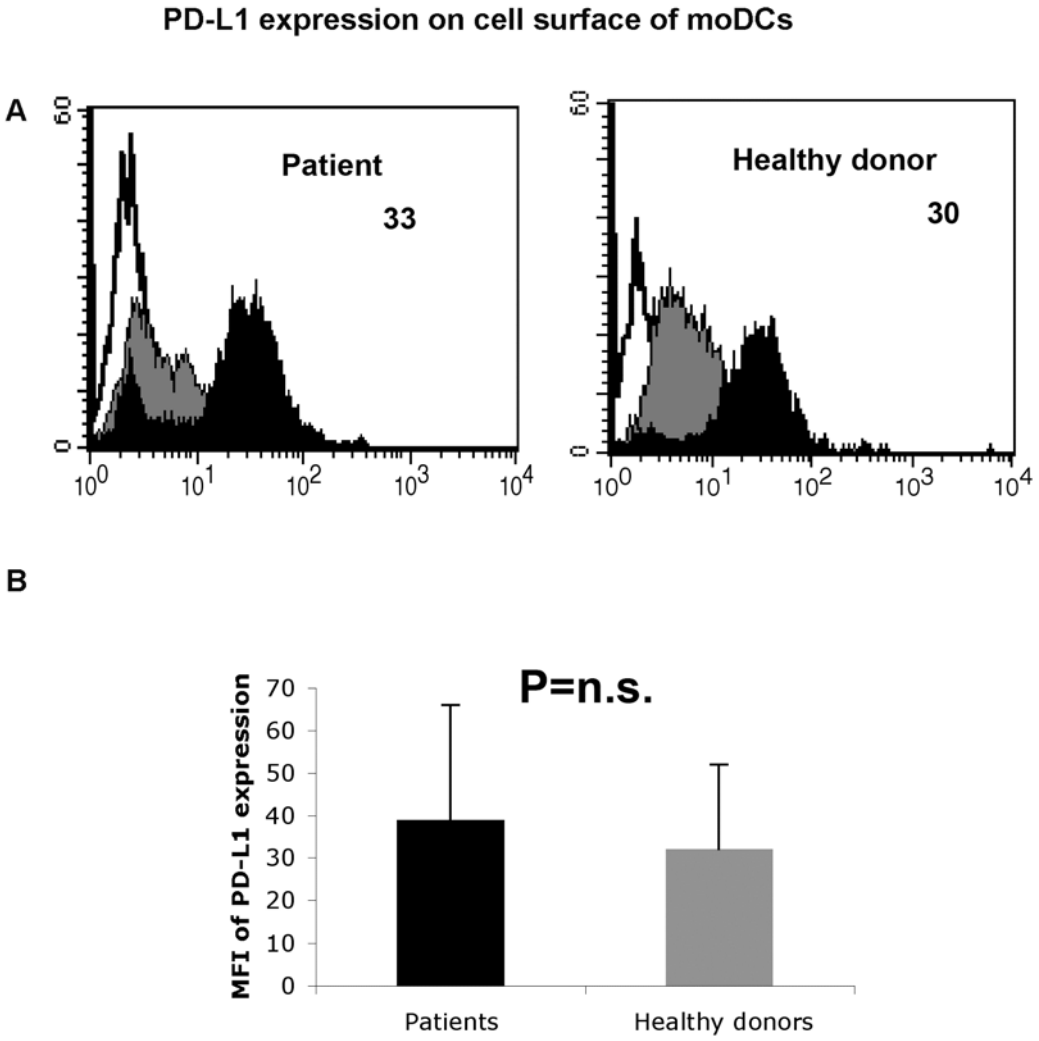



**Fig.3-3. moDCs from CHC patients express PD-L1 at a comparable level with moDCs from healthy donors.**

moDCs were prepared from adherent cells of PBMCs as described in *Materials and Methods*. The expression of PD-L1 on the cell surface of moDCs from 20 CHC patients and 20 healthy donors was determined. moDCs were stained with specific antibodies or isotype antibodies, and analyzed by flow cytometry. White indicates unstained control, gray indicates isotype control and black indicates specific staining. Numbers indicate the MFI of PD-L1 expression (**A**). Unpaired *t* test is used for statistical analysis (P=0.37) and mean ± SD is shown (**B**). *n.s.*, not significant.

**Table 3-2 Expression of TRAIL and BLyS on cell membrane of moDCs from CHC Patients and Controls**

| | HCV Patients | Controls | P value |
|---|---|---|---|
| mTRAIL | 31.2 ± 10.6 | 34.2 ± 17.4 | 0.86 |
| mBLyS | 25.5 ± 16.8 | 20 ± 13.1 | 0.48 |

NOTE. moDCs were stained with antibodies and then analyzed by flow cytometry. MFI is show. Results are expressed as means ± SD from experiments performed on 12 CHC patients and 12 controls.

**Table 3-3 Concentration of sTRAIL in serum and in culture supernatant of moDCs from CHC Patients and Controls**

| | HCV Patients | Controls | P value |
|---|---|---|---|
| sTRAIL in serum (pg/ml) | 868 ± 433 | 891 ± 324 | 0.98 |
| sTRAIL in culture supernatant (pg/ml) | 123 ± 85 | 52 ± 21 | 0.76 |

NOTE. The concentration of sTRAIL was determined by ELISA. Results are expressed as means ± SD from experiments performed on 12 CHC patients and 12 controls.

### 3.3.2 CHC patient moDCs induce the apoptosis of healthy CD4 T cells

The cell apoptosis was significantly increased where moDCs from CHC patients were co-cultured with healthy CD4 T cells compared to where moDCs from healthy controls were co-cultured with healthy CD4 T cells. This indicated that CHC patient moDCs were cytotoxic to healthy CD4 T cells. The optimal killing was observed at the moDCs: T cells (effector: target) ratio of 4:1 (Fig. 3-4 Panel A and B). As shown in the confocal microscopy picture, there were more apoptotic cells when patient moDCs were cocultured with CD4 T cells than that when healthy moDCs were cocultured with CD4 T cells (Fig. 3-4 Panel C).

Fig. 3-4A

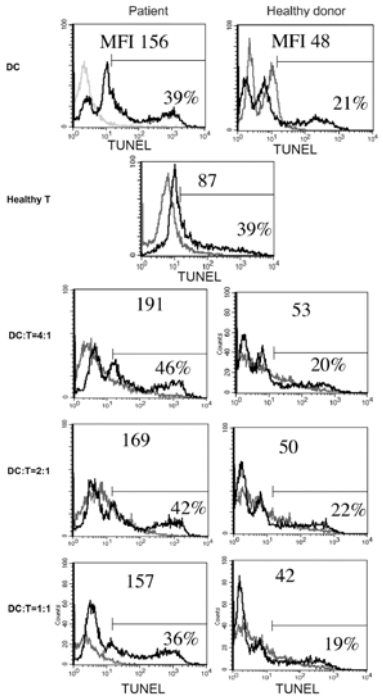



Fig. 3-4B
Increased apoptosis in the coculture of patient moDCs with allogeneic healthy T cells

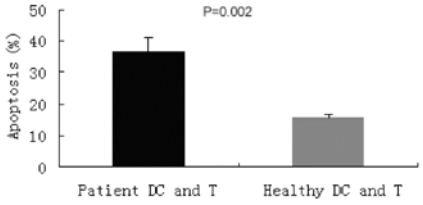



Fig. 3-4C

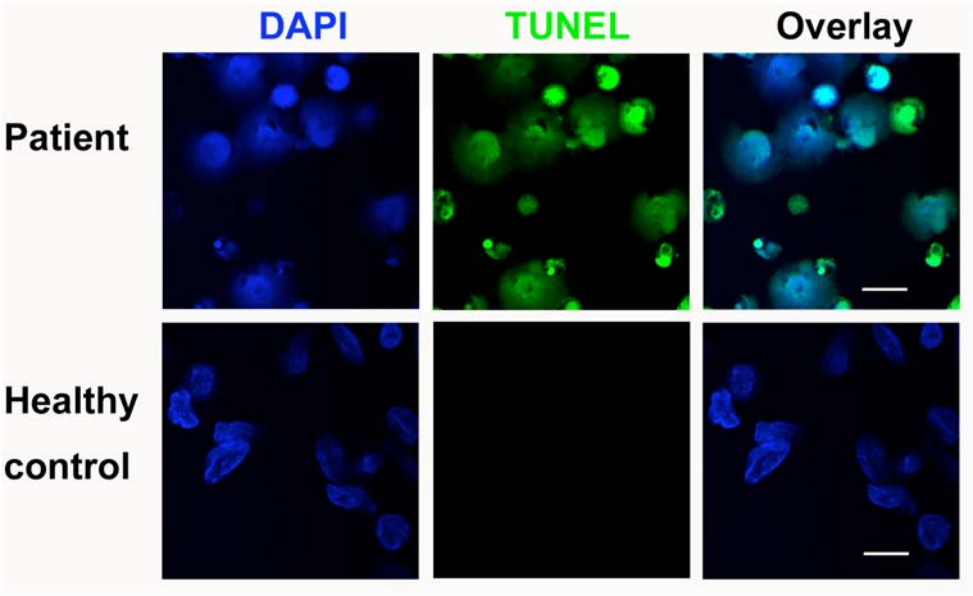



**Fig. 3-4. moDCs from CHC patients induce the apoptosis of healthy CD4 T cells.**

*In vitro* generated moDCs from CHC patients and healthy donors were incubated with allogeneic CD4 T cells from a third-party healthy donor. The cells were cocultured in AIM-V medium for 5 hours.

(**A**) Allogeneic CD4 T cells from a healthy donor were plated at the concentration of $1\times10^6$ cells/well. Then mature moDCs from CHC patients and healthy donors were co-cultured with allogeneic CD4 T cells at different ratios. Cells were co-cultured for 5 hours before TUNEL staining. Grey indicates unstained control and black indicates TUNEL staining. Numbers indicate the MFI of TUNEL-stained samples. The marker is set to make the percentage of positively-stained cells of unstained controls (background) < 1%. The percentage of positive cells of TUNEL-stained samples is shown. This figure is representative of five individual experiments.

(**B**) moDCs ($1\times10^6$ cells/well) from CHC patients or healthy donors were co-cultured with allogeneic healthy CD4 T cells ($2.5\times10^5$ cells/well) at a ratio of 4:1 for 5 hours. The overall apoptosis of cells was determined by TUNEL staining and flow cytometry analysis. The statistical data was analyzed by unpaired t-test (n=5). The statistical data is expressed as mean ± SD.

(**C**) Confocal microscopy image of cell apoptosis in coculture. *In vitro* generated moDCs ($1\times10^6$ cells/well) from CHC patients and healthy donors were incubated with allogeneic healthy CD4 T cells ($2.5\times10^5$ cells/well) for 5 hours. The ratio of moDCs to CD4 T cells was 4:1. The cells were stained with TUNEL and observed using confocal microscopy. DAPI (blue) indicates the nucleus and TUNEL (green)) indicates apoptosis. Scale bar =10 μm. This figure is representative of five individual experiments.

### 3.3.3 Both healthy CD4 T cells and CHC patient moDCs demonstrate more apoptosis after co-culture

To determine if the increased apoptosis in co-culture was due to moDCs and/or CD4 T cells, CFSE was used to label CHC patient moDCs before co-culture. $CFSE^+$ moDCs and $CFSE^-$ CD4 T cells were individually gated by flow cytometry. Interestingly, CD4 T cells demonstrated lower forward scatter (FSC) value (which indicates cell size) after co-culture (Fig. 3-5 A). This suggests cell shrinkage, which is one of the characteristics of apoptotic cells.

After moDCs were CFSE-labeled, CHC patient moDCs were co-cultured with healthy CD4 T cells. There were more apoptotic T cells when healthy CD4 T cells ($CFSE^-$ gating) were co-cultured with CHC patient moDCs compared to the same healthy CD4 T cells that were cultured alone, indicating CHC patient moDCs induced apoptosis of CD4 T cells (Fig.3-5 B, C and E). There were also more apoptototic moDCs when CHC patient moDCs ($CFSE^+$ gating) were in co-culture compared to the same moDCs that were not co-cultured (Fig. 3-5 B, D and E). This suggested that moDCs might depend on viable T cells to survive *in vitro*, and apoptotic T cells might induce moDCs to undergo apoptosis.

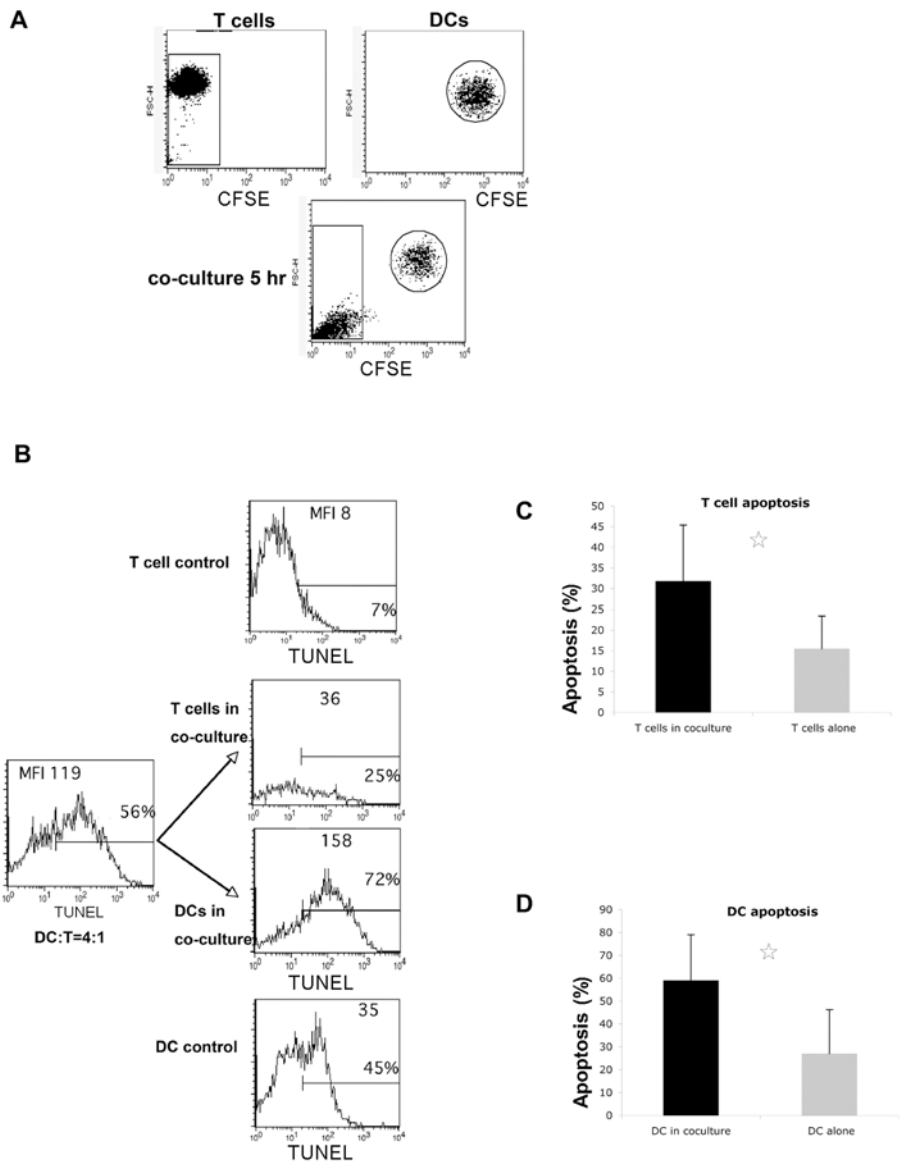



**Fig.3-5. Increased apoptosis of both healthy CD4 T cells and CHC patient moDCs after co-culture.**

**(A)** CFSE was used to label moDCs before co-culture. moDCs ($CFSE^+$) and CD4 T cells ($CFSE^-$) in the coculture were individually gated by flow cytometry. (**B)** CFSE-labeled moDCs from CHC patients were co-cultured with allogeneic healthy CD4 T cells ($2.5\times10^5$ cells/well) for 5 hours. The ratio of moDCs to CD4 T cells was 4:1. The cells cultured alone for 5 hours were used as controls. The apoptosis (TUNEL) of healthy CD4 T cells ($CFSE^-$ gating) and CHC patient moDCs ($CFSE^+$ gating) in the co-culture was analyzed individually. Numbers indicate the MFI of TUNEL-stained samples. The marker is set to make the percentage of positive cells of unstained controls (background) < 1%. The

percentage of positive cells of TUNEL-stained samples is shown. This figure is representative of five individual experiments.
(**C**) The apoptosis of T cells in coculture was compared to the apoptosis of T cells being cultured alone. The statistical result is analyzed by unpaired Student t test (P=0.039) and is expressed as means ± SD from five individual experiments.
(**D**) The apoptosis of moDCs in coculture was compared to the apoptosis of moDCs being cultured alone (P=0.018, unpaired Student t test). The statistical result is expressed as means ± SD from five individual experiments.

**E**

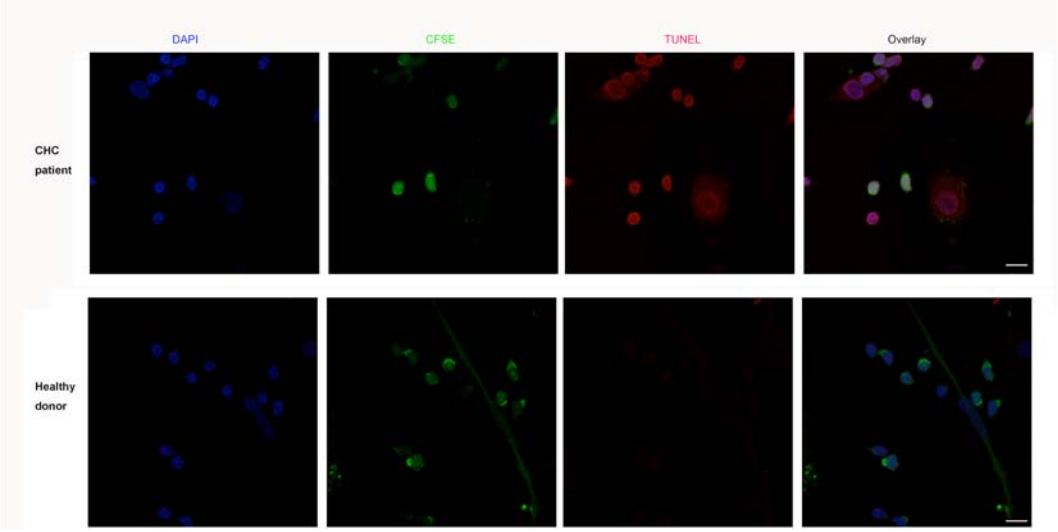



**Fig. 3-5 E.** Confocal microscopy image of cell apoptosis in coculture. CFSE was used to label moDCs before co-culture. *In vitro* generated moDCs from CHC patients and healthy donors were incubated with allogeneic healthy CD4 T cells ($2.5\times10^5$ cells/well) for 5 hours. The ratio of moDCs to CD4 T cells was 4:1. The cells were stained with TUNEL and observed using confocal microscopy. DAPI (blue) indicates the nucleus, CFSE (green) indicates moDCs and TUNEL (red) indicates apoptosis. Scale bar =10 μm. This figure is representative of five individual experiments.

### 3.3.4 Specific antibodies and transwell membranes can block the killing of CHC patient moDCs

CHC patient moDCs demonstrated increased expression of FasL (Fig. 3-1) and PD-L2 (Fig. 3-2), and had a killing effect on peripheral blood T cells (Fig. 3-4 and 3-5). It was hypothesized that the killing effect of patient moDCs may be mediated by the interactions between certain ligands (FasL, PD-L2) expressed on moDCs with the corresponding receptors (Fas, PD-1) expressed on target T cells, and if so, antibodies against these molecules may block the killing.

To test this hypothesis, antibodies against FasL and PD-L2 and their corresponding receptors were used to block the interaction between these molecules. It was found that antibodies against FasL, Fas, PD-L2 and PD-1 all partially abolished the killing effect, but isotype control antibodies did not (Fig. 3-6). These results suggested that the killing effect was mediated by the interactions between FasL/Fas and PD-L2/PD-1. Unexpectedly, antibody against TCR also partially blocked the killing (Fig. 3-6). This suggested that the killing effect might require cell-cell contact, since the interaction between TCR and HLA molecules is important for the contact between DCs and T cells [36].

Furthermore, the killing was almost totally blocked when moDCs and T cells were cultured in two chambers of transwell plates separated by polycarbonate membranes (Fig. 3-6), which confirmed that the killing of patient moDCs

primarily required cell-cell contact rather than being mediated by soluble effector molecules (e.g. TNFα).

These results demonstrated that the cytotoxic activity of CHC patient moDCs required cell-cell contact, and it was primarily mediated by the interactions between FasL/Fas and PD-L2/PD-1.

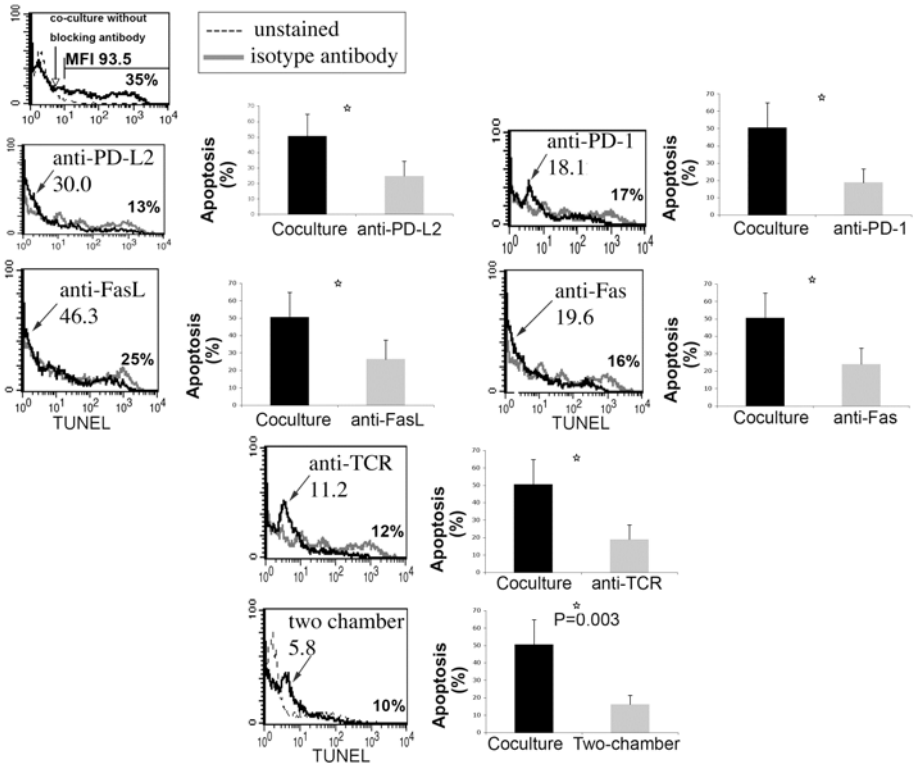



**Fig. 3-6. The cytotoxic activity of moDCs from CHC patients can be abolished either by a transwell membrane, or specific antibodies against Fas/FasL, PD-1/PD-L2 and TCR.**

In the blocking assay, cells were pre-incubated with antibodies for 45 minutes before mature moDCs from CHC patients and allogeneic CD4 T cells from healthy donors were co-cultured. In the transwell assay, CHC patient moDCs and healthy CD4 T cells ($2.5\times10^5$ cells/well) were cultured in two chambers isolated by a transwell permeable support (0.4 μm polycarbonate membrane). The effector: target (moDCs: CD4 T cells) ratio was 4:1. The cells were co-cultured for 5 hours before TUNEL staining. Numbers indicate the MFI of TUNEL-stained samples. The marker is set to make the percentage of positive cells of unstained controls (background) < 1%. The percentage of positive cells of TUNEL-stained samples is shown. This flow cytometry plot is representative of five individual experiments. The statistical data was analyzed by unpaired t test (n=5) and is expressed as mean ± SD (P=0.0094 for blocking PD-L2, P=0.0053 for blocking PD-1, P=0.016 for blocking FasL, P=0.0075 for blocking Fas, P=0.035 for blocking TCR, and P= 0.003 for blocking with two-chamber).

### 3.3.5 Effect of CHC patient moDCs on Jurkat T cells

To test if CHC patient moDCs can kill tumor cells, a human leukemia T cell line, Jurkat T cells, was selected as a target T cell line. Being sensitive to Fas-mediated apoptosis, Jurkat T cells have been widely used as a target in killing assays [37-39]. Surprisingly, when patient moDCs were co-cultured with Jurkat T cells, the apoptosis was comparable to that of healthy donor moDCs co-cultured with Jurkat T cells. The same result was observed at different effector: target ratios ranging from 8:1 to 1:4 (Fig. 3-7 A and B). These results suggest that Jurkat T cells are not sensitive to the killing of CHC patient moDCs.

As shown in Fig. 3-6, the killing of CHC patient moDCs requires cell-cell contact, and it was primarily mediated by the interactions between FasL/Fas and PD-L2/PD-1. It was hypothesized that Jurkat T cells express one or more important cell surface molecules at different levels, which leads to their poor cell-cell contact with moDCs and their lack of sensitivity to the killing of CHC patient moDCS. To test this hypothesis, the expression of PD-1, Fas and TCR on Jurkat T cells was examined. The expression of PD-1 and Fas on Jurkat T cells and healthy CD4 T cells was comparable, however, Jurkat T cells expressed TCR at a lower level than healthy CD4 T cells (Fig. 3-7 C).

There was the slight possibility that the Jurkat T cells used in my study were not sensitive to any apoptosis-inducing agents, or that the TUNEL method used to detect apoptosis was not appropriate for Jurkat T cells. To test these

possibilities, Jurkat T cells were exposed to UV light that leads to DNA damage and thus induces cells to undergo apoptosis. I found that Jurkat T cells were sensitive to UV light-induced apoptosis (Fig. 3-7 D). This result showed that Jurkat T cells used in my study could be induced to undergo apoptosis. The lack of sensitivity to the killing of CHC patient moDCs may due to their poor expression of TCR, which facilitates the cell-cell contact with moDCs.

Fig. 3-7 A

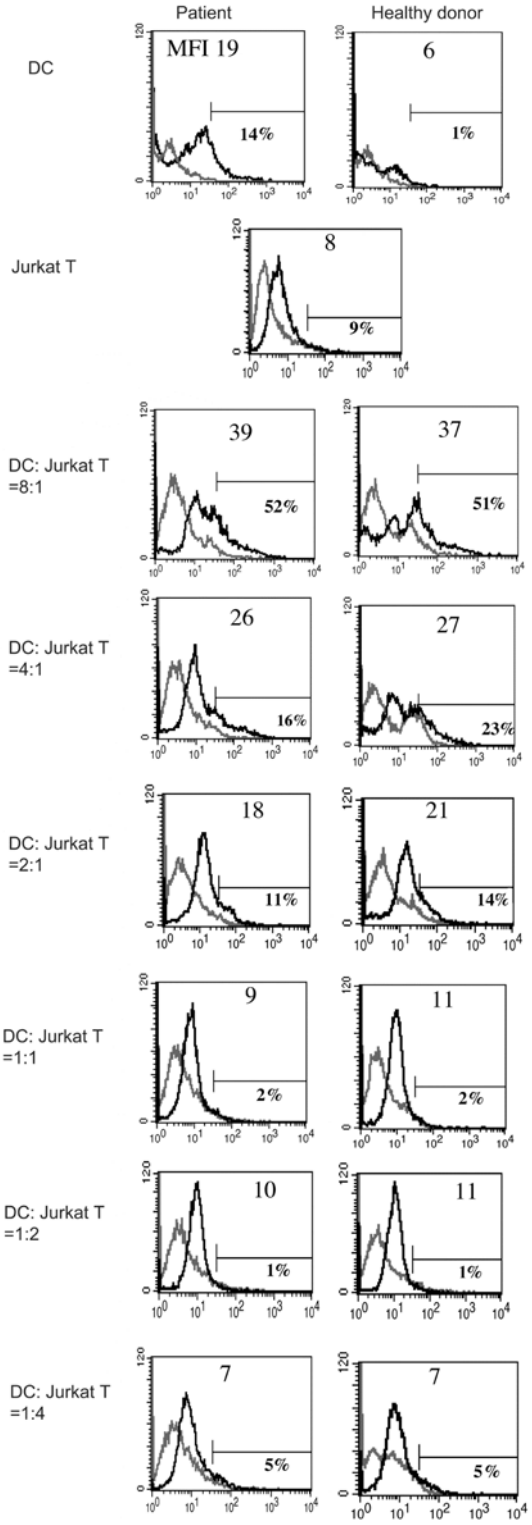



Fig. 3-7 B

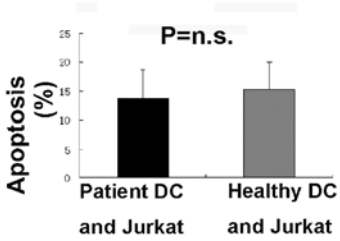


**Fig. 3-7 (A-B). moDCs from CHC patients have a poor killing effect on Jurkat T cells.**

**A.** Jurkat T cells were plated at the concentration of $1\times10^6$ cells/well. Then mature moDCs (irradiated) from CHC patients and healthy donors were co-cultured with Jurkat T cells at different ratios. Cells were co-cultured for 5 hours before TUNEL staining. Grey indicates unstained control and black indicates TUNEL staining. Numbers indicate the MFI of TUNEL-stained samples. The marker is set to make the percentage of positive cells of unstained controls (background) < 1%. The percentage of positive cells of TUNEL-stained samples is shown.
**B.** moDCs from CHC patients or healthy donors were co-cultured with Jurkat T cells ($2.5\times10^6$ cells/well) at a ratio of 4:1 for 5 hours. The overall apoptosis of cells was determined by TUNEL staining and flow cytometry analysis. The statistical data was analyzed by unpaired t-test (n=5).

**Fig. 3-7C**

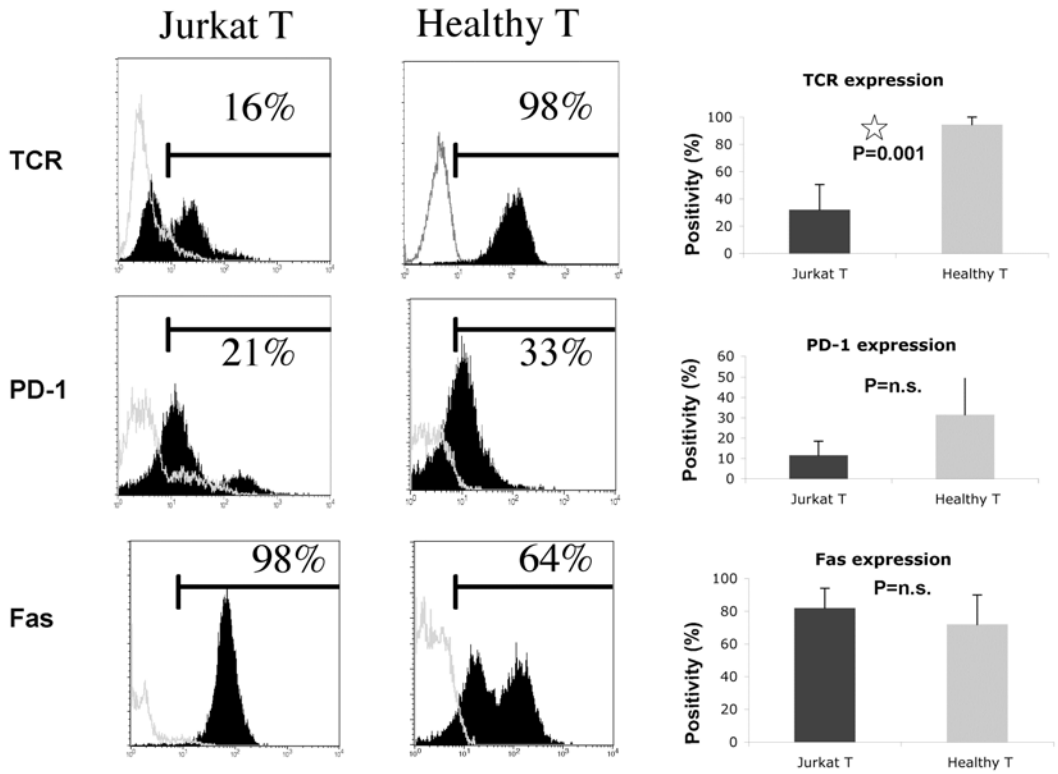



**Fig. 3-7 (C) Jurkat T cells used in this thesis express TCR at a lower level than healthy CD4 T cells.**

Jurkat T cells and healthy CD4 T cells were stained by antibodies against TCR, PD-1 and Fas, respectively. Cells were analyzed by flow cytometry. Grey indicates isotype control and black indicates specific staining. Percentage of positive cells is shown. The flow cytometry figures are representative of five individual experiments. The statistical data was analyzed by unpaired t test (n=5) and is expressed as mean ± SD. The positivity of PD-1 (P=0.13) and Fas (P=0.32) expression on Jurkat T cells and healthy CD4 T cells are comparable. Jurkat T cells express TCR at a lower level than healthy CD4 T cells (P=0.001).

**Fig. 3-7D**

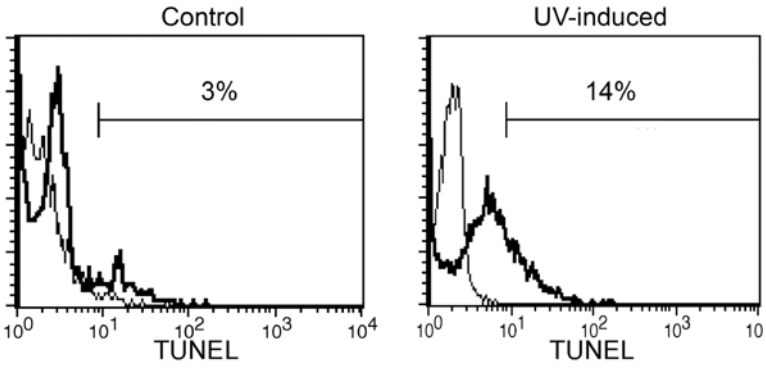



**Fig. 3-7 (D) Jurkat T cells can be induced to undergo apoptosis.**

To induce apoptosis by UV light, Jurkat T cells were exposed to UV light for 1 hour. The UV-induced cells and controls Jurkat T cells (without UV induction) were collected. Jurkat T cells were stained with TUNEL reagents and cell apoptosis was analyzed by flow cytometry. Fine line shows unstained control and thick line indicates TUNEL staining. The marker is set to make the percentage of positive cells of unstained controls (background) < 1%. The percentage of positive cells of TUNEL-stained samples is shown. This figure is representative of three individual experiments.

### 3.3.6 Patient moDCs have a killing effect on autologous patient T cells

To facilitate comparison of the tolerogenic activity of moDCs from CHC patients and healthy donors, allogeneic healthy CD4 T cells from a third party were used as target cells as described in the previous assays. I have found that CHC patient moDCs have up-regulated tolerogenic activity and were capable of killing healthy CD4 T cells (Fig. 3-4 and 3-5). However, autologous CHC patient T cells were the potential targets of the DCs in CHC patients. Therefore, I examined the apoptosis-inducing effect of moDCs from CHC patient on autologous patient CD4 T cells.

I found that CD4 T cells from CHC patient were undergoing more spontaneous apoptosis than CD4 T cells from healthy donors (Fig. 3-8 A and B). Patient moDCs could kill autologous patient T cells *in vitro*. The optimal effector: target (moDCs: T cells) ratio for autologous killing was 4:1 (Fig. 3-8 A), similar to that observed in allogeneic killing assay.

The increased total apoptosis in the patient co-culture system was not simply attributable to the spontaneous apoptosis of cells, since the total apoptosis (for example, MFI=550 when the moDCs: T cells ratio was 4:1, Fig. 3-8 A) in the patient co-culture system is much higher than the spontaneous apoptosis of the cells when they were cultured alone (MFI of patient T cells =239 and MFI of patient moDCs =120). This result (Fig. 3-8 A) supports the concept of CHC patient moDCs inducing apoptosis on patient CD4 T cells.

To determine if apoptosis of CHC patient CD4 T cells was significantly induced by autologous patient moDCs, patient CD4 T cells were cocultured with CFSE-labeled CHC patient moDCs. As shown by flow cytometry (Fig. 3-8 C), patient CD4 T cells ($CFSE^-$ gating) have a significantly higher apoptosis after being cocultured with autologous patient moDCs compared to CD4 T cells being cultured alone.

CHC patient CD4 T cells express PD-1 at an increased level compared to healthy CD4 T cells (Fig. 3-8 D and E). The PD-1 expression on target T cells may mediate the killing effect of CHC patient moDCs on patient CD4 T cells.

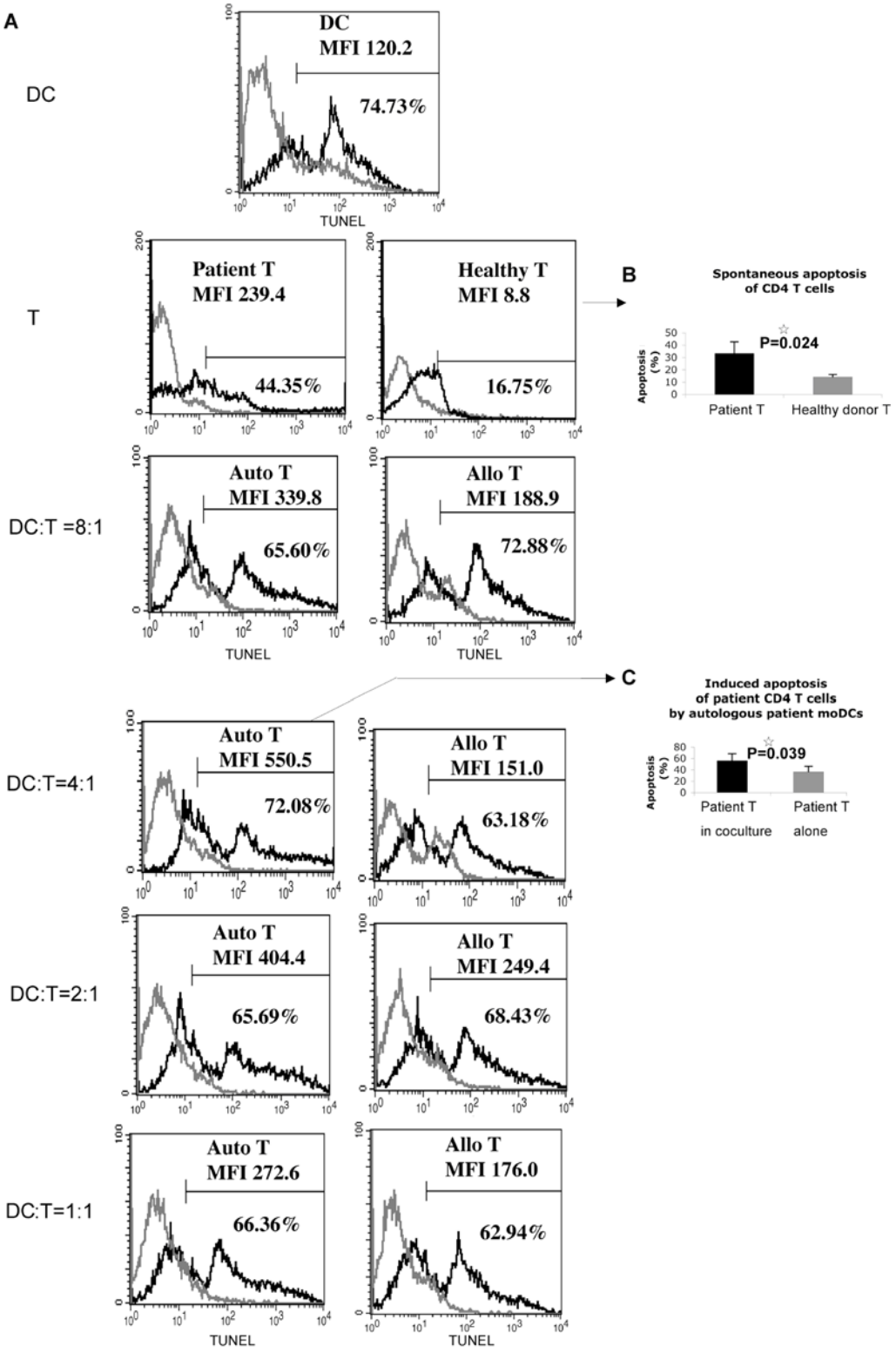



**Fig. 3-8. moDCs from CHC patients can induce the apoptosis of autologous patient CD4 T cells.**

**(A**) Mature moDCs from CHC patients were co-cultured with either autologous CD4 T cells from the same CHC patients, or allogeneic CD4 T cells from healthy donors. Cells were co-cultured at different ratios for 5 hours before TUNEL staining. Grey indicates unstained control and black indicates TUNEL staining. Numbers indicate the MFI of TUNEL-stained samples. The marker is set to make the percentage of positive-stained cells of unstained controls (background) < 1%. The percentage of positive cells of TUNEL-stained samples is shown.
**(B**) The spontaneous apoptosis of CD4 T cells from CHC patients is significantly higher than that of CD4 T cells from healthy donors. CD4 T cells were isolated by MACS, stained by TUNEL, and analyzed by flow cytometry. The statistical data was analyzed by unpaired t test (n=5) and is expressed as mean ± SD (P=0.024).
**(C**) CHC patient CD4 T cells have a significantly higher apoptosis after being cocultured with autologous patient moDCs. CD4 T cells were cultured with or without CFSE-labeled moDCs from the same CHC patient. In the coculture, the ratio of CHC patient moDCs to autologous patient CD4 T cells ($2.5 \times 10^5$ cells) was 4:1. After TUNEL staining, the apoptosis of CD4 T cells ($CFSE^-$ gating) with or without coculture with moDCs was examined by flow cytometry. The statistical data was analyzed by unpaired t test (n=5) and is expressed as mean ± SD (P=0.039).

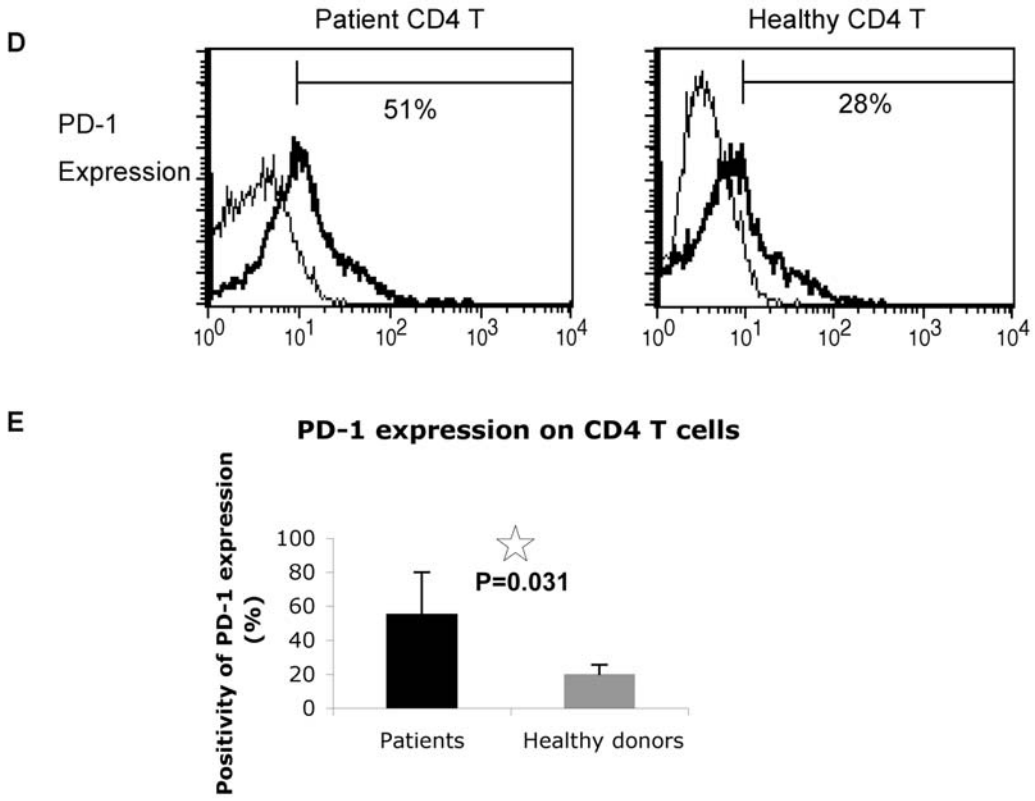



**Fig.3-8. (D**) CHC patient CD4 T cells express PD-1 at a higher level than healthy CD4 T cells. CD4 T cells were isolated from peripheral blood of donors by MACS. Cells were stained by an antibody against PD-1 and were analyzed by flow cytometry. Fine line indicates isotype control and thick line indicates specific staining. The gate is set to make the percentage of positive cells of isotype antibody control of healthy CD4 T cell (background) < 1%. The percentage of PD-1 positive cells of specific antibody-stained sample is shown. This figure is representative of five individual experiments.

**(E**) CHC patient CD4 T cells express PD-1 at a significantly higher level than healthy CD4 T cells. CD4 T cells were isolated by MACS, stained by an antibody against PD-1, and were analyzed by flow cytometry. The statistical data was analyzed by unpaired t test (n=5) and is expressed as mean ± SD (P=0.031).

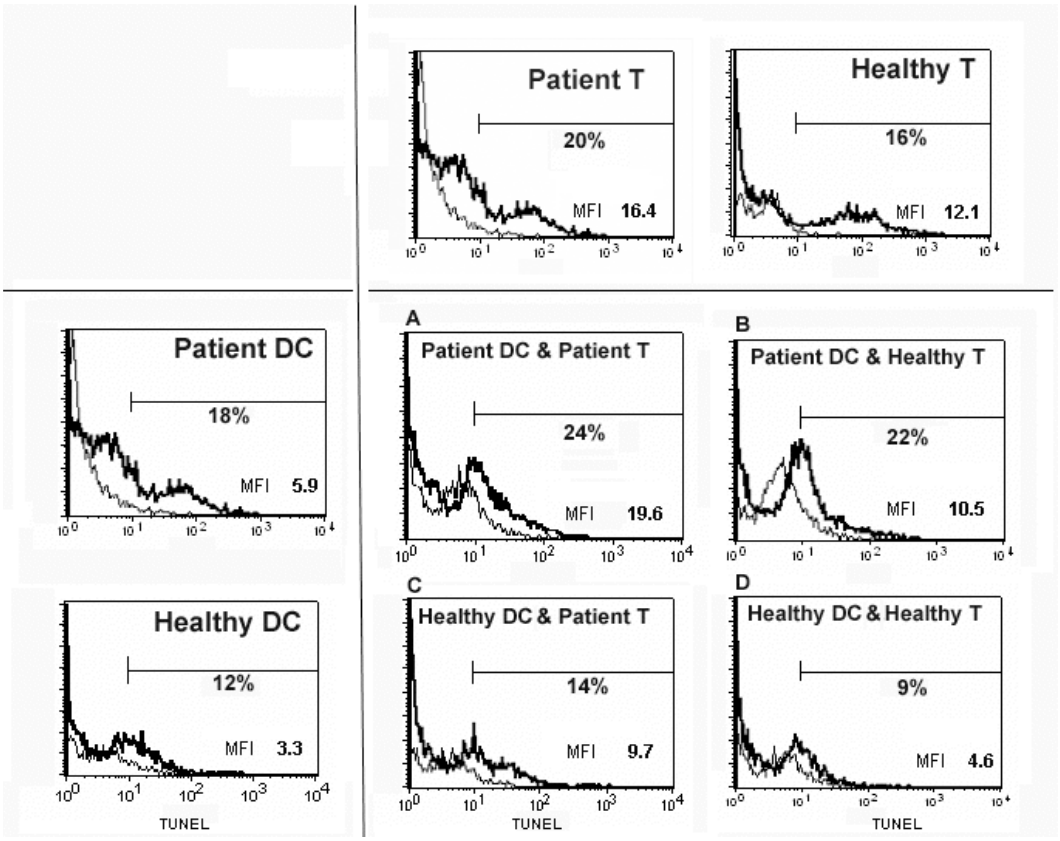
### 3.3.7 CHC patient moDCs induce the apoptosis of CD4 T cells, but healthy moDCs do not

Coculture of CHC patient moDCs with autologous CD4 T cells demonstrated increased apoptosis (Fig. 3-8). The coculture assays suggested that CHC patient moDCs were cytotoxic to CD4 T cells, which correlated with the result that CHC patient moDCs have up-regulated expression of FasL (Fig. 3-1) and PD-L2 (Fig. 3-2). However, there was a possibility that coculture of autologous immune cells may lead to the cytotoxic effect. To test the possibility that the cytotoxic effect was simply due to the co-culture of autologous immune cells, healthy donor moDCs were cocultured with autologous CD4 T cells. To ensure that the appropriate controls were used in the autologous and allogeneic coculture, two types of CD4 T cells (either from CHC patients or from healthy donors) were cocultured with two types of moDCs (either from the same CHC patients or from the same healthy donors).

Both groups in which T cells from patient (Fig. 3-9 A, 24%) and T cells from healthy donors (Fig. 3-9 B, 22%) were co-cultured with CHC patient moDCs demonstrated increased apoptosis compared to these cells being cultured alone (20% and 16% for patient and healthy T cells, and 18% and 12% for patient and healthy MoDCs, respectively). The highest percentage of cell apoptosis (Fig. 3-9 A, 24%) was observed in the group where patient moDCs were co-cultured with autologous patient T cells. The data support the concept that patient moDCs can kill autologous patient T cells *in vitro*.

Furthermore, both groups in which patient T cells (Fig. 3-9 C, 14%) and healthy donor T cells (Fig. 3-9 D, 9%) were co-cultured with healthy donor moDCs did not demonstrate increased apoptosis compared to these cells being cultured alone. The data further support the concept that patient moDCs have up-regulated cytotoxic activity compared to healthy moDCs.

I further analyzed the apoptosis of CD4 T cells with or without coculture with autologous moDCs. CHC patient CD4 T cells cocultured with autologous moDCs demonstrated higher apoptosis than these cells being cultured alone (Fig. 3-9 E), and the difference was significant (Fig. 3-9 F). Healthy CD4 T cells cocultured with autologous moDCs demonstrated apoptosis comparable to these cells being cultured alone (Fig. 3-9 G and H). This result excluded that possibility that coculture of moDCs and CD4 T cells always leads to increased apoptosis. This further supported my previous results that the CHC patient moDCs indeed had cytotoxic activity on CD4 T cells.


Patient T
20%
MFI 16.4
Healthy T
16%
MFI 12.1
Patient DC
18%
MFI 5.9
A
Patient DC & Patient T
24%
MFI 19.6
B
Patient DC & Healthy T
22%
MFI 10.5
Healthy DC
12%
MFI 3.3
C
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14%
MFI 9.7
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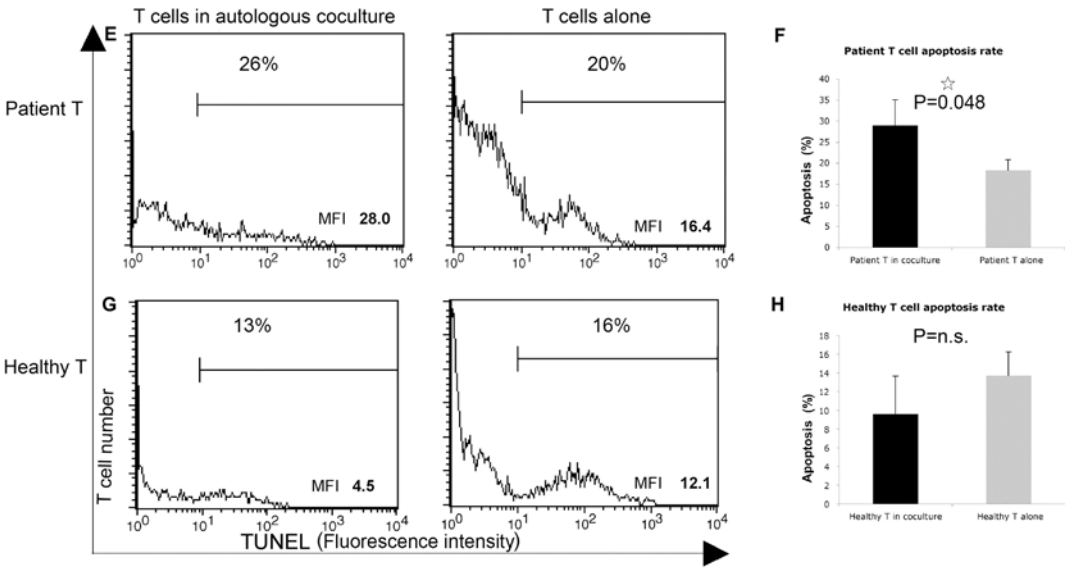
T cells in autologous coculture
T cells alone
E
Patient T
26%
MFI 28.0
20%
MFI 16.4
F
Patient T cell apoptosis rate
P=0.048
Apoptosis (%)
Patient T in coculture
Patient T alone
G
Healthy T
13%
MFI 4.5
16%
MFI 12.1
H
Healthy T cell apoptosis rate
P=n.s.
Apoptosis (%)
Healthy T in coculture
Healthy T alone
T cell number
TUNEL (Fluorescence intensity)

**Fig. 3-9. moDCs from healthy donors have a poor cytotoxic activity on autologous and allogeneic CD4 T cells.**

PBMCs were obtained from CHC patients and healthy donors. To obtain mature moDCs, monocytes from PBMCs were cultured in medium containing IL-4 and GM-CSF for 6 days to differentiate into moDCs and then were stimulated with LPS for 6 hours. CD4 T cells isolated from PBMCs were also cultured alone in medium for 6 days before being co-cultured. After washing with fresh medium, moDCs were co-cultured with either autologous or allogeneic CD4 T cells *in vitro* for 5 hours. The ratio of moDCs to CD4 T cells was 4:1. Overall cell apoptosis was determined by TUNEL staining and flow cytometry analysis **(A-D)**. Flow cytometry plots on the left and upper areas show the cell apoptosis of the control samples, moDCs or CD4 T cells, being cultured alone for 5 hours. Flow cytometry plots on the right lower area show the cell apoptosis of co-cultured samples. (**A)** moDCs from a CHC patient co-cultured with autologous T cells isolated from the same CHC patient. (**B)** moDCs from a CHC patient co-cultured with allogeneic T cells isolated from a healthy donor. (**C)** moDCs from the healthy donor co-cultured with allogeneic T cells isolated from the CHC patient. **(D)** moDCs from the healthy donor co-cultured with autologous T cells isolated from the same healthy donor. The marker is set to make the percentage of positive cells of unstained healthy CD4 T cell control (background) < 1%. The percentage of positive cells of TUNEL-stained sample subtracted by that of unstained control is shown. This figure is representative of five individual experiments performed on samples from five randomly chosen male CHC patients and their corresponding age-matched healthy male donors.

**(E-H)** moDCs from healthy donors have a poor cytotoxic activity on CD4 T cells. The apoptosis of patient CD4 T cells being co-cultured with autologous patient moDCs was compared with the apoptosis of patient T cells being cultured alone (**E**). The statistical result is expressed as mean ± SD from five individual experiments performed on 5 CHC patients and 5 healthy donors (P=0.048) (**F**). The apoptosis of healthy CD4 T cells being co-cultured with autologous healthy moDCs was compared with the apoptosis of healthy T cells being cultured alone (**G**). The statistical result is expressed as mean ± SD from five individual experiments (P=0.22) (**H**). The marker is set to make the percentage of positive cells of unstained controls (background) < 1%. The percentage of positive cells of TUNEL-stained samples is shown.

### 3.3.8 *Ex vivo* mDCs from CHC patients demonstrate up-regulated cytotoxic activity compared to *ex vivo* mDCs from healthy donors.

My previous studies have demonstrated that *in vitro* generated moDCs from CHC patients have a changed phenotype (increased expression of FasL and PD-L2) and changed tolerogenic activity compared to moDCs from healthy donors. To examine the phenotype and tolerogenic activity of *ex vivo* mDCs from peripheral blood of CHC patients and healthy donors, mDCs were isolated using MACS.

*Ex vivo* isolated mDCs from CHC patients have increased expression of FasL and PD-L2 compared to *ex vivo* isolated mDCs from healthy donors (Fig. 3-10 A). To determine if *ex vivo* isolated mDCs from CHC patients demonstrated changed tolerogenic activity compared to *ex vivo* isolated mDCs from CHC patients, the *ex vivo* mDCs were co-cultured with allogeneic healthy CD4 T cells and were examined for TUNEL signal by confocal microscopy. The cell apoptosis was increased when CHC patient *ex vivo* mDCs were co-cultured with allogeneic healthy CD4 T cells compared to healthy *ex vivo* mDCs co-cultured with the same allogeneic CD4 T cells (Fig. 3-10 B). Both CD4 T cells ($CFSE^-$) and the *ex vivo* mDCs ($CFSE^+$) in the patient co-culture had more apoptosis than the cells in the healthy donor co-culture. The result indicated that mDCs *ex vivo* isolated from CHC patients had up-regulated tolerogenic activity and were capable of killing CD4 T cells.

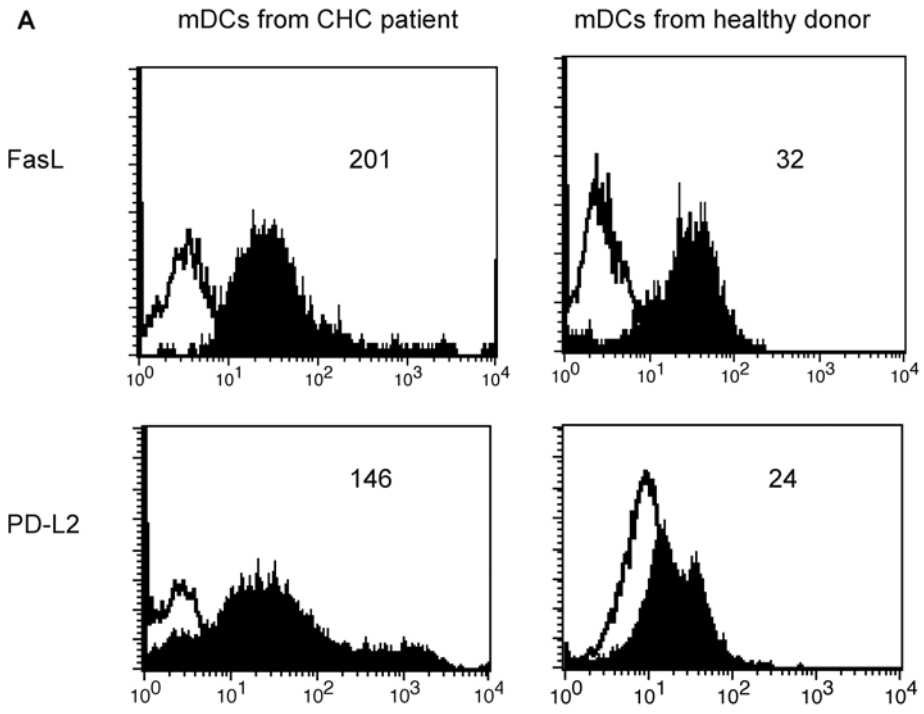


**Fig.3-10. The mDCs *ex vivo* isolated from peripheral blood of CHC patients induce the apoptosis of healthy CD4 T cells.**

(**A**) Expression of FasL and PD-L2 on *ex vivo* mDCs from CHC patients and *ex vivo* mDCs from healthy donors. *Ex vivo* mDCs were isolated from peripheral blood using BDCA-1 positive isolation kit by MACS. mDCs were stained with specific antibodies or isotype antibodies, and analyzed by flow cytometry. White indicates isotype control and black indicates specific staining. Numbers indicate the MFI of specific expressions. This figure is representative of five individual experiments.

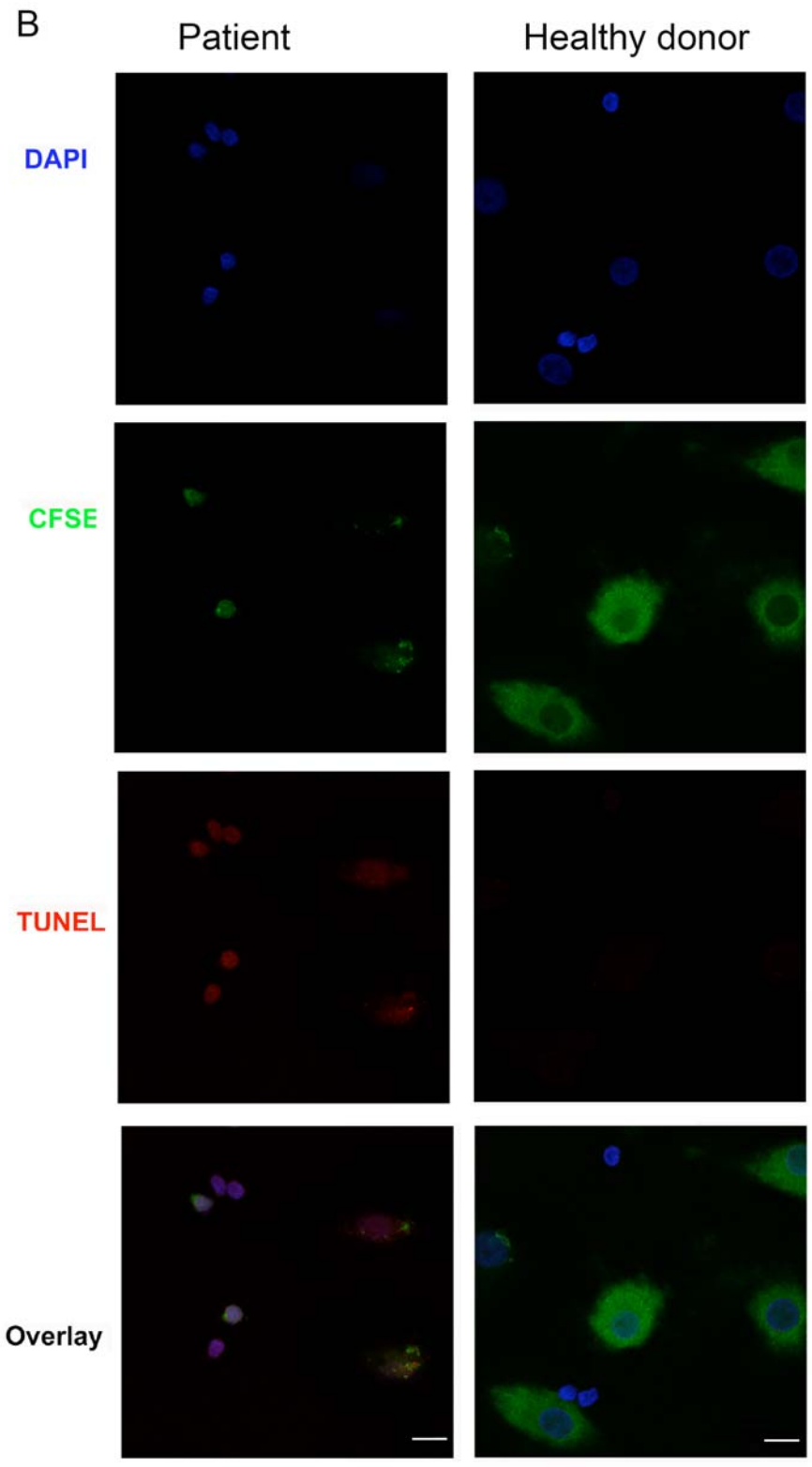
B
Patient
Healthy donor
DAPI
CFSE
TUNEL
Overlay

**Fig.3-10. The mDCs *ex vivo* isolated from peripheral blood of CHC patients induce the apoptosis of healthy CD4 T cells.**

(**B**) Confocal microscopy image of cell apoptosis in coculture. *Ex vivo* mDCs were isolated from peripheral blood using BDCA-1 positive isolation kit by MACS. CFSE was used to label mDCs before co-culture. *Ex vivo* mDCs from CHC patients and healthy donors were incubated with allogeneic healthy CD4 T cells for 5 hours. The ratio of mDCs to CD4 T cells was 2:1. The cells were stained with TUNEL and observed using confocal microscopy. DAPI (blue) indicates the nucleus, CFSE (green) indicates mDCs and TUNEL (red) indicates apoptosis. Scale bar =10 μm. This figure is representative of five individual experiments.

### *3.3.9 CD8 T cells are sensitive to apoptosis induced by moDCs from CHC patients*

Both CD4 [40] and CD8 T-cell responses [41] are important for HCV clearance. My study has demonstrated that CD4 T cells, both from healthy donor (Fig. 3-4) and CHC patient (Fig. 3-8), were sensitive to the cytotoxic activity of CHC patient moDCs. Since DCs also interact directly with CD8 T cells, I further examined whether moDCs from CHC patients have tolerogenic activity on CD8 T cells.

CD8 T cells from CHC patients were cocultured with autologous patient moDCs (Fig. 3-11 A) and demonstrated more apoptosis than the same CD8 T cells without coculture (Fig. 3-11 B), and the difference was significant (Fig. 3-11 C). This indicates that CD8 T cells were also sensitive to the tolerogenic activity of CHC patient moDCs.

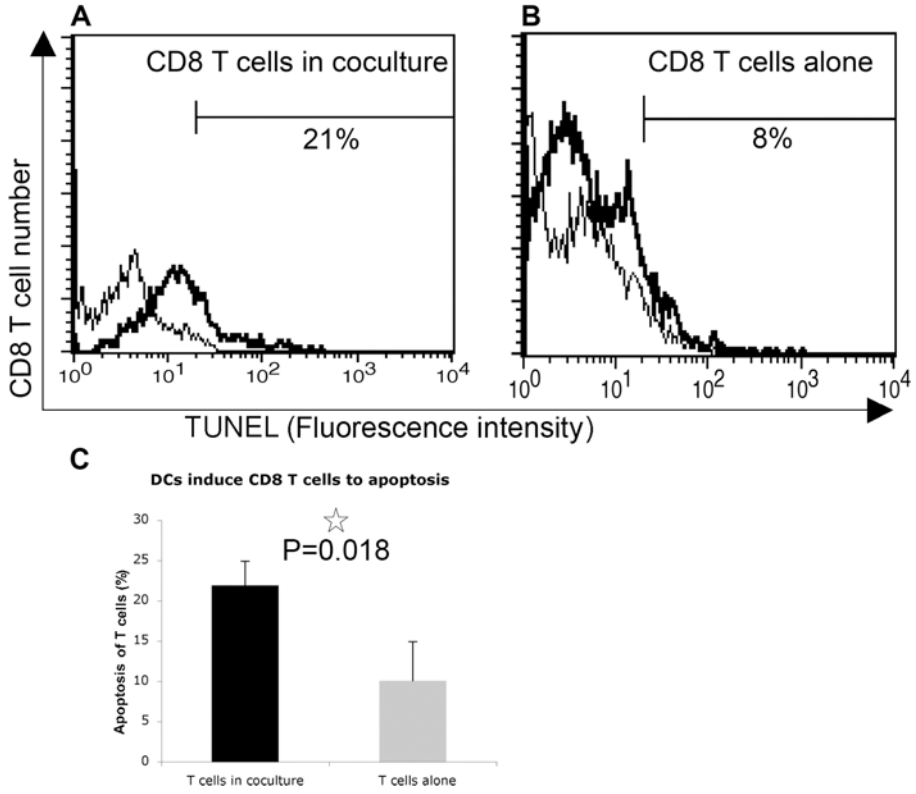


**Fig. 3-11. CD8 T cells are sensitive to apoptosis induced by moDCs from CHC patients.**

CD8 T cells were obtained from peripheral blood by MACS and then were cocultured with autologous moDCs from CHC patients. The apoptosis of CD8 T cells with (**A**) or without autologous coculture (**B**) were observed by TUNEL staining and flow cytometry analysis. Fine line shows unstained control and thick line indicates TUNEL staining. The marker is set to make the positivity of unstained controls (background) < 1%. The percentage of TUNEL-positive cells is shown. The statistical result is expressed as mean ± SD from three individual experiments (**C**).

### 3.4 Discussion

In this thesis, I found the presence of killer DCs during chronic HCV infection. Previous studies have shown that DCs can induce the apoptosis of CD4 T cells during MV [16, 17] and HIV [18, 19] infections. MV induces TRAIL expression in DCs and these DCs induce the apoptosis of activated T cells [16] and can kill tumor cells [17]. MV infection induces a profound immunosuppression which can lead to serious secondary infections. Fugier-Vivier I *et al*. [16] observed a significant apoptosis in both moDCs and CD3 T cells in the coculture of MV-infected moDCs and CD3 T cells, and suggested that DCs represent a major target of MV. Fugier-Vivier I *et al*. [16] believed that MV suppressed cell-mediated immunity by interfering with the survival and functions of DCs and T cells. Vidalain P *et al.* [17] demonstrated that MV infection induced TRAIL mRNA and protein expression in human moDCs, and MV-infected DCs were cytotoxic via the TRAIL pathway. However, TRAIL was not detected on the surface of DCs. Therefore, cytotoxic activity of MV-infected DCs is mostly mediated by sTRAIL. Vidalain P *et al.* [17] also suggested that either of the two functions could be assigned to human DCs *in vivo*: an antigen-presenting cell function which generate effector T cells, or cytotoxic activity upon infection with immunosuppressive viruses. During HIV infection, killer DCs produce TRAIL and induce the apoptosis of CD4 T cell lines [18, 19]. Stary G *et al.* [19] found the up-regulated expression of membrane-bound TRAIL (mTRAIL) on pDCs during HIV infection. HIV infection was also associated with the up-regulation of the apoptosis-transmitting

receptor TRAIL R1 on activated CD4 T cells and therefore these T cells became susceptible to TRAIL-dependent pDC-mediate killing. Therefore, Stary G *et al.* [19] defined pDCs as killers of CD4 T cells and believed that it implied a new mechanism of disease progression in HIV infection. In this thesis, my data demonstrated that DCs had up-regulated tolerogenic activity and were capable of inducing T-cell apoptosis, which supported the concept that the tolerogenic activity of DCs can be manipulated by viruses for immune evasion. However, my data revealed multiple inhibitory molecules which have up-regulated expression during virus infection. Different from MV and HIV infections during which TRAIL expression by DCs was up-regulated, I found that the expression of membrane-bound FasL and PD-L2 on mDCs was up-regulated but the expression of both mTRAIL and sTRAIL was unchanged in CHC patients.

This thesis shows that mDCs from peripheral blood of CHC patients can induce the apoptosis of CD4 T cells *in vitro*. I also demonstrate that CD4 T cells from CHC patients have more spontaneous apoptosis than CD4 T cells from healthy donors. This correlates with a study showing that CD8 T cells in CHC patients are highly apoptotic, which is associated with significant functional T-cell deficits [42]. CD8 T cells are important effector T cells, which kill virus-infected cells and thus are crucial for virus clearance. I suppose that there are two mechanisms leading to the poor CD8 T-cell response in CHC patients: (1) mDCs in CHC patients can directly kill CD8 T cells. Patient mDCs have up-regulated expression of FasL and PD-L2. Patient CD8 T cells express corresponding

receptors Fas and PD-1, and therefore are susceptible to the killer DCs. (2) CHC patient mDCs can affect CD4 T cells and thus weaken CD8 T-cell response indirectly. CD4 T helper cells are critical for the initiation and maintenance of CD8 T-cell response. Patient mDCs have decreased ability to stimulate CD4 T-cell proliferation [25], and patient mDCs have increased expression of inhibitory molecules (FasL and PD-L2) as well as increased tolerogenic activity that enables them to kill CD4 T cells. The poor CD4 T-cell response can also lead to poor CD8 T-cell response in CHC patients. In brief, DCs are pivotal in regulating T-cell responses, and HCV may develop multiple mechanisms to manipulate DCs and thus facilitate its evasion from host immune response.

My thesis demonstrates that mDCs from CHC patients may work as killer DCs to induce CD4 T-cell apoptosis. Although my study correlates with a previous report by Ciesek S *et al*. that *ex vivo* mDCs do not increase TRAIL production and can not kill Jurkat T cells, however, Ciesek S *et al*. concluded that *ex vivo* mDCs of patients with hepatitis C displayed no cytotoxic activity, which could represent a novel mechanism for the increased prevalence of autoimmunity in HCV infection [43]. This discrepancy between Ciesek S *et al.*'s and my studies may be explained by two factors, first, they used different target T cells and second, they only examined TRAIL molecules. Firstly, Ciesek S *et al.* used immortalized leukemia cell lines as target cells, including JY-EBV B cells, K562 cells, U937 and Jurkat T cells. In the present study I used primary CD4 and CD8 T cells freshly isolated from human blood as target cells. I demonstrated

that the cytotoxic activity of CHC patient mDCs was up-regulated, and CHC patient mDCs can induce the apoptosis of allogeneic healthy T cells and autologous CHC patient T cells. Since CD4 and CD8 T cells circulating in the blood of CHC patient are the real targets of DCs *in vivo*, I believe that assays using fresh T cells as targets are more representative of CHC than assays using immortalized leukemia cell lines in the term of clinical significance. Secondly, I studied multiple inhibitory molecules (FasL, PD-L1, PD-L2 and TRAIL) on the surface of mDCs whereas Ciesek *et al*. [43] focused only on TRAIL molecule. I found that CHC patient mDCs had up-regulated expression of FasL and PD-L2, which were not examined in the study by Ciesek *et al*. [43].

It has been reported that PD-1 is a potential target of immunotherapy for chronic viral infections, e.g. HCV infection [44, 45] and HIV infection [46]. In this study, I found that CHC patient CD4 T cells have a higher expression of PD-1 and more spontaneous apoptosis compared to healthy donor CD4 T cells. The increased expression of PD-1 on CD4 T cells from CHC patients indicates that T cells in the blood of CHC patients are more sensitive to apoptosis compared to healthy donor T cells. This correlates with previous study that HCV-specific CD8 T cells undergo significant apoptosis in the peripheral blood during acute HCV infection and in the liver during the chronic HCV infection [42].

The two ligands for PD-1, PD-L1 and PD-L2, differ in their expression. PD-L1 is expressed much more broadly than PD-L2. While PD-L1 is expressed on multiple blood cells and a wide variety of non-hematopoietic cells, PD-L2 is only

expressed on DCs, macrophages and bone marrow-derived cultured mast cells [47]. Blocking PD-L2 on DCs results in enhanced T-cell proliferation, while blocking PD-L1 results in similar, but more modest effects [31]. In a previous study on HCV, blocking PD-L1 restored T-cell function in HCV-infected patients [34]. However, my studies suggest that PD-L2 and FasL may be more important targets to regulate CD4 and CD8 T-cell apoptosis during HCV infection.

Full-length BLyS molecule is expressed on the plasma membrane of immune cells as mBLyS. After cleavage by polyprotein convertases, the extracellular C-terminal fragment containing amino acids 134-285 is released as soluble BLyS (sBLyS). BLyS was first reported to be a cytokine critical for B-cell maturation, function, and survival [20-22]. BLyS-knockout mice have significantly reduced spleen weight, markedly reduced numbers of peripheral blood B cells, and a profound reduction in total serum immunoglobulin (Ig) [22]. Furthermore, mBLyS expressed on DCs regulates T-cell responses [23, 24]. BLyS is predominantly produced by myeloid cells, including monocytes, macrophages, DCs and neutrophils [48, 49]. It has been reported that sBLyS levels in serum of HCV-infected patients were significantly higher than those in healthy controls [50] , and serum sBLyS level predicts the outcome of acute HCV infection [51]. sBLyS level is significantly increased in acute HCV-infected patients evolving to chronicity than in those with a self-limited course, and thus a higher sBLyS level is associated with persistence of HCV infection [51]. mBLyS expressed on APCs provides a complete and potent second signal for T cell activation, which leads to T cell division and cytokine secretion [52, 53]. In this chapter I found

that mBLyS expression on DCs was not changed during CHC infection, which indicates that mBLyS on DCs may not be one of the molecules leading to impaired T-cell response during CHC. However, mBLyS expression on other immune cells is to be determined in future studies.

HCV may infect mDCs but this does not result in a productive infection. Genomic RNA of HCV has been detected in mDCs *ex vivo* isolated from the blood of HCV-infected patients [36, 54], suggesting that mDCs are susceptible to HCV infection. However, the replication intermediate was observed in DCs isolated from only 3 out of 24 patients infected with HCV, indicating that HCV replication occurs at a much lower frequency in DCs than in hepatocytes [55]. Furthermore, a study using UV-treated HCV showed that HCV-mediated mDC maturation was independent of virus replication and, using strand specific PCR, there was no evidence for HCV replication within mDCs [56]. As such, the up-regulated cytotoxic activity of mDCs from CHC patients is probably not the result of HCV replication in mDCs.

Killer B cells with up-regulated expression of FasL can regulate CD4 T cell apoptosis during *Schistosomal* infection [57]. This along with our study and the reports of killer DCs during MV [16, 17] and HIV infections [18, 19] suggests a model in which apoptosis of CD4 T cells is mediated by APCs which could regulate T-cell responses against infections. In conclusion, mDCs from chronic HCV-infected patients express increased levels of inhibitory ligands, FasL and PD-L2, and can induce the apoptosis of CD4 and CD8 T cells. This

characteristic of mDCs may contribute to the impaired protective T-cell responses of CHC patients. The up-regulated cytotoxic activity of CHC patient mDCs may be another mechanism of HCV-induced immune dysfunction in CHC patients.

In my thesis, the apoptosis of T cells is due to direct killing by DCs, but not of activation induced cell death (AICD). AICD is a form of T cell apoptosis that deletes autoreactive T cells. After activation, peripheral T cells express both Fas and FasL, and apoptosis is induced by FasL/Fas interaction with other activated T cells [58]. The method used to induce AICD is complicated and time-consuming, including incubation of T cells with concanavalin A for 24 hours, followed by rIL-2 for 24 hours and finally anti-CD3ε + rIL-2 for 24 hours [59]. In my thesis, however, I do not believe the apoptosis of T cells occurs as a result of AICD for the following reasons. (1) The co-culture time of DCs with T cells was short (only 5 hours). It is much shorter than the time required for AICD (3 days). (2) T cells are not pre-activated by concanavalin or IL-2, and are not pre-stimulated by anti-CD3. (3) To obtain high level of T-cell apoptosis in my studies, T cells need to interact with DCs. Healthy T cells do not undergo massive apoptosis when they were cultured alone in my studies, which is a characteristic of AICD. (4) T cells are not autoreactive. The target T cells used in my study are healthy T cells or CHC patient T cells, neither of which are induced to be autoreactive. The characteristics of T-cell apoptosis in my studies support the concept that DCs of CHC patients directly kill T cells.

My study demonstrates a mechanism of HCV-induced immune dysfunction during chronic hepatitis C infection. In this model, the function of DCs during chronic HCV infection may be switched from immunogenic to tolerogenic. Such a switch in DC function may contribute to the impaired T-cell responses in CHC patients.

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# Chapter 4

# Summary

# And

# Future Directions

## 4.1 Summary

In this thesis, I compared the function of mDCs from patients with CHC infection with the function of mDCs from healthy age- and gender-matched uninfected controls. My studies showed that the ability of mDCs to stimulate T-cell proliferation was impaired in CHC patients compared to mDCs from uninfected controls [1], and the ability of mDCs to induce T-cell apoptosis was up-regulated in CHC patients compared to uninfected controls (manuscript in preparation).

HCV infection remains a huge public health burden. mDCs are potent professional APCs that initiate and maintain specific immune responses against infection [2, 3]. Changes of mDC function result in altered T-cell responses and affect the outcome of HCV infection.

As demonstrated earlier in this thesis, the immunogenic function of mDCs from CHC patients on T cells has been studied (Chapter 2) [1]. In brief, the ability of mDCs to stimulate T-cell proliferation was impaired in CHC patients. Patient mDCs demonstrated decreased expression of HLA-DR and CD86, up-regulated spontaneous apoptosis, and diminished NF-κB activity [1]. Previous studies on mDC function during chronic HCV infection have yielded conflicting results [4-12]. Some studies report that the function of mDCs in CHC patients [10-12] and chimpanzees [9] is comparable to the function of mDCs in healthy controls, while other studies indicate that mDC function is significantly inhibited in CHC patients [4-8]. Of note, in the previous studies demonstrating mDC function is

inhibited in CHC patients [4-8], the mechanism of the inhibition of mDC function in CHC patients has yet to be determined. The discrepancies of previous studies might be the result of slight differences in study designs, e.g., using heterologous sera [10-12] or using unpurified PBMCs as target T cells [12]. The first difference lies in the culture condition of mDCs. For example, Barnes E. *et al.* [12] used RPMI-1640 media supplemented with 10% FCS. However, heterologous serum has immunosuppressive activity on mDC function [13] and may affect results of the experiment. The second difference between the previous studies is the responder cells (or, target T cells) used in MLR. Averill L. *et al*. [8] worked on T cells isolated using a $CD3^+$ T cell isolation kit and declared that the kit yielded > 98% purity, while Barnes E. *et al*. [12] used PBMCs as responder cells. Using PBMCs as target T cells may not have been the best choice for these studies, as the components of PBMCs are not pure T cells. I have focused my research on studying mDC while autologous serum was used to culture DCs and MACS was used to isolate T cells as target cells in MLR. My studies correlated with previous studies showing that the ability of mDCs to stimulate T cell proliferation was impaired in CHC patients [4-8], and my studies further showed the mechanisms for the impaired ability of mDCs.

Furthermore, my studies demonstrated that the tolerogenic activity of mDCs on T cells from CHC patients was up-regulated (Chapter 3). CHC patient mDCs demonstrated increased expression of inhibitory ligands, FasL and PD-L2. The cell apoptosis was significantly increased when mDCs from CHC patients were

co-cultured with healthy T cells compared to when mDCs from healthy controls were co-cultured with T cells. Both T cells and mDCs demonstrate more apoptosis compared to either being cultured alone. This suggested that mDCs from CHC patients could directly kill T cells. Furthermore, antibodies against FasL and PD-L2 partially abolished the killing effect, and culture of mDCs and T cells in two chambers separated by transmembranes almost completely abolished the killing. These results suggested that the killing effect was mediated by the interactions between FasL/Fas and PD-L2/PD-1. Conclusion of my studies are different from the previous study which reported that mDCs have an impaired cytotoxic activity during HCV infection [14]. This discrepancy may be explained by two differences in study design, target T cells and examined molecules. Firstly, Ciesek S *et al.* [14] used immortalized leukemia cell lines (including JY-EBV B cells, K562 cells, U937 and Jurkat T cells) as target cells. In the present study I used primary CD4 and CD8 T cells freshly isolated from human blood as target cells. Since CD4 and CD8 T cells circulating in the blood of CHC patient are the real targets of DCs *in vivo*, assays using fresh T cells as targets are more important than assays using immortalized leukemia cell lines in terms of clinical significance. Secondly, I examined the expression of multiple inhibitory molecules (FasL, PD-L1, PD-L2 and TRAIL) on the surface of mDCs whereas Ciesek *et al*. [14] only focused on TRAIL molecule. The molecules demonstrate up-regulated expression on mDCs during CHC infection in my study were FasL and PD-L2, which were not examined in the study by Ciesek *et al.*

[14]. These two differences between the study design of the earlier study and my study may account for the discrepancies in results.

Taken together, my thesis demonstrated that mDCs from CHC patients have phenotypical and functional changes compared to mDCs from healthy donors (Fig. 4-1). These changes might contribute to the poor T-cell responses seen in CHC patients.

My thesis provided novel information about the immune responses during chronic HCV infection. New questions are raised and should be answered in the future.

The studies in my thesis suffered from a lack of human liver samples. Immune cells isolated from human liver samples might be different from those immune cells circulating in human peripheral blood. Due to technical difficulties to obtain human liver samples, the study on immune cells from human liver was not performed in my thesis, however, and is expected to be addressed in the future.

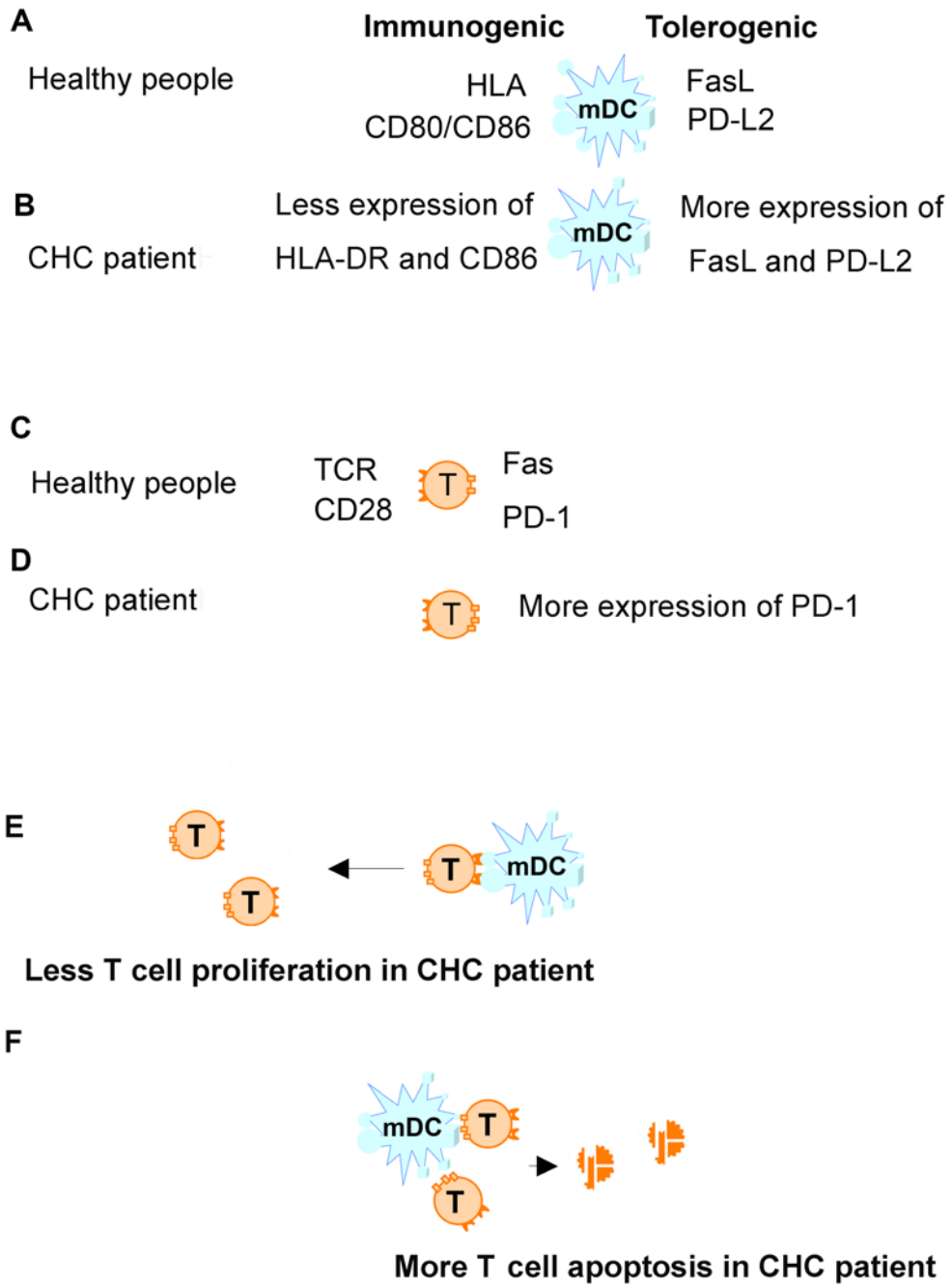


**Fig. 4-1. The model of mDC function during CHC as demonstrated in my thesis.**

mDCs in CHC patients express less activating molecules, HLA and CD86 [1]. mDCs in CHC patients express more inhibitory molecules, FasL and PD-L2 compared to mDCs in healthy donors (**A** and **B**). CD4 T cells in CHC patients have up-regulated expression of the receptor, PD-1 (**C** and **D**).

mDCs in CHC patients are less immunogenic in stimulating T-cell proliferation [1] (**E**) and are more tolerogenic in inducing T-cell apoptosis (**F**).

## 4.2 Future directions

### 4.2.1 Future research on immune cells isolated from liver samples

An obvious disadvantage of my thesis is that the immune cells used were obtained from peripheral blood of human. Because human liver is the primary site of HCV replication and disease pathogenesis, studies on immune cells obtained from human liver might be more convincing than those obtained from human peripheral blood.

During HCV infection, immune cells are enriched in human liver and the liver-infiltrating immune cells could have different phenotype from those in peripheral blood. For example, it has been reported that HCV-specific CD8 T cells were enriched in liver of CHC patients by 10-fold more than peripheral blood [15]. PD-1 expression is markedly increased in HCV-specific CD8 T cells in the liver of CHC patients compared with PD-1 expression in peripheral blood CD8 T cells. Influenza and EBV-specific CD8 T cells were also detected in the liver of CHC patients; however, they displayed similar PD-1 expression in both the liver and blood. Thus Nakamoto *et al*. concluded that the increased expression of PD-1 on intrahepatic T cells was HCV-specific [15]. Furthermore, Nakamoto *et al*. found that intrahepatic PD-$1^+$ CD8 T cells expressed less CD28 (co-stimulatory protein) and CD127 (IL-7 receptor which is essential for mature lymphocyte survival), but more expression of cytotoxic T-lymphocyte antigen-4 (CTLA-4, also known as CD152, which transmits an inhibitory signal to T cells) than peripheral PD-$1^+$ CD8 T cells. These observations suggest that intrahepatic

PD-1$^{+}$ CD8 T cells displayed a highly activated but also more exhausted phenotype, as CD28 and CD127 are positive receptors but CTLA-4 is a negative co-stimulatory receptor for CD80/CD86 [15].

Besides phenotypical changes, liver-infiltrating immune cells could have functional changes compared to those in peripheral blood during HCV infection. For example, intrahepatic HCV-specific CD8 T cells expanded poorly *in vitro* and expressed very little perforin and IFN-γ compared to peripheral HCV-specific T cells from the same patients following antigenic stimulation. This difference is not specific to the liver site since liver-derived influenza-specific CD8 T cells expanded efficiently with a high level of perforin expression similar to those peripheral influenza-specific CD8 T cells [15].

Liver-infiltrating T cells and mDCs could have different function or apoptosis from those cells obtained from peripheral blood. However, the samples of human livers have been difficult to obtain. Firstly, the natural host of HCV infection is human. Obtaining human liver samples requires collaboration with surgeons and complicated ethical consent. Secondly, neither of the two animal models of HCV infection, chimpanzees and Alb-uPA/SCID mice transplanted with human hepatocytes, is suitable for providing liver samples. Chimpanzees are endangered species and chimpanzee experiments are restricted in Canada. Alb-uPA/SCID mice transplanted with human hepatocytes lack specific immune cells and HCV infection occurs only in transplanted human hepatocytes, but not

in the mouse hepatocytes [16]. SCID mice do not have T and B cells, and thus demonstrate the combined immunodeficiency of both specific cellular immunity and specific humoral immunity. The human hepatocytes grow in small red nodules scattered in yellow mouse livers, and mouse immune cells in mouse livers may not have the same characteristics as human immune cells in HCV-infected patients. These restrictions have made experiments on liver samples difficult.

Recently some studies have used explant liver [15] or liver biopsy samples [17, 18] to obtain immune cells from human liver. Explant liver tissue was processed usually within 1-3 hours of explant. Tissue was diced into pieces and incubated with collagenase and deoxyribonuclease for 30 minutes. Immune cell marker expression was maintained after 30 minutes of collagenase digestion. Digested liver samples were washed in RMPI 1640 medium, mechanically dissociated, and passed through a nylon mesh filter before Ficoll-Hypaque density centrifugation [15]. For liver biopsy samples, liver infiltrating lymphocytes were purified by washing liver tissue extensively in RPMI 1640 plus 1% FCS and then digested with collagenase and DNase. The cell suspension was washed, and liver infiltrating lymphocytes were recovered by centrifugation over a Ficoll-Hypaque density gradient [17, 18]. The methods could be adopted to obtain human immune cells from livers particularly from the explant tissue following liver transplantation.

In future studies, the phenotype, function and apoptosis of the T cells and mDCs from CHC patients and healthy donors could be compared. These cells isolated from human livers could also be compared to the cells isolated from peripheral blood of the same patients. These assays may include:

(1) The expression of activating molecules (HLA-DR, CD80, CD86) and inhibitory molecules (FasL, PD-L1 and PD-L2) on mDCs as detected by antibody-staining and flow cytometry.

(2) MLR will be used to determine the ability of mDCs to induce T-cell proliferation as detected by flow cytometry.

(3) The expression of PD-1 on CD4 and CD8 T cells as detected by antibody-staining and flow cytometry.

(4) The spontaneous apoptosis of these mDCs, CD4 T cells and CD8 T cells as stained by TUNEL and detected by flow cytometry.

(5) The coculture of mDCs (CFSE-labeled) with CD4 T cells (or CD8 T cells) and the apoptosis detection to study the cytotoxic activity of mDCs.

(6) Cytokine production by mDCs, CD4 T cells, and CD8 T cells by ELISA, enzyme-linked immunosorbent spot (ELISPOT), or intracellular antibody staining and flow cytometry analysis.

### 4.2.2 Future research on the interaction between mDCs and Treg during HCV infection

Treg are a component of the immune system that can suppress responses of other immune cells. This is an important mechanism of the immune system to prevent

excessive immune reactions. Humans with a genetic deficiency in Treg develop severe and fatal autoimmune disorders [19].

Treg come in many forms. While most studies have focused on $CD4^+$ $CD25^+$ $Foxp3^+$ Treg, $CD8^+$ Treg have also been identified recently [20]. Similar as $CD4^+$ $CD25^+$ $Foxp3^+$ Treg, the $CD8^+$ Treg can also suppress immunity. Genetic disruption of the inhibitory interaction between $CD8^+$ Treg and their target follicular T helper cells results in the development of a lethal autoimmune disease. Treg are essential for the maintenance of self-tolerance and prevention of autoimmune disease [20].

It is hypothesized that upon encounter with infectious microorganisms, the immunosuppressive activity of Treg may be down-regulated to facilitate elimination of the infection. Studies suggested that some pathogens may have evolved to manipulate Treg to suppress the host immune response and thus facilitate pathogen survival. Prior studies have reported higher Treg levels in patient blood during chronic HCV infection compared with who resolved infection [21]. Similar phenomenon has been reported for other pathogen infections. For example, the frequency and the proliferation state of Treg are higher in HIV-infected patients than those in normal adults [22].

In my project, mDCs in CHC patients have down-regulated immunogenic function and up-regulated cytotoxic activity on T cells. It raised the question whether mDCs from CHC patients can kill Treg cells.

Treg in peripheral blood and particularly those infiltrating the liver have a high expression of PD-1 in CHC patients compared to healthy donors. The frequency of PD-$1^+$ Treg was greater than the frequency of PD-$1^+CD4^+$ effector T cells [23]. Since PD-1 is an important molecule mediating the tolerogenic activity of mDCs from CHC patients, it is possible that patient mDCs could interact with and kill Treg. The interaction between mDCs and Treg should be studied in the future.

### 4.2.3 Future research on effects of cytokines on mDCs

The changes in the phenotype and function of DCs during CHC as described in my thesis is not due to the direct effect of HCV, as proved by several studies on JFH-1 [24, 25]. JFH-1 is a HCV strain with a full-length HCV genome, and it can be actively produced and secreted *in vitro* [26]. By releasing infectious particles, JFH-1 can be passed effectively in cell culture [26]. JFH-1 has been used to study the direct effects of HCV on immune cells. For example, monocytes showed a tendency to shift to immature DCs when cultured with JFH-1 [24]. However, JFH-1 did not alter the phenotype of moDCs [24], and JFH-1 did not alter the capacity of *ex vivo* isolated mDCs or *in vitro* generated moDCs to induce CD4 T-cell proliferation [25]. These studies indicate that HCV may not directly change the phenotype or function of DCs. I did a

preliminary experiment exposing healthy moDCs to JFH-1 ($10^7$ copies/mL) for 1-7 days, and did not see changed phenotype of moDCs. At that time the above studies [24, 25] were published, and so I did not pursue the study further.

Since cytokines can regulate the function of immune cells, it is possible that changed level of cytokines may lead to the phenotypical and functional changes of DCs during HCV infection. Among the Th1 cytokines (IFN-γ and IL-2) and Th2 cytokines (IL-10 and IL-4) that have been reported to be associated with the outcome of HCV infection [27], IL-10 plays a central role in the suppression of antiviral immunity and thus should be studied in more details. IL-10 could possibly affect mDC phenotype and functions as a result of indirect effects of HCV infection.

IL-10 is critical for viral infections to persist [28-30]. Viral infection results in a significantly increased expression of IL-10 by DCs, B cells and macrophages, and leads to impaired T-cell response. Genetic removal of *IL10* in mice leads to the maintenance of robust T-cell responses and rapid elimination of virus [30]. IL-10 is associated with HIV control, since IL-10 expression in plasma and PBMCs in HIV-infected patients correlated positively with viral load. Although IL-10 can be produced in T, B, and NK cells, monocytes are a major source of IL-10 [31]. In HCV-infected patients, early IL-10 response determines infection outcome [27]. The production of IL-10 by PBMCs in acute HCV infection is particularly important. Patients demonstrate high IL-10 production in PBMCs is

associated with progression to CHC. IL-10 production correlates positively with HCV-RNA level in CHC patients [27]. Furthermore, HCV-specific T-cell responses in CHC patients were restored by usage of neutralizing anti–IL-10 monoclonal antibodies to block IL-10 [32]. On the other hand, administration of recombinant IL-10 (rIL-10) inhibits the proliferation of HCV-specific T cells and enhanced HCV replication. A neutralizing antibody to rIL-10 completely abrogated the inhibition of rIL-10 [33]. These studies demonstrated that IL-10 is critical for immuno-suppression during HCV infection.

Although these studies have demonstrated the association of IL-10 with impaired HCV-specific T-cell responses and HCV persistence [32, 33], there are important questions to be answered in this area. First, the exact source of IL-10 is unclear. Previously study demonstrated high IL-10 production by PBMCs in CHC patients [27]. The production of IL-10 by T cells, B cells, NK cells, DCs and monocytes in CHC patients and healthy donors could be compared. HCV-infected hepatocytes could be a source of IL-10, and intracellular staining of IL-10 in hepatocytes from liver biopsy samples from CHC patients and healthy donors should be determined. JFH-1 could be cultured with the immune cells and hepatocytes described above and the production of IL-10 by these cells could be observed. Second, whether or not the changes observed in DCs of CHC patients are due to IL-10 over-expression needs to be determined. IL-10 could be applied to DCs from healthy donors and the phenotype and function of DCs will be compared to those without IL-10 treatment. If IL-10 can directly inhibit

the ability of DCs to stimulate T cell responses, antibodies against IL-10 or against IL-10 receptor should be able to block the inhibitory effect of IL-10. Such approaches to treat chronic HCV have been attempted [32]. Furthermore, as the Th1 cytokines (IFN-γ and IL-2, which inversely correlate with HCV RNA level in patients) are competing and interacting with IL-10 [27], administration of IFN-γ and IL-2 to DCs from CHC patients could probably counteract the inhibitory effect of IL-10 on these cells.

### 4.2.4 Future research on the tolerogenic activity of pDCs during HCV infection

Being similar to mDCs in certain aspects, pDCs are innate immune cells that circulate in the blood and are found in peripheral lymphoid organs. However, their surface marker and function distinguish them from the conventional mDCs. Human pDCs express surface markers CD123, BDCA-2 and BDCA-4. Upon stimulation, pDCs produce large amounts of type I IFN, which is a critical anti-viral cytokine [34]. In addition to their major function in producing cytokines, pDCs can stimulate T-cell proliferation by expressing surface molecules including HLA-DR, CD80 and CD86 [35].

HCV-infected cells trigger a robust production of IFN in pDCs since the IFN-inducing ability of the HCV-infected cells correlated directly with the level of HCV-RNA. The IFN production by pDCs also requires direct cell-cell contact, as the production was triggered by co-culture of HCV-infected cells with

pDCs, and was abrogated when pDCs and HCV-infected cells were separated in a transwell plate. Furthermore, supernatant from the activated pDC inhibited HCV infection in target cells [36]. The study demonstrated that pDCs are an important immune component regulating HCV clearance. During chronic HCV infection, however, pDCs have decreased production of IFN and impaired activity in stimulating T-cell proliferation, which have been attributed to HCV core and NS5 proteins [35, 37].

During HIV infection, pDCs express more TRAIL on cell surface than pDCs from uninfected patients, and they induce the apoptosis of CD4 T cell lines. The expression of TRAIL on pDCs was dependent on the IFN produced by pDCs, since it was significantly reduced after addition of antibodies against IFN [38, 39]. With these studies focused on killer pDCs during HIV infection, however, little attention has been given to examine the possibility of killer pDCs during HCV infection.

While the immunogenic function of pDCs during HCV infection has been studied [25], the tolerogenic function of pDCs during HCV infection has not been reported yet. Future studies are expected to determine if pDCs express TRAIL, FasL or PD-1 ligands at changed levels, and if they can regulate the apoptosis of T cells during HCV infection.

**4.3 Conclusion**

In this thesis, the studies on mDCs have yielded data that support the altered function of mDCs during CHC. The altered function of mDCs contributes to impaired T-cell responses in CHC patients. Further studies on mDCs may have the ability to restore/improve immune function in CHC patients.

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