# The Association of Interleukin-27 and HIV Infection in Chinese

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Abstract of thesis entitled:

"The Association of Interleukin-27 and HIV Infection in Chinese"

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Human Immunodeficiency Virus (HIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS); HIV/AIDS caused 1.8 million deaths world-widely in 2010 and became a major global health challenge. HIV co-infections with Hepatitis B virus (HBV), Hepatitis C virus (HCV) are common and have emerged into new health problems with severe clinical consequences. Since the discovery of HIV, massive progress in understanding of the pathogen has been achieved. Due to the restriction of research model, how human immune system responds to HIV infection, particularly, to HBV or HCV co-infections is still worthy further elucidation.

A cohort study was first conducted in Shenzhen regarding the seroprevalence of HBV, HCV infections among HIV-infected population. Totally 914 HIV positive individuals were recruited in the study and tested for HBsAg and anti-HCV antibodies. The results showed a 10.9% (100/914) HIV/HBV co-infection rate, 14.6% (133/914) HIV/HCV co-infection prevalence and 3.7% (34/914) HIV/HBV/HCV triple-infection prevalence. Multivariate logistic regression revealed that HIV transmission risk behavior was significantly associated with HIV, HBV, HCV co-infections. Most HIV/HBV co-infection cases got HIV through sexual contact including heterosexual and homosexual behaviors (95/100, 95%); while most HIV/HCV co-infection subjects were injection drug users (IDUs) (89/133, 66.9%). In the case of HIV/HBV/HCV triple-infection, IDUs accounted for a large ratio (28/34, 82.4%). Among IDUs, most of them were male (108/122, 88.5%) and nearly half were aged 27 to 32 years old (56/122, 45.9%). Near half people who got HIV through blood and blood products were HIV/HCV co-infected (10/23, 43.5%).

Gender has a significant correlation with HIV risk behavior and most IDUs were male.

Next, we applied ELISA to test HIV positive clinical samples and proved that plasma interleukin-27 (IL-27) level was significantly elevated in HIV mono-infected, HIV/HBV co-infected and HIV/HCV co-infected subjects when compared with healthy controls. Later, we further revealed that plasma IL-27 titer was not significantly varied among HIV, HBV and HCV co-infections except between HIV/HCV co-infections and HIV/HBV/HCV triple-infections. We also observed a significant positive correlation between $CD4^+$ T cell counts and plasma IL-27 titer within HIV mono-infected group ($r = 0.177$, $P = 0.034$).

We further analyzed the impact HIV and HCV viral loads on plasma IL-27 titer. We found there was no significant correlation between HIV viral load and IL-27 titer among HIV mono-infected individuals ($r = - 0.063$, $P = 0.679$); while a significant positive correlation was observed between HIV viral load and IL-27 titer in HIV/HCV co-infected individuals ($r = 0.362$, $P = 0.049$). In the case of HIV/HCV co-infection, there was no significant linear correlation between HIV and HCV viral loads ($r = - 0.072$, $P = 0.704$) but exist obvious subdivision of samples in terms of HIV and HCV viral loads with significant IL-27 titer variance ($P = 0.014$). No correlation was observed between HCV viral load and IL-27 titer ($r = - 0.119$, $P = 0.530$).

IL-27 p28 polymorphisms were genotyped with TaqMan® Allelic Discrimination Assay in Chinese men who have sex with men (MSM) population in Shenzhen and the results revealed that proportions of IL-27 p28 -964A/G and 4603G/A genotypes were not significantly different from the healthy controls; IL-27 p28 -964A/G and 4603G/A allele frequencies were similar between HIV positive MSM group and healthy control MSM group. Results also showed that for IL-27 p28 2905T/G

polymorphism, TG genotype has a 2.77-fold decreased risk of HIV susceptibility and subjects with G allele has a 2.72-fold decreased risk of HIV susceptibility. Linkage disequilibrium (LD) coefficients were observed between IL-27 p28 -964A/G and 2905T/G (|D'| = 0.942), -964A/G and 4603G/A (|D'| = 0.930). Haplotype analyses revealed that haplotype GGG has a protective role against HIV infection in MSM population (OR = 0.313; $P < 0.001$).

In conclusion, the seroprevalences of HBV and HCV infection among HIV positive population in Shenzhen were surveyed and risk factors associated with co-infections were analyzed. Plasma IL-27 titer was significantly elevated in HIV mono-infected, HIV/HBV co-infected and HIV/HCV co-infected individuals. IL-27 level was correlated with $CD4^+$ T cell counts within HIV mono-infected people. A significant positive correlation was found between HIV viral load and IL-27 titer in HIV/HCV co-infected individuals (r = 0.362, $P = 0.049$). IL-27 p28 2905T/G was associated with individual susceptibility to HIV infection and haplotype GGG showed a protective role in restricting HIV infection in MSM population.

# 摘 要

人類免疫缺陷病毒 (HIV) 是人獲得性免疫缺陷綜合征 (愛滋病，AIDS) 的致病原，2010 年全球有 180 萬人死於愛滋病，HIV/AIDS 已成為全球健康的嚴重挑戰。人類免疫缺陷病毒與乙型肝炎病毒 (HBV) ，丙型肝炎病毒 (HCV) 的合併感染非常普遍，已演變成具有嚴重臨床後果的新健康問題。儘管對於人類免疫缺陷病毒的研究已有很大的進展，但由於受研究模型的限制，人體免疫系統對人類免疫缺陷病毒感染的應答，特別是對乙型肝炎病毒，丙型肝炎病毒與人類免疫缺陷病毒合併感染的免疫應答，仍值得進一步的闡明。

在本研究中，我們首先對深圳人類免疫缺陷病毒，乙型肝炎病毒，丙型肝炎病毒合併感染的流行情況進行研究。共選取 914 份人類免疫缺陷病毒感染者的血漿，經過對乙型肝炎病毒表面抗原 (HBsAg) 和抗丙型肝炎病毒抗體 (anti-HCV) 的檢測，發現 10.9% (100/914) 的被檢測者是人類免疫缺陷病毒/乙型肝炎病毒合併感染， 14.6% (133/914) 為人類免疫缺陷病毒/丙型肝炎病毒合併感染，3.7% (34/914) 為人類免疫缺陷病毒/乙型肝炎病毒/丙型肝炎病毒三重感染。多元邏輯回歸分析證明人類免疫缺陷病毒傳染的危險行為與合併感染顯著相關聯。大多數的人類免疫缺陷病毒/乙型肝炎病毒合併感染者都是通過性接觸感染人類免疫缺陷病毒，包括異性傳播與同性傳播 (95/100, 95%); 大多數的人類免疫缺陷病毒/丙型肝炎病毒合併感染者是靜脈注射吸毒者 (89/133, 66.9%); 人類免疫缺陷病毒/乙型肝炎病毒/丙型肝炎病毒三重感染者中，大多數是靜脈注射吸毒者 (28/34, 82.4%)。靜脈注射吸毒人群中，大部分是男性 (108/122, 88.5%)，約半數人的年齡介乎 27 至 32 歲 (56/122, 45.9%) 。有接近一半的經過血液和血液製品傳播人類免疫缺陷病毒的人是人類免疫缺陷病毒/丙型肝炎病毒合併感染者 (10/23, 43.5%) 。性別與人類免疫缺陷病毒感染的危險行為有顯著關係，大部份的靜脈注射吸毒者是男性。

進一步，我們利用酶聯免疫吸附測定法 (ELISA) 檢測深圳愛滋病陽性樣本血漿中白細胞介素 27 (IL-27) 的濃度。結果顯示，對比健康參照者，人類免疫缺陷病

毒單獨感染者，人類免疫缺陷病毒/乙型肝炎病毒合併感染者，人類免疫缺陷病毒/丙型肝炎病毒合併感染者的血漿 IL-27 濃度顯著升高。隨後我們進一步發現，人類免疫缺陷病毒單獨感染組，人類免疫缺陷病毒/乙型肝炎病毒，人類免疫缺陷病毒/乙型肝炎病毒/丙型肝炎病毒合併感染組之間的血漿 IL-27 濃度沒有顯著差異，而人類免疫缺陷病毒/丙型肝炎病毒合併感染組與人類免疫缺陷病毒/乙型肝炎病毒/丙型肝炎病毒三重感染組的血漿 IL-27 濃度差異顯著。我們還發現人類免疫缺陷病毒單獨感染組中，血漿 IL-27 濃度與 $CD4^+$ T 淋巴細胞數量顯著正相關 (r = 0.177, *P* = 0.034)。

我們進一步分析了人類免疫缺陷病毒和丙型肝炎病毒的病毒載量對血漿 IL-27 濃度的影響，發現 HIV 單獨感染組中人類免疫缺陷病毒載量與血漿 IL-27 濃度沒有顯著相關 (r = - 0.063, *P* = 0.679)，而人類免疫缺陷病毒/丙型肝炎病毒合併感染組中，人類免疫缺陷病毒載量與血漿 IL-27 濃度顯著正相關 (r = 0.362, *P* = 0.049)。人類免疫缺陷病毒/丙型肝炎病毒合併感染組中，人類免疫缺陷病毒載量與丙型肝炎病毒載量缺少顯著線性關聯 (r = - 0.072, *P* = 0.704)，而人類免疫缺陷病毒/丙型肝炎病毒合併感染組可根據人類免疫缺陷病毒與丙型肝炎病毒的病毒載量再細分成血漿 IL-27 濃度差異顯著的三組 (*P* = 0.014) ， 丙型肝炎病毒載量與血漿 IL-27 濃度缺少顯著關聯 (r = - 0.119, *P* = 0.530) 。

我們利用 TaqMan®等位基因分型技術測定深圳男同性戀人群中 IL-27 p28 基因的單核苷酸多態性 (SNP)。結果顯示，人類免疫缺陷病毒感染組 IL-27 p28 -964A/G 和 4603G/A 的基因型與健康男同性戀參照組的基因型沒有顯著差異，IL-27 p28 -964A/G 和 4603G/A 的等位基因比率也沒有顯著差異。結果也顯示，IL-27 p28 2905T/G 的 TG 基因型可減少 2.77 倍的人類免疫缺陷病毒感染風險，等位基因 G 可減少 2.72 倍的人類免疫缺陷病毒感染風險。連鎖不平衡在 IL-27 p28 -964A/G 和 2905T/G 中存在 (|D'| = 0.942), -964A/G 和 4603G/A 中也存在 (|D'| = 0.930) 。單型分析結果表明，GGG 單型可減少男同性戀人群的人類免疫缺陷病毒感染風險 (OR = 0.313; *P* < 0.001)。

綜上所述， 在本研究中，我們首次調查了深圳人類免疫缺陷病毒，乙型肝炎病毒，丙型肝炎病毒合併感染的流行情況，並分析了合併感染的風險因素。 發現人類免疫缺陷病毒單獨感染者，人類免疫缺陷病毒/乙型肝炎病毒合併感染者，及人類免疫缺陷病毒/丙型肝炎病毒合併感染者的血漿 IL-27 濃度比健康參照組顯著地升高；人類免疫缺陷病毒單獨感染組中，血漿 IL-27 濃度與 $CD4^+$ T 淋巴細胞數量顯著正相關。人類免疫缺陷病毒/丙型肝炎病毒合併感染組中，人類免疫缺陷病毒載量與血漿 IL-27 濃度顯著正相關 ($r = 0.362$, $P = 0.049$) 。分析深圳男同性戀人群 IL-27 p28 基因的單核苷酸多態性，發現 IL-27 p28 2905T/G 與人類免疫缺陷病毒感染相關，GGG 單型可降低男同性戀人群人類免疫缺陷病毒感染的風險。

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# Abbreviations

| | |
|---|---|
| ADE | antibody-dependent enhancement |
| ALT | alaine aminotransferase (ALT) |
| APOBEC | apolipoprotein B mRNA-editing enzyme catalytic-polypeptide |
| APECED | Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy |
| ARS | acute retroviral syndrome |
| AST | aspirate aminotransferase (AST) |
| CCR5 | CC chemokine receptor type 5 |
| COPD | chronic obstructive pulmonary disease |
| CTLs | cytotoxic T lymphocytes |
| CXCR4 | CXC chemokine receptor 4 |
| DCs | dendritic cells |
| EBI3 | Epstein-Barr virus-induced gene 3 |
| ELISA | Enzyme-linked immunosorbent assay |
| FPDs | former plasma donors |
| FPV | Fowl plague virus |
| GWAS | genome-wide association studies |
| HAART | highly active antiretroviral therapy |
| HBV | hepatitis B virus |
| HCV | hepatitis C virus |
| HPV | human papilloma virus |
| HIV | human immunodeficiency virus |
| ICAM-1 | intracellular adhesion molecule-1 |
| IL-27 | interleukin-27 |
| IBD | inflammatory bowel disease |
| IFIGs | IFN-inducible genes |
| IFN- α | interferon alpha |
| IFN- β | interferon beta |
| IFN- γ | interferon gamma |
| LCs | langerhans cells |
| LFA-1 | leukocyte function antigen-1 |
| LTRs | long terminal repeats |
| MAPK | mitogen-activated protein kinase |
| MBLs | mannose-binding lectins |
| MDMs | monocyte-derived macrophages |
| MSM | men who have sex with men |
| NK | natural killer |
| NSCLC | non-small-cell lung cancer |
| ORF | open reading frame |
| PBMCs | peripheral blood mononuclear cells |
| TCR | T cell receptor |
| VLPs | virus-like particles |

| | |
|---|---|
| SD | standard deviation |
| SDF-1 | stromal cell-derived factor-1 |
| SIV | simian immunodeficiency virus |
| SNPs | single-nucleotide polymorphisms |
| STDs | sexually transmitted diseases |
| WHO | world health organization |

# Chapter 1

# Introduction

## 1.1 Human Immunodeficiency Virus

The human immunodeficiency virus (HIV), a member of *Retroviridae* family, mainly infects cells of immune system. HIV infection causes human acquired immunodeficiency syndrome (AIDS), which is characterized by progressively impaired immune system and life-threatening opportunistic infections.

AIDS was first reported in 1981 from homosexual men with *Pneumocystis carinii* pneumonia symptoms (Gottlieb, Schroff et al. 1981); later HIV was identified as the causative agent in 1983 (Barre-Sinoussi, Chermann et al. 1983; Gallo, Sarin et al. 1983). Since then, significant progresses have been achieved in the understanding of HIV/AIDS; in 1996, highly active antiretroviral therapy (HAART) was introduced to treat HIV infection. HIV/AIDS remains to be a severe global health problem as of its continuing transmission. In 2009, there were an total estimated 33.3 million people living with HIV/AIDS; among them, 2.5 million were children (UNAIDS 2010). The estimated number of new HIV infection was 2.6 million, about 14.2% were children, the AIDS-related deaths was 1.8 million in 2009 (UNAIDS 2010).

### 1.1.1 HIV virology

HIV is a member of the genus *Lentivirus* in the *Retroviridae* family. HIV has two species: HIV-1 and HIV-2. HIV-1 is more virulent and infective than HIV-2 and causes the majority of HIV infections globally (Gilbert, McKeague et al. 2003). HIV-1 infection is epidemic while HIV-2 infection is mainly restricted to West Africa because of its low virulence (Reeves and Doms 2002).

#### 1.1.1.1 HIV structure and Genome organization

HIV is an enveloped, positive single-stranded RNA virus (Fig. 1-1). Follow the typical pattern of retrovirus family, mature HIV virions have spherical morphology

of 100-120 nm in diameter and the virion envelop consists of a bilayer lipid membrane derived from host cell membrane during budding of the newly formed viral particle (Sierra, Kupfer et al. 2005). Each viral envelop subunit is composed of two non-covalently linked membrane proteins: glycoprotein 120 (gp120), the outer envelop glycoprotein; and gp41, the transmembrane glycoprotein that anchors the glycoprotein complex to the surface of virion. The dense truncated cone-shaped nucleocapsid (core) is located just below the bilayer lipid membrane and is composed of 2,000 copies of p24 viral proteins. The viral genome is located inside the core and contains two identical copies of 9.2 kb single-stranded, positive-sense RNA.

The HIV genome contains two identical 9.2 kb RNA molecules. Structurally, the HIV genome can be divided into four main regions: (1) long terminal repeats (LTRs), which functions as the regulatory regions at both ends of HIV RNA molecules through the U3, R and U5 regions for cellular transcriptional factors binding and *trans*-activation (Starcich, Ratner et al. 1985; Berkhout 1992; Richter, Cao et al. 2002); (2) The gag-pol gene region, which encodes two polyproteins that will be catalyzed into catalytic and structural proteins; (3) the env gene region, which encodes the gp160 precursor will further be divided into gp120 and gp41; (4) HIV tat, rev, nef, vif, vpr, and vpu accessory genes region, which encodes all regulatory and accessory proteins for HIV replication. The 9.2 kb HIV genome contains nine open reading frames (ORFs), which are gag, pol, env, vif, vpr, nef, tat, vpu and rev ORF (Fig. 1-2). The nine genes totally encode 19 proteins which can be broadly classified into structural, catalytic, regulatory, and accessory proteins. Gene gag, pol and env encode the Gag, Pol and Env polyproteins, which are subsequently proteolyzed into individual proteins common to all retroviruses. The Gag polyprotein is proteolyzed into four Gag proteins, MA (matrix, p17), CA (capsid, p24), NC (nucleocapsid, p7), and p6; Env polyprotein proteolyzed into 2 proteins, surface protein gp120 and transmembrane protein gp41. Gag, MA, CA, NC, p6, gp120 and gp41 are structural proteins which bind together to form the core of HIV virion and outer membrane envelop. Three catalytic proteins, i.e., protease, reverse transcriptase and integrase, provide essential enzymatic functions to HIV life cycle and are

encapsulated within HIV viral particle. Three accessory proteins (Vif, Vpr and Nef) present in viral particle. Another accessory protein Vpu indirectly assists the HIV virion assembly. Another two regulatory proteins (Tat and Rev) provide essential gene regulatory functions (Frankel and Young 1998).

#### 1.1.1.2 HIV life cycle

HIV, a prototype *retrovirus*, is an obligatory intracellular parasite. HIV can not replicate outside human cells. The HIV life cycle can be divided into four stages, including binding and entry, reverse transcription and integration, transcription and translation, assembly and secretion (Fig. 1-3).

The HIV life cycle begins with the binding of HIV particle to target cells. The binding occurs through a specific interaction between the HIV viral envelop protein gp120 and the CD4 molecular on host cells. Upon gp120 binding to CD4, a conformational change happens within gp120 leading to the exposes of co-receptor binding site in gp120. The gp120 co-receptor binging site then recognizes according to the structure and then binds to CXC chemokine receptor 4 (CXCR4) or CC chemokine receptor type 5 (CCR5). HIV X4 isolates preferentially uses CXCR4 as co-receptors, while HIV R5 isolates utilize CCR5 as co-receptors. HIV R5X4 isolates use both CCR5 and CXCR4 as co-receptors. After gp120 binds to co-receptors, the HIV envelope protein gp41 then binds to host heparan sulfate, triggering fusion of the viral envelope lipid bilayer with host cell membrane which enables the viral nucelocapsid (core) gain access to host cytoplasm. The viral lipid envelop is left outside the host cell lipid bilayer. The viral nucleocapsid then uncoats to expose viral nucleocapsid complex, which contains matrix, reverse transcriptase, integrase, Vpr and gnomic RNA (Frankel and Young 1998).

After binding and entering into the host cells, the released nucleocapsid complex starts reverse transcription and HIV genome will integrates into the host genome. Reverse transcription proceeds in a series of steps that utilize several *cis*-acting

elements encapsulated in the viral genome to convert the HIV RNA genome into double-stranded DNA. A DNA/RNA hybrid molecule is first synthesized, and then the RNA portion of DNA/RNA hybrid is degraded by the RNase H activity of reverse transcriptase and left the minus-strand DNA fragment. The minus-strand DNA jumps from the 5' to the 3' end of the genome through the first strand transfer process, then minus-strand DNA synthesis occurs followed by plus-strand DNA synthesis and second-strand transfer which is ended with plus-strand DNA synthesis, and finally forms double-stranded DNA (Frankel and Young 1998). The newly synthesized double-stranded viral DNA is transported into the host cell nucleus with the assistance of accessory proteins Vpr and Vif. Follow nuclear entry, the integrase catalyzes the insertion of double-stranded viral DNA into host chromosome. In the integration process, HIV integrase first clips off several nucleotides in the 3' end of HIV double-stranded DNA and cleave host target DNA, then joins both ends together between HIV DNA and host DNA, followed by host cellular enzymes to fill the gaps (Brown 1997).

The integrated double-strand HIV DNA is referred to as "provirus", which is passively replicated along with host genome replication and is passed into host cell offsprings. In latent infection, the HIV provirus is transcriptionally silent and no HIV virion is produced. In response to host environmental changes or immunity alterations, the latent HIV provirus can be activated. In the productive infection, the HIV provirus serves as template for synthesis of HIV viral RNAs which encodes the full complement of structural, catalytic, regulatory, and accessory proteins used for the replication of HIV. The HIV LTR region serves as transcription initiation site and harbors *cis*-acting elements essential for HIV RNA synthesis. During transcription, Tat greatly enhances the transcription rate and promotes RNA synthesis (Dayton AI 1986). The transcription produces a large number of HIV RNAs. They belong to three main categories: unspliced RNAs serve as genomic RNA for production of progeny HIV virions and Gag, Gag-Pol polyprotein precursors; partially spliced

mRNAs encodes Env, Vif, Vpu and Vpr; multiply spliced mRNAs, are translated into Rev, Tat, and Nef proteins (Purcell and Martin 1993). Transcripted HIV mRNAs are relocated from nucleus to cytoplasm. They are translated or packaged into new virions. Rev plays an important role in the process of RNA export to cytoplasm (Pollard and Malim 1998). Gag and Gag-Pol polyproteins are localized to cell membrane.

After HIV mRNAs are transcripted and translated into proteins, HIV progeny particles start to be produced. This process can be further subdivided into three steps: assembly, budding and maturation. Gag precursor polyprotein is the major player of virus assembly which happens on host cell plasma membrane. Gag interacts with packaging signal to encapside two copies of single-stranded HIV genomic RNA into virus particle, where HIV core particle (nucleocapsid) is formed after genomic RNA package with Gag, Gag-Pol polyproteins, Vif, Vpr and Nef. Env precursor protein, gp160, is synthesized in endoplasmic reticulum (ER) and cleaved into gp120 and gp41. The newly formed gp120 and gp41 are then transported to host cell membrane. Progeny HIV particle buds to host plasma membrane, where is coated with gp120 and gp41. Newly produced HIV releases from host cell membrane, during or shortly after release, Gag and Gag-Pol polyproteins are cleaved by Protease to generate mature Gag and Pol proteins. This process named as maturation. After maturation, the progeny HIV particles are capable of infect susceptible cells through the interaction between gp120, gp41 and CD4 and CCR5 or CXCR4 molecules (Freed 2001).

#### 1.1.1.3 HIV genotypes

HIV has high genetic variability, because of its rapid replication cycle with the generation of $10^9$ to $10^{10}$ progeny virions per day; high mutation rate that about 3 x $10^{-5}$ per nucleotide base per cycle in replication and as of reverse transcriptase's recombinogenic properties (Rambaut, Posada et al. 2004). The high genetic

variability results in many different strains of HIV, even simultaneous infection of the same cell with different strains. Based on genetic similarities, HIV strains can be clustered into types, groups and subtypes. Two types of HIV exist, namely HIV-1 and HIV-2. HIV-1 is the predominant virus worldwide, while HIV-2 is concentrated in West Africa. HIV-1 is further classified as group M, O, N and P according to the differences in the envelope region. Group M is the most prevalent HIV, accounts for more than 90% of HIV infections worldwide. Group M HIV-1 was further subdivided as subtypes, including subtype A, B, C, D, F, G, H, J, K and circulating recombinant forms (CRFs) raised from recombination between different subtypes (Hemelaar, Gouws et al. 2006). Group O is restricted to west-central Africa, mainly in Cameroon. A survey on 1997 revealed that 2% HIV-positive samples belonged to group O (Peeters, Gueye et al. 1997). Group N, which was discovered in 1998, is extremely rare and only seen in Cameroon (Yamaguchi, Coffey et al. 2006). Group P was discovered in Cameroon in 2009 and reported to have great similarity to a simian immunodeficiency virus (SIV) discovered in wild gorillas (Plantier, Leoz et al. 2009).

### 1.1.2 Epidemiology

HIV primarily infects cells of human immune system, causing systematic impairment of immune system and leading to susceptible condition towards opportunistic infections. HIV infection in human is regarded as pandemic by the World Health Organization (WHO).

#### 1.1.2.1 Global HIV epidemiology

Since 1981 when AIDS was first reported, HIV/AIDS has lead to more than 30 million deaths globally (UNAIDS 2011). In 2009, the estimated number of people living with HIV was 33.3 million worldwide, accounts for 0.49% of world's total population. The number was 16.5% higher than year 2000, and the prevalence was roughly three-folds higher than in 1990 (Fig. 1-4). The continuing rise of people

living with HIV is caused by high new infection rate and the beneficial outcome of improved life span after HAART. The newly infected people were about 2.6 million; and AIDS-related death were approximately 1.8 million in 2009 (Fig.1-5). Among them, about 370,000 new infections occurred among children under age 15. The number was roughly 26% lower than 2001, most of them were infected in utero during delivery and breastfeeding post-partum (UNAIDS 2010). After the introduction of antiretroviral therapy, significant progress has achieved, especially in resources-limited regions. Till 2009, about 5 million people with HIV infection had received antiretroviral therapy in low- and middle-income countries. The number has increased 13 folds in the past five years (UNAIDS 2010).

The HIV pandemic is not homogeneous between different regions as of social and economic diversity. The HIV epidemic appears to be stabilized in most regions, including Western and Central Europe, North America, Caribbean, South and South-east Asia; while the number of people living with HIV/AIDS has been dramatically increased in countries of Eastern Europe and Central Asia, Central and South America, Sub-Saharan Africa (Fig. 1-6) (UNAIDS 2010). For HIV incidence, it has been decreased more than 25% since 2001 in 33 countries, most of them are Sub-Saharan Africa countries; countries in North America, Western and Central Europe, Caribbean, Central and South America, Oceania maintain a relatively similar prevalence compared to year 2001; while for countries in East Asia, Middle East and North Africa, the prevalence continues to increase due to high rates of new HIV infection among injection drug users and their sexual partners (Fig. 1-7) (UNAIDS 2010).

As of unprotected sexual activity, women and girls are more vulnerable to HIV infection. For young women aged 15-24 years in sub-Saharan Africa, they are eight times more likely to be HIV positive than male (UNAIDS 2010). In sub-Saharan Africa, unprotected sex with multiple partners is the major risk factor for HIV

infection. A vast majority of new infections were through unprotected heterosexual intercourse and onward transmission to newborn babies and breasted babies. HIV prevalence in discordant couples, which defined as one person is infected with HIV among a couple, reached as high as 85% (Lingappa, Lambdin et al. 2008). In Asia, commercial sex activity is the centre of HIV epidemic as condom use is infrequent in paid sex and IDUs also buy or sell sex. The interplay of paid sex and illicit drug injection also accelerate the HIV transmission in Eastern Europe and Central Asia (UNAIDS 2010).

Injection Drug Users (IDUs) are easily to be infected by HIV as they may share syringe and needles during injection of illicit drugs, which passes HIV pathogen through polluted blood. In year 2008, according to a systematic review report, there were about 15.9 million (range 11.0 - 21.2 million) people inject drugs worldwide. Among them, about 3.0 million (range 0.8 – 6.6 million) people might be HIV positive. In China, the USA and Russia (which held the three largest number of drug injectors), the HIV prevalence among IDUs were 12%, 16% and 37%, respectively (Mathers, Degenhardt et al. 2008). Although injection drug use is a relatively new phenomenon in sub-Saharan Africa, it fuels new HIV epidemics in Asia with an estimated average HIV prevalence of 16% (UNAIDS 2010). In Asia countries, China, Myanmar, Thailand, Indonesia and Viet Nam, the HIV prevalence ranges from 7% to 50%. In some parts of Viet Nam, the prevalence even sky-rocketed to 58% (Tran, Nguyen et al. 2006; National AIDS Commission 2008; National AIDS Prevention and Alleviation Committee 2008; Wang, Wang et al. 2009).

AIDS cases were first reported from 5 men who have sex with men (MSM) in year 1981 and accounted for 44% to 65% AIDS cases in United States, Canada and Australia (Jaffe, Valdiserri et al. 2007). Due to unprotected sex activities, MSM has re-emerged as a high-risk population for HIV infection. What make the situation even worse is that the majority of men who have sex with men also have sex with

women. In sub-Saharan Africa, 15% to 20% new HIV infections were attributed to MSM, 33% to 82% of surveyed MSM also have sex with women (Lowndes, Alary et al. 2008; Asiimwe, Koleros et al. 2009; Gelmon, Kenya et al. 2009; Halimatou, Coumba et al. 2009; Ndiaye, Toure-Kane et al. 2009; Beyrer, Trapence et al. 2010). High HIV prevalence among MSM also reported in Asia countries, which ranges from 5% in Indonesia to 29% in Myanmar (National AIDS Commission 2008; National AIDS Programme 2009). For North America and Western and Central European countries, HIV epidemic has been resurged among MSM population with the annual number of HIV diagnosed among MSM rose 86% in year 2006 compared to year 2000 due to unprotected sex (Likatavicius, Klavs et al. 2008).

Globally, there were an estimated 370 000 children infected with HIV during perinatal and breastfeeding periods in year 2009 (UNAIDS 2010). In sub-Saharan Africa, there were an estimated 130 000 (range 90 000 – 160 000) children newly infected with HIV, a vast majority of them were resulted from HIV positive mother as of unprotected heterosexual intercourse.

#### 1.1.2.2 HIV epidemiology in China

With the development of globalization and implementation of the Reform and Opening-up Policy, China experienced its first diagnosed AIDS case in 1985, which was a foreign traveler abroad (Zhang Kl and Ma 2002). Since then, China experienced 3 phases HIV/AIDS epidemics (Zhang Kl and Ma 2002).

The first phase, from 1985 to 1988, reported cases were imported and composed of foreigners and oversea Chinese. Followed was the discovery of 146 HIV/AIDS cases in Yunnan province in injection drug users (Ma, Li et al. 1990; Duan, Shen et al. 2010), mainly composed by illicit injection drug users in drug trafficking road. The third phase, started from 1994, when cases were reported from IDUs and commercial plasma donors. By 1998, all 31 provinces, autonomous regions and municipalities under central government were reported of HIV infection cases (Zhang and Ma

2002). At that time, injection drug users account for 60% to 70% of reported HIV infections, while heterosexual transmission emerged to account for 7% (Zhang and Ma 2002).

In year 2011, there were totally 780 000 people living with HIV/AIDS, among them 154 000 were AIDS patients; 28 000 deaths were attributed to HIV/AIDS and there were 48 000 new HIV infections nationwide (Ministry of Health of China, UNAIDS et al. 2011). According to the 2011 national report, among the 780 000 people living with HIV/AIDS, nearly half (46.5%) were infected through heterosexual activities, 17.4% were via homosexual transmission, 28.4% were infected during injecting illicit drugs, 6.6% were through blood products and blood transfusion, and the left 1.1% were mother-to-child transmissions (Ministry of Health of China, UNAIDS et al. 2011). Yunnan, Guangxi, Henan, Sichuan, Xinjiang and Guangdong provinces were reported with high HIV/AIDS figures and accounted for 75.8% of the national data (Ministry of Health of China, UNAIDS et al. 2011).

Drug abuse has emerged as a serious social and public health challenge in China. As syringe and needle sharing is common in IDUs, low awareness of HIV risk and paucity of HIV/AIDS knowledge together aggravate HIV transmission among IDUs. HIV/AIDS epidemic among IDUs in China starts from Yunnan province boarding to the “Golden Triangle” (Thailand, Laos, Viet Nam and Myanmar), then spread along with major drug trafficking roads to Guangxi, Xinjiang, Sichuan and Guangdong provinces, followed with the spread to other provinces. The first reported HIV epidemic in IDUs was the 146 HIV/AIDS cases in Yunnan province (Ma, Li et al. 1990; Duan, Shen et al. 2010); till 2007, an estimated national wide 7% - 13% IDUs were living with HIV/AIDS, among 2011 new HIV infections, 18% were attributed to illicit drug injection (Wang, Wang et al. 2009; Ministry of Health of China, UNAIDS et al. 2011). Regionally, the HIV prevalence among IDUs can be skyrocketed to 80% which mainly scattered in Guangxi, Xinjiang, Sichuan, Guizhou,

Jiangxi and Yunnan provinces (China Ministry of Health and UN Theme Group on HIV/AIDS in China 2004). Recent data showed that the HIV prevalence among IDUs is gradually decreasing. In 2011, among people living with HIV/AIDS, 28.4% were IDUs, while the portion were 32.2% in year 2009, 38.1% in year 2007 and 44.3% in year 2005. Among the new infections in China, 18.0% were IDUs in year 2011, 24.3% in year 2009, 42.0% in year 2007 and 48.6% in year 2005, respectively (Wang, Wang et al. 2010; Ministry of Health of China, UNAIDS et al. 2011). Although much have been done for promoting HIV/AIDS knowledge and reducing the risk factors, 25% IDUs were still sharing syringe and needles (Ministry of Health of China, UNAIDS et al. 2011).

Comparing to other countries, what makes China special in terms of HIV/AIDS epidemic is blood products and blood transfusion as a main HIV transmission way in a specific period. Since 1994, HIV outbreaks among commercial plasma donors in poor rural communities in Henan, Anhui, Hubei and Shanxi provinces have been reported as of small commercial plasma collection. After plasma were collected from donors, the left blood cells, which may be a mixture of several people or even several villages, and were often contaminated with HIV, returned to the donors. Commercial plasma donation, together with needle sharing and inadequate sterilization has promoted HIV transmission among these plasma donors, which made former plasma donors (FPDs) a high risk population of HIV/AIDS epidemic. The seroprevalence of HIV among FPDs is positively correlated to plasma donation frequencies, range from 9.1% in one county to 17% in another, even as high as 74% (Liu, Zheng et al. 2001). Until recently, many FPDs who may have been infected with HIV but never had been tested for HIV infection were developed into AIDS. In year 2011, among those living with HIV/AIDS, 6.6% were transmitted through blood products and blood transfusion due to commercial plasma donation in Henan, Hubei, Anhui and Shanxi provinces. Among alive AIDS patients, 26.6% were caused by blood; and among HIV/AIDS-related deaths, 10.1% were through blood transmission (Ministry of

Health of China, UNAIDS et al. 2011). After the implementation of blood screening and prohibition of commercial blood donation, seldom HIV infection cases have been reported recently, the number of people living with HIV/AIDS due to blood transmission has been gradually decreasing these years, from 70 000 in year 2005, to 60 000 in year 2007, to 57 700 in year 2009, to 51 500 in year 2011 (Wang, Wang et al. 2010; Ministry of Health of China, UNAIDS et al. 2011).

Based on national surveillance studies, the overall prevalence of HIV among female sex workers was 0.6% in year 2009, double the figure in year 2007 (State Council AIDS Working Committee Office China 2010). In high HIV/AIDS prevalence provinces, such as Yunnan and Guangxi, as more IDUs are females, and most of them are commercial sex workers, they form a special group with high HIV risk as they can be infected from illicit drug injection and commercial sex. This special group functions as a bridge from high-risk groups to the general population of men, which transmit HIV to commercial sex clients, their extramarital partners and their spouses. Since 2007, sexual transmission has replaced injection drug users as the dominant mode of HIV transmission. In year 2011, among 780 000 people who living with HIV/AIDS, nearly half (46.5%) were attributed to heterosexual transmission; among 154 000 AIDS patients, 46.8% were infected through heterosexual transmission; and among 48 000 new HIV infections, more than half (52.2%) were transmitted via heterosexual behaviors (Ministry of Health of China, UNAIDS et al. 2011). Comparing to previous data, HIV transmission via heterosexual activities has surged in recent years; the portion of heterosexual transmission among people living with HIV/AIDS in year 2005 was 36.3%, to 40.6% in year 2007, to 44.3% in year 2009, then 46.5% in year 2011. Among newly HIV infected people, the percentage also elevated, from 44.7% in year 2007, to 42.2% in year 2009, to 52.2% in year 2011 (Wang, Wang et al. 2010).

Men who have sex with men (MSM) has emerged as a new HIV high-risk group as

of its common unprotected sexual behaviors and also have sex with women. In China, although MSM is not accepted in traditional culture, it gradually improves its popularity and acceptance in general population, especially in the young generation due to western culture influences. An estimated 4.1 (range 2.7 – 5.4) millions MSM were existed in China. More publications have focused on MSM in China and its role in HIV transmission since 2000. HIV prevalence among MSM is rocketing recently, raised from 1.0% in year 2003, to 1.5% in year 2005, to 2.1% in year 2007, then to 5.0% in year 2009 (State Council AIDS Working Committee Office China 2010). In some specific regions, the HIV prevalence among MSM can score high to 9.1% in Chengdu (van Griensven, de Lind van Wijngaarden et al. 2009). Among people living with HIV/AIDS, the percentage of homosexual transmission has been climbing all the way, from 7.3% in year 2005, to 11.0% in year 2007, to 14.7% in year 2009 (Wang, Wang et al. 2010). For newly infected HIV cases, the portion has jumped from 12.2% in year 2007, to 32.5% in year 2009 and 29.4% in year 2011 (Wang, Wang et al. 2010; Ministry of Health of China, UNAIDS et al. 2011).

Due to mother-to-child transmission, HIV infection in children also appeared in China. Among people living with HIV/AIDS, 1.1% was infected during perinatal and breastfeeding periods. Among AIDS patients, 1.3% were attributed to mother-to-child transmission; In new HIV infections, the percentage has dropped from 1.6% in year 2005, to 1.0% in year 2007 and 2009, then further decreased to 0.4% in year 2011 (Wang, Wang et al. 2010; Ministry of Health of China, UNAIDS et al. 2011).

### 1.1.3 HIV pathogenesis

#### 1.1.3.1 Natural history of HIV infection

HIV primarily infects human immune cells, including helper T cells, macrophages and dendritic cells. Among these target cells, $CD4^+$ T lymphocytes are preferential target cells (Klatzmann, Barre-Sinoussi et al. 1984). Infection with HIV generally leads to the progressive destruction of $CD4^+$ T cell and increase of viral load (Fig. 1-

8) (Pantaleo, Graziosi et al. 1993). According to $CD4^+$ T cell counts and blood viral loads, the typical pattern of HIV infection *in vivo* can be divided into three phases: the acute or primary infection, the latency stage, and the symptomatic phase.

Immediately follows the transfer of body fluids from HIV infected individuals to healthy individuals, the primary HIV infection starts. Primary infection generally lasts for 2 to 8 weeks, and is characterized by high viral load in blood and marked decline of $CD4^+$ T cells. The level of viremia, which is measured by the amount of HIV RNA in the plasma, accurately reflects the extent of virus replication in an infected person as HIV RNA in plasma is contained within HIV particles or virions (Feinberg 1996). During primary infection, when there are numerous susceptible target cells without countervailing host immune responses, the concentration of plasma HIV RNA can exceed $2.2 \times 10^7$ copies per milliliter, corresponding to $1 \times 10^7$ virions per milliliter (Piatak, Saag et al. 1993). Soon after the emergence of antiviral immune responses, of which the antiviral cytotoxic T lymphocytes (CTLs) response is believed to play a pivotal role, the HIV RNA concentration declines sharply. During the decline of HIV RNA concentration, the HIV RNA titer generally fluctuates for a period of time, often lasting for 6 months or so, and then become stabilized around a specific titer, which is called set point. During the process of HIV RNA decline from peak to set point, the amount of $CD4^+$ T cells increase to form an inverse correlation with HIV RNA titer (Mellors, Rinaldo et al. 1996). During primary infection, the number of $CD4^+$ T cells declines dramatically but returns to a near normal level when HIV RNA arrives set point stage. The decline of $CD4^+$ T cells after HIV infection is mainly caused by three pathways: direct cytopathic consequences of HIV infection, HIV infection mediated apoptosis and being killed by $CD8^+$ CTLs. During the primary infection stage, normally between 1 to 4 weeks, newly infected individuals may present a virus-like illness called acute retroviral syndrome (ARS). The symptoms of ARS include fever, rash, muscle aches, sore throat, swollen lymph nodes and joint pains (Levy 2007).

About 6 months after the primary infection, most infected individuals enter into the asymptomatic stage, a latency phase of infection in which HIV viremia reaches the set point as induction of strong immune responses against HIV. These innate and adaptive immune responses includes circulating mannose-binding lectins (MBLs), complement, anti-HIV antibodies, natural killer cells (NK) and T cells. HIV replication during the asymptomatic phase is low in lymph nodes and other tissues. Even in some cases, strong antiviral immune responses lead to latent infection in which the HIV genome is integrated into host genome and no HIV replication or protein expression happens.

As the decline of $CD4^+$ T cells in the chronic asymptomatic phase appears to be approximately linear and constant over the time, when the $CD4^+$ T cell counts is below 200 cells/$mm^3$, the HIV infection enters the final stage, the symptomatic AIDS stage which is characterized by dramatic decline of $CD4^+$ T cells and rapid HIV replication with significantly improved chances of opportunistic infections. The median time from initial HIV infection to the development of AIDS in adults of developed countries without antiviral therapy is about 10 to 11 years (Touloumi and Hatzakis 2000). During this stage, the HIV-specific cellular immunity is exhausted, a direct T-cell killing mechanism dominate and may accelerate the destruction of $CD4^+$ T cells which normally controls the opportunistic infections and tumors, leading to the infection of Mycobacterium tuberculosis, herpes simplex virus, and occurrence of Epstein-Barr virus-induced B-cell lymphomas and Kaposi's sarcoma.

#### 1.1.3.2 HIV transmission

The transmission of HIV is influenced by the amount of infectious agent in a body fluid and the extent of an individual's contact with the body fluid. From classical epidemiological triangle, the establishment of an infection depends on three points: characteristics of the infectious agent; host-related factors like susceptibility,

contagiousness and immune response; and environmental factors including social, cultural and political influences (Levy 2007). According to the amount of HIV in body fluid and the extent of contact with the body fluid, the HIV transmission is categorized into three types: blood and blood products, sexual transmission and mother-to-child transmission.

HIV transmission by blood and blood products: As free infectious HIV particles and HIV-infected cells are present in blood, especially HIV-infected cells appears to be more numerous than infectious virus, blood and blood products becomes the main source of HIV transmission. Blood and blood products transmission can be further divided into three subgroups as of pathways: injection drug users, transfusion recipients and hemophiliacs, needle-stick and other injuries. The first group of individuals in the United States and Europe showing evidence of HIV infection are injection drug users with an estimated 50% to 60% seroprevalence. AIDS in injection drug users further confirmed the presence of HIV in blood through shared needles and syringes (Levy 2007). Before the screening of blood and blood products, HIV could be transmitted to transfusion recipients and hemophiliacs. For transfusion recipients, the risk of HIV infection depends on virus load, the amount of infectious agent including free infectious viruses and HIV-infected cells in blood. The most infectious material is blood from acute infection cases and patients advanced to AIDS. Health care workers such as nurses, laboratory workers and doctors could be infected by needle-stick or mucosal membrane and skin exposure to HIV contaminated blood or blood products. The estimated risk of HIV infection after percutaneous exposure to HIV infected blood is 1 in 300 to 400 (Gerberding 1995). People receiving medical care in developing countries without standard hygiene of medical instruments could also be infected by HIV as contact with HIV-infected blood.

Sexual transmission of HIV: AIDS was first reported as a disease most likely

transmitted by sexual route among male homosexual behaviors. And later studies provided that heterosexual activity is responsible for the majority of infections worldwide (Quinn 1996). As the number of HIV-infected cells is markably higher than free infectious HIV in genital fluids, the transmission of HIV through sexual contact is mainly depends on HIV infected cells while not free HIV particles. There is greater potential of HIV transmission through genital fluids when the donor developed into diseases from an study on homosexual men (Osmond, Bacchetti et al. 1988). And the possibility of sexual transmission is associated with lack of circumcision (Halperin and Bailey 1999).

Mother-to-child transmission: The transmission of HIV from infected mother to child mediated via *in utero, intrapartum* and breastfeeding. The transmission rate between untreated mother and child up to birth is around 25% (Coovadia 2004). This transmission is associated with several factors: a low $CD4^+$ T cell counts, high virus load, p24 antigenemia of mother at the time of delivery, and a large number of HIV-infected cervicovaginal cells (Levy 2007). And a long duration of membrane rupture before delivery enhances the risk of mother-to-child transmission (Kuhn, Abrams et al. 1997). After delivery, HIV can be transmitted to child from HIV infected mother through breastfeeding, as there are HIV-infected cells and absence of anti-HIV IgM and IgA antibodies in milk (Becquart, Petitjean et al. 2006).

#### 1.1.3.3 HIV tropism

HIV tropism generally refers to cell types which HIV can infect and replicate. HIV can infect a variety of immune cells, such as $CD4^+$ T cells, macrophages, microglial cells, langerhans cells (LCs), dendritic cells (DCs) and $CD8^+$ T lymphocytes. HIV entering into target cells is mediated through the interaction of HIV gp120 with CD4 molecule and chemokine co-receptors of target cell (Chan, Fass et al. 1997).

LCs and DCs are located in genital tract and express CD4, CCR5 and CXCR4 molecules, they are considered as the initial targets of HIV infection (Ayehunie,

Garcia-Zepeda et al. 1997; Dittmar, Simmons et al. 1997).

$CD4^+$ T lymphocytes are the major targets of HIV infection; both activated and resting memory ($CD45RO^+$) $CD4^+$ T cells are susceptible to HIV infection *in vivo*. *In vivo*, the activated $CD4^+$ T cells produce the largest amount of infectious virus, while there is limited HIV production in resting memory $CD4^+$ T cells (Cayota, Vuillier et al. 1993).

Macrophages are susceptible to HIV infection and replication through its CD4 and CCR5 molecules. Generally, in macrophage infections, the production of progeny HIV is low and mainly sequesters in intracellular vacuoles (Gendelman, Orenstein et al. 1989). Monocytes differentiate into macrophages is needed for efficient HIV infection (Rich, Chen et al. 1992).

After the identification of CD4 as the major HIV receptor, it is soon appreciated that CD4 is not sufficient for HIV Env-mediated membrane fusion and virus entry. Two co-receptors appear to be predominately important *in vivo*: the $\alpha$-chemokine receptor CXCR4 and the $\beta$-chemokine receptor CCR5. The basis for differential cell-type tropism is based on co-receptors: T-cell lines typically express CXCR4 but not CCR5; primary lymphocytes express both CXCR4 and CCR5, and macrophages express CCR5. To reflect the importance of co-receptors in HIV biology, a system of nomenclature is developed base on co-receptor usage: strains (generally T-tropic) that preferentially use CXCR4 are named X4 viruses, isolates (generally M-tropic) that utilize CCR5 are denoted R5 isolates, and dual-tropic strains that utilize both CCR5 and CXCR4 are termed R5X4 isolates (Freed 2001). CCR5 co-receptor is used by almost primary HIV isolates regardless of viral genetic subtype. Activation of the macrophages with CD40 ligand induces the cells susceptible to replication of X4 viruses (Bakri, Mannioui et al. 2002). Sexual transmission is the major mode of HIV transmission. During sexual intercourse, both X4 and R5 HIV are present in the

seminal fluid, after a selection process, R5 HIV predominant the transmission (Zhu, Mo et al. 1993; van't Wout, Kootstra et al. 1994). After primary infection with R5 HIV, there is often a co-receptor switch in late-stage disease and T-tropic X4 HIV variants appear which can infect a variety of T cells through CXCR4 co-receptor (Clevestig, Maljkovic et al. 2005). These X4 HIV variants replicate more aggressively than R5 HIV, more virulent which cause rapid $CD4^+$ T cell depletion, immune system collapse, and opportunistic infections that characterize the advent of AIDS (Moore 1997). In total, during HIV infection process, HIV adapts to use CXCR4 as co-receptor instead of CCR5 might be a key regulatory mechanism in the progression to AIDS.

### 1.1.4 Immune responses to HIV infection

Upon HIV infection, human immune system is activated to prevent, control and eliminate HIV. Innate immune response and adaptive immune response are involved in the battle against HIV infection.

#### 1.1.4.1 Innate immune response

The innate immune system is the first line of defense against infectious organisms. As innate immune system has earlier origin and presence in primordial organism, the innate immune response also functions as the precursor of adaptive immune response. Being the first reaction to entry of pathogens, innate immune response is rather important in the prevention of HIV infection and controling of HIV infection.

Innate immune system responds rapidly and acts without MHC restriction. It is non-specific by recognizing a conformational pattern of organism while not specific epitope. Components of innate immune system include anatomical barriers, inflammation, complement system and innate leukocytes include NK cells, mast cells, eosinophils, basophils, and phagocytic cells composed of macrophages, neutrophils and DCs. These immediate innate responses to HIV infection help to prevent infection and dramatically reduce the progression to AIDS after HIV

infection (Levy 2007).

Acute-phase proteins and cytokines: Studies with HIV infected plasma donors revealed that just before HIV RNA was first detectable (100 copies per ml) in plasma, the first detectable innate immune response was the increased acute-phase proteins, e.g., the serum amyloid A (McMichael, Borrow et al.). A further wave of acute-phase protein production and cytokine response were coincided with rapid increase of plasma viremia. As viremia increased, cytokines and chemokines in plasma increased relatively. Acute HIV infection witnessed a systematic cytokine cascade and the correlation between plasma viremia increase and multiple cytokines/chemokines elevation, including rapid and transient elevation of IFN-α and IL-15, rapid and sustained increase of TNF-α, slowly elevated pro-inflammatory cytokines IL-6, IL-8, IL-18 and IFN-γ, then followed with peak upregulation of immunoregulatory cytokine IL-10 (Stacey, Norris et al. 2009). Some of these cytokines have antiviral activity; some enhance innate and adaptive immunity. IFN-α, -β, and -γ inhibit HIV-1 replication in primary human macrophages by reducing viral DNA synthesis during early virus life cycle (Meylan, Guatelli et al. 1993); IFN-λ induces the expression of CC chemokines and upregulate type I IFNs and cytidine deaminase apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G/3F (APOBEC3G/3F) to inhibit HIV-1 infection and replication of monocyte-derived macrophages (Hou, Wang et al. 2009); human CC chemokines RANTES, MIP-1 alpha, and MIP-1 beta are also HIV suppressors, they are produced by $CD8^+$ T cells and inhibit different strains of HIV-1 in a dose-dependent manner (Cocchi, DeVico et al. 1995); IL-16 is also found to prohibit HIV-1 replication at level of RNA expression by repressing HIV-1 promoter activity (Baier, Werner et al. 1995; Maciaszek, Parada et al. 1997; Zhou, Goldstein et al. 1997).

DCs: The presence of DCs in the genital mucosa identifies them as potentially the first cells infected by HIV. DCs are important APCs; they take up antigens and

present them to the immune system via MHC peptide complexes. The antigen presenting property of DCs make them essential for the activation of adaptive immune response against HIV. After recognize foreign antigens, Myeloid DCs also activate T-cell immunity (Connolly, Colleton et al. 2007). HIV infected plasmacytoid DCs (pDCs) also produce type I IFNs, which have been shown to directly inhibit HIV replication through IFN-stimulated genes and enhance the antiviral activity of NK cells (Biron 1998; Samuel 2001).

NK cells and NK-T cells: During acute HIV-1 infection, NK cells and NK-T cells are activated. NK cells and NK-T cells produce antiviral cytokines and chemokines, like IFN-γ and TNF-α to control HIV replication (Alter, Malenfant et al. 2004). NK cells and NK-T cells eliminate the HIV-infected cells through antibody-dependent cell-mediated cytotoxicity (ADCC). They also interact with DCs and therefore induce T cell responses.

Macrophages: macrophages are a type of white blood cells differentiated from monocytes. Macrophages act in innate immune response in a non-specific way, also act in adaptive immune response by initiating antigen-specific mechanisms. Macrophages also have APC capacity by phagocytosing antigens and presenting them on cell surface to stimulate lymphocytes and other immune cells to respond to pathogen. During HIV infection, macrophage heterogeneity and differentiation has been highlighted by three major types of macrophage activation: M1, M2 and deactivated macrophages. After HIV infection, in the presence of Th1 and proinflammatory cytokines, M1 macrophage is classically activated and accelerates the formation of HIV reservoirs. Then, IL-4/IL-13 activated M2 macrophages differentiate and committee to tissue repair, MHC II-mediated antigen presentation, increased T cell activation and clearance of opportunistic infections. In late stage of HIV infection, IL-10 deactivate macrophages appearances, which generally leads to immune failure characterized with diminished Ag-mediated T cell response and

accelerated depletion of $CD4^+$ T cells and $CD8^+$ T cells (Herbein and Varin 2010).

#### 1.1.4.2 Adaptive immune response

After immediate and nonspecific innate immune responses took place, the specific adaptive immune responses begin to take effect. Adaptive immune system recognize foreign invaders through specific binding of cell surface receptors, then B lymphocytes begin clonal expansion and differentiate into plasma cells to produce antibodies specific against the recognized antigen, and T lymphocytes are divided into $CD4^+$ T cells and $CD8^+$ T cells. Part of these differentiated B lymphocytes and T lymphocytes will survive for a long period as memory cells rapidly responds to infections when repeated infection occurs. The B lymphocytes mediated adaptive immune responses is humoral immunity, and T lymphocytes mediated adaptive immunity is cellular immune responses.

Humoral immunity generally presents about 2 weeks after HIV infection. They are mainly mediated through specific antibodies against HIV proteins. Structural protein antibodies appear first, including Gag, p24 and p17, but they are non-neutralizing and last shortly; neutralizing antibodies appear later and last for a long time. They mediate through four processes, neutralizing antibodies, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cytotoxicity (ADC) and complement binding antibodies to react against HIV and HIV-infected cells.

Neutralizing antibodies: neutralizing antibodies is a conventional response of the host against virus infection by attaching and neutralizing the virus. Generally, IgG has higher neutralization activity than IgA. Antibodies against HIV are mainly focusing on three domains of HIV envelop protein: a variable region of gp120, the binding sites for CD4 and chemokine receptors, and transmembrane protein gp41. The underlying mechanism of how neutralizing antibodies inactivates HIV has not been well defined, it may involve virus attachment, fusion or virus: cell fusion (McInerney, McLain et al. 1997). Enhancing antibodies: two types of antibody-

dependent enhancement (ADE) exist: complement mediated and Fc-mediated. Both types of ADE involve binding of antiviral antibodies to virion and infected cells by this immune complex via complement or Fc receptor. Mediated through enhancing antibodies, HIV can be facilitated to infect susceptible cells. ADCC and ADC: antibodies against envelop protein gp120 and gp41 can trigger ADCC. In ADCC, the antibody-antigen-coated cells are recognized by effector NK cells or by monocytes/macrophage bearing Fc receptors. The formed antibody-antigen cell complex then be killed by perforin or apoptosis mediated cytolytic mechanism (Levy 2007). In chimpanzees, antibody and complement attach to virus-infected cells and trigger cell death without effector cells, this process is called antibody-dependent cytotoxicity (ADC). Neutralizing antibodies and nonneutralizing antibodies can lyse HIV via complement fixation (Spear, Sullivan et al. 1990).

Cellular immune response plays a pivotal role in restricting and eliminating infectious agent. All T lymphocytes are originated from hematopoietic stem cells in bone marrow. After positive selection and negative selection, survived T lymphocytes are migrating into thymus and maturing into immunocompetent T cells. T lymphocytes differ from other lymphocytes by the presence of specific receptor on their cell surface called T cell receptor (TCR). T lymphocytes can further be categorized into $CD4^+$ T cells and $CD8^+$ T cells as they respectively harbor CD4 and CD8 proteins on their cell surface. $CD4^+$ T cells and $CD8^+$ T lymphocytes contain two major categories: antigen-naive (naive) and antigen-experienced (memory) cells. Memory T cells are a subset of antigen-specific T lymphocytes and live a long-time after infection. Memory T cells can further be divided into central memory T cells (Tcm) and effector memory T cells (Tem) according to their resident locations. Tem can quickly expands to a huge amount of effector T cells when re-challenged by memorized past antigens. Antigens are processed into antigen peptide by APCs and presenting on cell surface, either coupled with MHC II molecules for exogenous antigens, or MHC I molecules for endogenous antigens, respectively activate $CD4^+$ T

cells and $CD8^+$ cytotoxic T cells through TCR.

$CD4^+$ T cells: $CD4^+$ T cells are T helper cells (Th) as they assist other white blood cells in immune response. According to cytokine production and function, Th cells are further divided into Th1 and Th2 subsets. Th1 cells secrete IL-2, IFN-γ and TNF-α to mediate strong cell-mediated immunity; Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 to increase antibody production in humoral immunity. Th cells play an important regulatory role in HIV infection and affect progression to AIDS. Th1 responses are primarily found in healthy asymptomatic HIV-infected individuals, while Th2 predominate the response during symptomatic stage of disease (Clerici, Hakim et al. 1993; Clerici and Shearer 1994). $CD4^+$ T cells also have cytotoxic activity during HIV infection. $CD4^+$ T cells mediate through MHC II molecules to kill HIV infected, uninfected $CD4^+$ T cells or cells expressing HIV peptides by perforin or Fas/FasL-induced apoptosis (Piazza, Gilardini Montani et al. 1997).

$CD8^+$ T cells: $CD8^+$ T cells have cytotoxic (CTL) and non-cytotoxic anti-HIV activity. Upon HIV infection, naive $CD8^+$ T cells are differentiated into effector cells when there are high antigen expression levels, and then developed into Tem and Tcm $CD8^+$ cells and respond to exposed antigens. Tcm $CD8^+$ T cells produce IL-2 to provide protective immunity and produce IFN-γ when they become Tem cells after activation. The $CD8^+$ cytotoxic (CTL) response is MHC restricted, antigen-specific and need cell-to-cell contact. $CD8^+$ CTL response involves perforin production and Fas/FasL apoptosis (Isaaz, Baetz et al. 1995; Kagi, Seiler et al. 1995). $CD8^+$ T cells can also suppress HIV replication in infected $CD4^+$ T cells without killing the cells. This $CD8^+$ T noncytotoxic anti-HIV activity, which is believed as innate immune response, is correlate with cell activation and production of antiviral factors (Landay, Mackewicz et al. 1993; Wilkinson, Zaunders et al. 1999).

### 1.1.5 Diagnosis

HIV/AIDS is a severe health crisis globally. Early diagnosis and early intervention

can prolong the survival time and improve quality of life for people living with HIV/AIDS. Diagnosis of HIV infection can be carried out through detection of antibodies to HIV, p24 HIV antigen, and HIV nucleic acid (RNA/DNA).

HIV antibodies: Detection of HIV antibodies in serum or plasma is the most commonly used method as it is economical, rapid and easy to be performed. About 4 to 6 weeks after HIV infection, HIV antibodies appear and can be detected by ELISA or rapid anti-HIV tests based on immunofilteration, immunochromatography and dot immunoassay. Western blot is used to detect HIV antibodies in clinical samples and can be used as confirmatory assay after antibody test. Diagnosis of HIV infection in babies from HIV-infected mothers can not be dependent on conventional antibody tests as antibodies in babies may be transmitted from mother. HIV DNA or HIV RNA assay are golden standard for diagnosing of HIV infection in children (WHO 2009).

### 1.1.6 HIV prevention

HIV/AIDS caused 1.8 million deaths in 2009, as lack of effective medicine to cure the disease, HIV prevention is thus becoming rather crucial for control of HIV/AIDS epidemic. As HIV was transmitted through blood and blood products, sexual contact and mother-to-child transmission, preventions can be based on these main routes.

Prevent HIV transmission through blood and blood products: Make sure blood and blood products are free of HIV contamination by HIV screening; avoid contact or be well protected before exposing to blood or blood products of HIV-infected individuals; prevent percutaneous injuries for health care workers; prevent needle exchange for IDUs; provide maintenance therapy to minimize risks with needle injection.

Prevent sexual transmission: for commercial sex workers and their clients, promoting condom use and safe sex, educating HIV related knowledge; promote male circumcision. For MSM, safe sex should be promoted.

Prevent mother-to-child transmission: as HIV-infected mother can pass HIV to her babies during pregnancy, childbirth and breastfeeding, effective prevention contains HIV testing, mainly by taking antiretroviral therapy and safer infant feeding procedures.

### 1.1.7 Anti-HIV therapy

Massive progress has been achieved since the discovery of HIV, studies in animal models have brought thorough understanding of how HIV replicate, which shed light on the development of anti-HIV therapy. Multiple drugs have developed to arrest HIV replication and treat AIDS patients.

Reverse transcriptase is essential for HIV replication and is chosen to design drugs. Nucleoside and Nucleotide reverse transcriptase inhibitors (NRTI) interferes reverse transcriptase by directly incorporating into newly synthesized viral DNA and terminating viral DNA production, commercial products includes AZT and Tenofovir; Non-nucleoside reverse transcriptase inhibitors (NNRTI) is directly binding to reverse transcriptase and blocking its function, products includes Nevirapine and Efavirenz.

Protease Inhibitors (PIs) block HIV protease function and arrest new virions assembly. Saquinavir, Ritonavir and Nelfinavir are PIs. Integrase inhibitors interfere with HIV DNA integrate into cell DNA to block HIV replication.

Combination therapy: To improve antiretroviral effects, two or more drugs are combined to treat AIDS patients, this combination antiretroviral therapy is known as highly active antiretroviral therapy, HAART. Most current HAART regiments consist of three drugs: 2 NRTIs + 1 PI/NNRTI.

Other steps of HIV life cycle are also drug development targets, such as entry

inhibitors and maturation inhibitors. Immune system based therapies are focus on restoring immune functions of AIDS patients.

### 1.1.8 Hepatitis B virus, Hepatitis C virus infection

Hepatitis B virus (HBV) and Hepatitis C virus (HCV) are blood-borne pathogens, infect human liver and cause hepatitis.

#### 1.1.8.1 HBV infection natural history, diagnosis, disease progression and epidemiology

Hepatitis B virus (HBV) is an enveloped partially double-stranded DNA virus belongs to family *Hepadnaviridae*. HBV predominantly infects human liver and causes the disease hepatitis B. HBV genome is 3 200 base pair and contains two unsymmetrical DNA strands, the minus strand is a complete circle while the plus strand is shorter with variations in length. The 3 200 bp genome contains four open reading frames (ORFs), which separately encoding the envelope proteins (Large (L), middle (M), small (S) surface proteins), the pre-core/core protein, the polymerase and the transactivating X protein. HBV infects human liver hepatocytes, after a specific reverse transcription, produces progeny genomes and packing into progeny HBV virions.

HBV is blood-borne pathogen; it can be transmitted via contaminated blood and body fluids including saliva, breast milk through perinatal infection and horizontal infection in early childhood, or horizontal infection in adult via injection drug use or sexual transmission. HBV infection can be classified into acute infection and chronic infection with diverse outcomes. For acute HBV infection, its incubation period ranges from 1 to 4 months depends on the HBV transmission mode and quantity of HBV inoculated. Acute HBV infection can be either asymptomatic or symptomatic. In symptomatic acute HBV infection, the symptoms can be fever, fatigue, vomiting and nausea. During acute infection, hepatitis B surface antigen (HBsAg) is detect first, and followed by antibodies against hepatitis B core antigen (HBcAb), and

circulating of hepatitis B e antigen (HBeAg). With the clearance of HBV infection by immune system, HBV replication ends and results HBsAg and HBeAg disappearance. The outcome for perinatally acquired HBV infection is associated with 90% risk of chronicity, which is chronic HBV infection; infections between infancy and younger than 5 years generally have a 20% - 50% risk of chronic HBV infection; infections for adults generally have no jaundice during the entire illness, which indicate complete immune clearance and lifelong immunity, the risk of developing into chronic HBV infection is less than 5% (Gordon 2001).

During acute HBV infection, if the presence of HBsAg, HBeAg and high level of HBV DNA for greater than 6 months, chronic HBV infection established. Chronic HBV infection is usually divided into an early HBV replication phase characterized with acute liver disease and a later non-replication phase with remission of liver disease. In chronic HBV infection settings, HBeAg can be positive or negative. For HBeAg positive chronic HBV infection, HBV replication continues, Alanine transaminase (ALT) levels elevated and active liver disease existed. But for perinatally and early childhood acquired chronic HBV infection, high level of HBV replication exist, normal ALT levels and usually minor liver biopsy changes; they only change into HBeAg positive chronic HBV infection with elevated ALT levels after 10 – 30 years of infection (Pan and Zhang 2005). Among the natural history of HBeAg chronic HBV infection, one key event is HBeAg seroconversion characterized with HBeAg positivity converts into HBeAb and marked reduction of HBV production. The mean annual rate of spontaneous seroconversion is 8% to 15% among individuals with active liver diseases, while for patients with normal ALT levels, the rate is dramatically drop to 2% to 5% (Pan and Zhang 2005). When patients move into HBeAg negative chronic HBV infection stage, HBV replication decreased, HBsAg and HBeAb positive, HBeAg negative, elevated ALT levels. A part of HBeAg seroconversion was attributed to HBV variants which unable to express HBeAg, including G1896A precore mutation, A1762T and G1762A core

promoter mutation. Except HBeAg positive and negative chronic HBV infection, there is another chronic HBV infection phenomena, inactive HBsAg carrier state characterized with HBeAg negativity, HBeAb positivity, low or undetectable HBV DNA, normal ALT levels, minor changes of liver biopsy. The prognosis of the inactive HBsAg carrier usually benign, rarely develop into cirrhosis or hepatocellular carcinoma (HCC).

Detection of hepatitis B virus infection mainly relies on viral antigens or host produced specific antibodies in serum or blood. HBsAg is widely applied in HBV infection detection, mainly for screening purpose as HBsAg is the first detectable antigen during HBV infection. Later, antibody to hepatitis B core antigen (HBcAb, IgM) appears and can be apply as serological evidence of HBV infection, especially helpful for window period of HBsAg seroconversion. HBeAg appears shorter after HBsAg, indicating high level of HBV replication and enhanced infectivity. Aside from serum diagnosis, HBV DNA can also be the target for detection using PCR, Real-time fluorescent quantitative PCR (FQ-PCR) for clinical samples diagnosis, HBV replication monitoring and treatment evaluation (Gitlin 1997). Real-time PCR for quantification of covalently closed circular DNA (cccDNA) can be applied in liver biopsy of HBV patients for long-term anti-HBV therapy evaluation (He, Wu et al. 2002). For patients with chronic HBV infection, HBeAg, HBeAb and HBV DNA should be tested for viral replication (Gordon 2001).

When entering into chronic HBV infection stage, cirrhosis and hepatoma are two major long-term complications. HBeAg negative chronic HBV patients have higher rate of cirrhosis than HBeAg positive chronic HBV patients. The mean duration for onset of cirrhosis among chronic HBV infection acquired in childhood is around 40 years (Guan and Lui 2011). For untreated HBeAg positive chronic HBV patients, the incidence of cirrhosis ranges between 2% to 5.4%, the 5 years cumulative incidence rate is 8% to 20% (Pan and Zhang 2005). The mortality rate at 5 years for

compensated cirrhosis patients is 16% and 65% - 86% for decompensated cirrhosis (De Jongh, Janssen et al. 1992). Development into HCC is a main causality for chronic HBV patients. HBsAg positivity increased the risk of HCC more than 10 folds and HBeAg positivity significantly increased the risk by 60 folds, detectable HBV DNA increased the risk for 4 folds (Yang, Lu et al. 2002). Each year, over 1 million people died of HBV-related liver disease, among them 30% to 50% were HCC-related (Parkin, Bray et al. 2001).

HBV infection is a global public health challenge. The HBV prevalence rate varies among different geographic regions. Base on HBsAg carrier rate, three categories existed: high endemicity regions with HIV prevalence more than 8%, intermediate endemicity regions with HIV prevalence range between 2% to 7%, low endemicity with HIV prevalence lower than 2% (World Health Organization 2001). Developing regions such as China, Southeast Asia, and Africa are with high HBV endemicity as most infections are acquired during infancy and early childhood due to lack of proper medical services and diseases prevention knowledge. Intermediate endemicity regions are scattered in Eastern, Southern and Central Europe, the Middle East, Japan, India and part of South Africa, where HBV infection occurs in infancy, adolescents and adults. For most developed countries, include Western and Northern Europe, North America and Australia, they belong to low endemicity category as most infections are in adults and through horizontal transmission.

Globally, there were about 2 billion people have been infected with HBV; among them 350 millions are remain chronical with HBV (World Health Organization 2001). In China, more than 130 million people are suffering from chronic HBV infection, accounts for one-third of the world's infected population (Custer, Sullivan et al. 2004). The HBsAg carrier rate among the general population is 9.09%, 4.51% among vaccinated population, and 9.51% among unvaccinated (Liang, Chen et al. 2005).

#### 1.1.8.2 HCV infection natural history, diagnosis, disease progression and epidemiology

Hepatitis C virus (HCV) belongs to family *Flaviviridae* and is a small enveloped positive-sense single-stranded RNA virus, which infects human liver and causes disease hepatitis C. HCV was first discovered in year 1989 from parenterally transmitted viral hepatitis cases (Choo, Kuo et al. 1989). HCV genome is single-stranded RNA molecule composed of 9 000 nucleotides and consists of a unique ORF, which encodes a single polyprotein subsequently processed into capsid protein, two envelope proteins E1 and E2, nonstructural proteins NS2, NS3, NS4 and NS5. HCV infects human liver hepatocytes through viral binding, attachment and fusion, to RNA translation, HCV replication and HCV assembly and release. Persistent HCV infection rely on rapid HCV replication and cell-to-cell spread, HCV turnover rate in hepatocyte is $10^{10}$ to $10^{12}$ virions per day.

HCV infection can be further divided into acute HCV infection and chronic HCV infection. Among acute HCV infection, 70% to 80% cases were asymptomatic, only about 20% to 30% adults may develop clinical symptoms including fatigue, jaundice, malaise, and anorexia 3 – 12 weeks after exposure. Serum alaine aminotransferase (ALT) elevated. HCV RNA can be detected in serum 1 to 2 weeks after exposure, while anti-HCV antibody only appears approximately 1 to 3 months after exposure.

Chronic hepatitis C is defined as the persistence of HCV RNA in blood for more than 6 months after acute HCV infection. It is generally believed that approximately 85% of acute HCV infection will develop into chronic HCV infection and at risk for development of compensated and decompensated cirrhosis, and hepatocellular carcinoma (HCC) (Chen and Morgan 2006). The rate of chronic HCV infection is affected by the age of HCV infection, development of jaundice during acute infection. The younger, the lower chronicity rate of HCV; lower chronic HCV

infection rate in patients developed jaundice and showed symptoms during acute infection (Chen and Morgan 2006). 10% to 15% chronic HCV infected patients will develop into cirrhosis. The risk of progression into advanced liver fibrosis is affected by external and internal factors, including alcohol use, age of infection, degree of inflammation and fibrosis, and co-infection with HIV or HBV (National Institutes of Health Consensus Development Conference Statement 2002). The estimated 3, 5 and 10-year survival rate of compensated cirrhosis were 96%, 91% and 79%, respectively (Fattovich, Giustina et al. 1997). Once cirrhosis is established, HCC will develop at an annual rate of 1% to 4%. The risk to HCC in HCV-infected patients is 17 folds higher than HCV negative controls (Donato, Boffetta et al. 1998).

After HCV infection, the antibody to HCV, namely anti-HCV to be detected by enzyme immunoassay only appears near the onset of symptoms which is 1 to 3 months after HCV exposure. As nearly 30% of patients will have negative result for anti-HCV at onset of their symptoms, making anti-HCV testing unreliable for the diagnosis of acute HCV infection. The anti-HCV assay detects greater than 90% of HCV infections 3 months after HCV exposure (Chen and Morgan 2006). As serologic marker in acute HCV infection is slow and inaccurate, reverse-transcriptase PCR is needed for the detection of HCV RNA in chronic HCV infection and early acute HCV infection (Gretch 1997). Liver biopsy is also an important parameter for diagnosis of chronic HCV infection (Castillo, Bartolomé et al. 2010).

Approximately 175 million people are affected with HCV worldwide, comprising around 3% of the global population (Chen and Morgan 2006). Each year 3 to 4 million new HCV patients are diagnosed (Munir, Saleem et al. 2010). HCV transmission is primarily through exposure to infected blood. Risks for transmission include blood transfusion, injection drug use, high-risk sexual activity, solid organ transplantation from an infected donor, occupational exposure, birth to infected mother. 90% injection drug users are at high risk of getting HCV, 5% HCV infections

are caused by needle stick injury, and 3% to 5% infants acquire HCV from infected mother through perinatal transmission (Munir, Saleem et al. 2010). The estimated global HCV prevalence is 2.2%, with lowest prevalence in United Kingdom (0.01%-0.1%) to the highest prevalence in Egypt (15%-20%); among HCV-infected patience, the estimated cirrhosis and HCC prevalence are 27% and 25% globally (Alter 2007). In patterns of HCV infection, there are geographic and temporal differences among different countries. Occult HCV infection is defined as HCV RNA exists in liver and peripheral blood mononuclear cells (PBMCs), and negative for anti-HCV and HCV RNA in serum. One study reported a 3.3% occult HCV infection rate in a population unselected for hepatic disease (De Marco, Gillio-Tos et al. 2009). In China, the estimated national HCV seroprevalence was 3.2% in general population, 8.68% in blood donors (Liu and Wei 2007; Gao, Cui et al. 2011).

### 1.1.9 HIV, HBV, HCV co-infections

HIV is the prototype virus of family *Retroviridae* and infects cells of immune system. According to the amount of HIV in body fluid and the extent of contact with body fluid, HIV transmission is categorized into three types: blood and blood products, sexual transmission and mother-to-child transmission. Hepatitis B virus (HBV), a species of family *Hepadnaviridae*, infects human liver and causes the disease hepatitis B. HBV can be transmitted via contaminated blood, sweat, tears, saliva, semen, vaginal secretions, menstrual blood and breast milk. Hepatitis C virus (HCV) belongs to family *Flaviviridae* and is a small enveloped positive-sense single-stranded RNA virus, which infects human liver and causes disease hepatitis C. HCV transmission mainly via contaminated blood and associated with risk factors including injection drug use, inadequate sterilized medical equipment, blood transfusion and sexual intercourse. As HIV, HBV and HCV are blood-borne pathogens and share routes of transmission, HIV, HBV and HCV co-infections are common.

Worldwide, there were an estimated 370 millions chronic HBV infections, 130 millions chronic HCV infections and 33.3 millions HIV infections; among HIV positive persons, 90% of them have anti-hepatitis B core antibodies (anti-HBcAb) which is a biological marker of prior HBV infection; based on the presence of serum hepatitis B surface antigen (HBsAg), 7% - 10% HIV-infected individuals were HBV chronic infected (Alter 2006). HBV transmission mode in HIV positive persons varies among different geographical regions. In low HBV prevalence area, where less than 2% population is HBsAg positive, including the United States and Western Europe, injection drug use and unprotected sex are the primary mode of HBV transmission and mainly affect adult population. As HBV is around 100 folds more likely to be transmitted than HIV, HBV infection precedes HIV infection (Chapman, Sullivent et al. 2008). In this setting, the HIV/HBV prevalence is range from 5% to 10%, which are 10 folds higher than general population. In intermediate and high HBV prevalence regions, which mainly in Africa and Asia, HBV transmission mainly via perinatal, close contact within households and contaminated medical instruments, and affect children; in this setting, the HIV/HBV co-infection prevalence can score to 20% (Diop-Ndiaye, Toure-Kane et al. 2008; Lee, Ko et al. 2008; Nyirenda, Beadsworth et al. 2008). Except for geographic regions, HBV prevalence also varies among transmission modes. According to report regarding Western Europe and the United States, the overall chronic HBV prevalence is 6% - 14%, 4% - 6% for heterosexual activities, 9% - 17% for MSM and 7% - 10% for IDUs (Alter 2006). Occult HBV infection is defined as infection of HBV with the detection of HBV DNA while lack of HBsAg. The estimated occult HBV infection prevalence in HIV positive people is vary between 2% and 85% (Hofer, Joller-Jemelka et al. 1998; Tsui, French et al. 2007).

HCV share transmission routes with HIV, while HCV is more efficiently transmitted via exposure to contaminated blood and needle-sharing in IDUs. Globally, there is an estimated 4 - 5 millions who have HIV/HCV co-infections (Alter 2006). The

seroprevalence of HCV in HIV-infected persons varies between 15% and 30% depending on population characteristics (Sherman, Rouster et al. 2002). HCV prevalence varies among transmission modes. According to report about Western Europe and the United States, the overall HCV prevalence is 25% - 30%, 9% - 27% for heterosexual activities, 1% - 12% for MSM and 72% - 95% for IDUs (Alter 2006). An Iran study revealed a 72% HIV/HCV prevalence, together with 7.9% HIV/HBV/HCV prevalence (Mohammadi, Talei et al. 2009).

For patients who acquired HIV prior to HBV, HIV adversely affects the natural history of HBV infection, including 6 folds increased chance of progression into chronic HBV infection, decreased HBeAg clearance rate and increased HBV replication rate. All together will promote to HBV-related liver diseases -- cirrhosis and liver cancer, and increase a 3.6-folds risk of liver-related mortality compared to HBsAg-negative. The risk of liver-related mortality for HIV/HBV co-infections doubled after highly active antiretroviral therapy was introduced (Bodsworth, Cooper et al. 1991; Hadler, Judson et al. 1991; Gilson, Hawkins et al. 1997; Colin, Cazals-Hatem et al. 1999; Thio, Seaberg et al. 2002; Konopnicki, Mocroft et al. 2005; Thio 2009). For HIV/HCV co-infection patient, progression to chronic HCV infection has significantly elevated from 70% - 85% in HIV-negative to over 90%, especially among AIDS patients with advanced immunosuppression (Thomas, Astemborski et al. 2000; Mehta, Cox et al. 2002). HIV/HCV co-infection also showed higher HCV RNA levels in plasma, higher risk of HCV transmission, and impaired antibody responses (Cribier, Rey et al. 1995). Among HIV/HCV co-infections, there were 2-folds and 6-folds higher risk of progression to cirrhosis and liver failure, increase liver-related morbidity and mortality (Graham, Baden et al. 2001). Although HCV sero-status has not affected the overall virological and immunologic responses to HAART and the disease progression of HIV infection, the liver-related mortality was markedly elevated (Rockstroh, Mocroft et al. 2005).

After 14 years implementation of hepatitis B vaccination to infants, HBsAg prevalence in China has dropped from 9.75% in year 1992 to 7.2% in year 2009; HBsAg was greatly diminished for less than 15 years old youths and only 1.0% among younger than 5 years old children (Liang, Bi et al. 2009). According to a report, the prevalence of chronic HBV infection among HIV positive people was similar to general population (Liang, Chen et al. 2005). HCV infection was transmitted along with drug trafficking routes and together with HIV spread into China. HIV/HCV co-infection prevalence in China varies in different geographic regions and transmission modes. A systematic review summarized the overall prevalence of HIV/HCV co-infection in IDUs was 6.45%, ranging from 2.48% to 15.23% among different provinces; while for Southeastern region of Yunnan province, the HIV/HCV prevalence among IDUs was 99.3% (Zhang, Yang et al. 2002; Bao and Liu 2009). For former plasma donors (FPDs), the prevalence of HIV/HCV varies in different regions. A study in Shanxi revealed the HIV/HCV prevalence was 85.0% among IDUs, other studies revealed the prevalence of HIV/HCV co-infection were 68.0% and 87.0% (Yan, Zheng et al. 2000; Zhang 2001; Qian, Vermund et al. 2006).

## 1.2 Interleukin-27

Interleukin-27 (IL-27), a heterodimeric cytokine, composed of Epstein-Barr virus-induced gene 3 (EBI3) protein and p28 protein, belongs to IL-6/IL-12 cytokine family. Since the first description of IL-27 in year 2002, numerous progresses have been achieved on the understanding of IL-27. IL-27 has been demonstrated to modulate immune system, posses anti-tumors and antiviral features, it also participate in the pathogenesis of hepatitis.

### 1.2.1 Biology of IL-27

IL-27 was first identified in 2002 by computational technology (Pflanz, Timans et al. 2002). A new protein was identified and named as p28 as of its molar mass on SDS-

PAGE, evolutionary dendrogram then classified p28 as an IL-6/IL-12 family cytokine. Co-immunoprecipitation assay revealed p28 form a soluble complex with EBI3 protein and the heterodimeric complex was designated as IL-27. EBI3 and p28 were mainly co-expressed on APCs. IL-27 induces proliferation of naive $CD4^+$ T cells, synergize with IL-12 to induce high levels of IFN-γ production, which prompt naive $CD4^+$ T cells transfer into Th1 phenotype cells. IL-27 promotes vigorous growth of activated naive $CD4^+$ T cells, which suggests IL-27 is essential for the initiation of immune response to inflammatory challenges (Pflanz, Timans et al. 2002).

To exert its function, IL-27 has to interact with its receptor(s). Orphan WSX-1/TCCR was first identified as IL-27 receptor subunit and essential for the initiation phase of Th1 responses (Pflanz, Timans et al. 2002); gp130 was later demonstrated to interact with WSX-1/TCCR to constitute a functional signal-inducing receptor for IL-27. WSX-1/TCCR and gp130 are required to mediate IL-27-induced signal transduction, while subunits itself is insufficient. WSX-1/TCCR and gp130 are widely co-expressed in many immune cells, includes monocytes, DCs, T and B lymphocytes, NK cells, mast cells and endothelial cells. The wide distribution of IL-27Rs indicates IL-27 has an active role in the coordination of innate as well as adaptive immune responses (Pflanz, Hibbert et al. 2004).

### 1.2.2 IL-27 on immune system

IL-27 exerts immune modulate function through its receptor. After elicitation by APCs upon stimuli, IL-27 binds to its functional signal-inducing receptor WSX-1/TCCR and gp130, induces phosphorylation of Jakl, STAT1, STAT3, STAT4, and STAT5, initiate intracellular signaling cascade and play roles in immunity.

Pro-inflammatory functions: IL-27 can promote Th1 responses. IL-27 augments proliferation of naive $CD4^+$ T cells and secretion of IFN-γ, which was the signature cytokine of Th1 response (Pflanz, Timans et al. 2002). IL-27 also synergizes with IL-

12 to produce IFN-γ in NK cells (Pflanz, Timans et al. 2002; Lucas, Ghilardi et al. 2003; Takeda, Hamano et al. 2003). Naive WSX-1-deficient $CD4^+$ T cells produce less IFN-γ than wide-type counterparts, further stressed the essential role of IL-27Rs in Th1 immune response (Chen, Ghilardi et al. 2000). IL-27 promotes IFN-γ production was mainly attributed to transcription factor T-bet. Activation of STAT1 can directly induces T-bet expression, which will promote production of IFN-γ and induction of IL-12R$\beta$2. IL-27 also phosphorylates STAT4 to enhance IFN-γ transcription and secretion (Villarino, Huang et al. 2004). Furthermore, IL-27 drives Th1 differentiation can also mediate through p38 mitogen-activated protein kinase (MAPK) and intracellular adhesion molecule-1/leukocyte function antigen-1 (ICAM-1/LFA-1) signaling pathways. IL-27 stimulation leads to STAT1 dependent and independent IFN-γ expression through p38 MAPK (Owaki, Asakawa et al. 2005; Owaki, Asakawa et al. 2006).

Anti-inflammatory functions: IL-27, like IL-6, signals through gp130, can also suppresses inflammatory response *in vivo* (Taga and Kishimoto 1997; Ishihara and Hirano 2002). Several studies reported increased proliferation of WSX-1-deficient $CD4^+$ T cells during culture *in vitro*; both $CD4^+$ T and $CD8^+$ T cells of WSX-$1^{-/-}$ mice displayed enhanced proliferation and secretion of IFN-γ after infection with *Toxoplasma gondii* (Chen, Ghilardi et al. 2000; Yoshida, Hamano et al. 2001; Villarino, Hibbert et al. 2003). The possible mechanism of IL-27 suppresses Th1 immunity is STAT1-dependent inhibition of T cell responses (Villarino, Huang et al. 2004). IL-27 signal through WSX-1 to suppress Th1 response, it can also negatively regulates the generation of Th2 inflammatory responses. Elevated expression of IL-4, IL-5 and IL-13 in WSX-$1^{-/-}$ $CD4^+$ T cells than wild-type counterparts were observed after *T.cruzi* infection (Hamano, Himeno et al. 2003). The inhibitory function of IL-27 may mediate through down-regulation of GATA3 in T cells (Lucas, Ghilardi et al. 2003).

To summarize, IL-27 prompts Th1 response in early stage of infection, it negatively regulates Th1 activity in later stages and suppresses Th2 and Th17 differentiation. *In vivo*, the dominant role of IL-27 is negatively regulate the immune system (Batten and Ghilardi 2007).

### 1.2.3 IL-27 anti-tumor properties

IL-27 has immune modulation property; it also plays a crucial role in tumor prevention and treatment. Hisada first described IL-27 has anti-tumor activity in a murine tumor model (Hisada, Kamiya et al. 2004). Murine colon carcinoma C26 cells were transduced with the single-chain IL-27 cDNA and secreting IL-27 (C26-IL27). Mice inoculated with C26-IL27 exhibits complete tumor remission, recovered mice from inoculation were shown tumor-specific protective immunity to following challenges with C26. The antitumor activity of IL-27 was mainly mediated through induction of IFN-γ production, enhancement of $CD8^+$ cytotoxic T-lymphocyte activity and T-bet (Hisada, Kamiya et al. 2004).

Apart from CTL activity and IFN-γ, IL-27 also activates NK cells and suppresses NK-resistant head and neck squamous carcinoma (Matsui, Kishida et al. 2009). IL-27 induces expression of T-bet and granzyme B in murine NK cells, and enhances NK cells cytotoxic activity both *in vitro* and *in vivo*. Therapeutic administration of IL-27 gene successfully suppressed the growth of NK-unsusceptible squamous cell carcinoma (SCC) VII tumors via its antibody-dependant cellular cytotoxicity machinery. IL-27 thus can be used as therapeutic tool to eradicate head and neck squamous cell carcinoma by sensitizing NK cells and producing tumor-specific immunoglobulin, which cooperatively activate antibody-dependent cellular cytotoxicity activity (Matsui, Kishida et al. 2009).

IL-27 also exerts anti-proliferative activity on melanomas through WSX-1/STAT1 signaling (Yoshimoto, Morishima et al. 2008). IL-27 augments IRF-1 and IRF-8 expression, both of which possess tumor suppressor activities. IL-27 also exerts

direct anti-proliferative effect *in vivo*.

IL-27 modulates DCs functions *in vivo* (Shinozaki, Wang et al. 2009). During generation of tumor immunity, IL-27 promotes CTL generation while suppresses DCs functions through the suppression of cytokine production and antigen-presenting functions (Shinozaki, Wang et al. 2009).

IL-27 receptor WSX-1 is essential for its biological functions. Controversial to previous ideas that WSX-1 itself was highly expressed in multiple types of epithelial tumor cells when compared to normal epithelial cells (Dibra, Cutrera et al. 2009). The highly expressed exogenous WSX-1 in epithelial tumor cells suppresses tumorigenicity *in vitro* and inhibits tumor growth *in vivo*. This antitumor property of WSX-1 is not mediated through IL-27 dependent signaling pathway while through enhanced NK cytolytic activity dependant on NKG2D ligand up-regulation (Dibra, Cutrera et al. 2009).

Together, IL-27 exerts antitumor immunity through induction of IFN-γ, enhancement of $CD8^+$ CTL function; activate NK cells and antibody-dependent cellular cytotoxic activity; exerts anti-proliferative activity through WSX-1/STAT1 signaling; suppress DCs functions. WSX-1 also exerts antitumor activity through IL-27 independent NK cytolytic activity.

### 1.2.4 IL-27 antiviral features

Beside IL-27's features on immune modulation, IL-27 also demonstrated antiviral capabilities. Studies have revealed that IL-27 can inhibit the replication of R5 and X4 strains HIV-1, Hepatitis C Virus (HCV), Fowl plague virus (FPV), and Influenza virus *in vitro*.

Human papilloma virus (HPV)-like particles (VLPs) has been widely used as vaccine for preventing infection of HPV. Study found that HPV-VLPs can elicits antiviral

cytokines, which later proved HPV-VLPs can suppresses the replication of R5 and X4 HIV replication in PBMCs, $CD4^+$ T cells and macrophages (Fakruddin, Lempicki et al. 2007). The inhibitory function of VLPs was mainly mediated by soluble factors secreted from HPV-VLPs treated PBMCs and macrophages. Among these soluble factors, IL-27 was later proved to be important in suppressing HIV-1 replication. IL-27 inhibits HIV-1 replication in a dose-dependent manner and does not affect CD4, CCR5 and CXCR4 expression levels (Fakruddin, Lempicki et al. 2007). IL-27 activates STAT1 in T cells and STAT1 and STAT3 in monocyte-derived macrophages (MDMs). Microarray assay later indicated that the inhibition of HIV replication by IL-27 in MDMs involves activation of multiple Interferon-inducible genes (IFIGs) which was similar to IFN-α. DNA microarray demonstrated that IL-27 also induced multiple genes associated with the defense response, innate immune response and responds to virus infection (Imamichi, Yang et al. 2008). APOBEC cytidine deaminases, is a class of proteins which can interfere with the replication of HIV (Mangeat, Turelli et al. 2003). Greenwell-Wild reported IL-27 induces an intermediate production of type I interferon in macrophages and $CD4^+$ T cells, which in turn trigger synthesis of APOBEC cytidine deaminases to fend off advances by HIV (Greenwell-Wild, Vazquez et al. 2009).

IFN-α has been widely used to treat HCV and HIV/HCV co-infection patients, as IL-27 induced IFIGs which was similar to IFN-α did and intermediate induction of IFN-α, it was speculated that IL-27 has therapeutic functions on HCV. Frank and colleagues demonstrated that IL-27 induced STAT1 and STAT3 in a HCV permissive cell line Huh7.5, and inhibited HCV replication in a dose-dependent manner, 100 ng/ml IL-27 can suppresses nearly 50% HCV replication (Frank, Zhang et al. 2010).

It has been proven that IL-27 has functions on T cells, and signals via common IL-6 cytokine family receptor gp130 (Pflanz, Timans et al. 2002). As IL-6 is an important mediator of acute phase response in liver damage and liver regeneration (Heinrich,

Castell et al. 1990; Fausto 2004), people suspect the potential role of IL-27 on liver cells. Bender and colleagues described for the first time that IL-27 displayed IFN-γ-like functions in human hepatoma cells and hepatocytes. IL-27 induces a sustained phosphorylation of STAT1 and STAT3 in HepG2 cells and PH5CH8 cells. Pretreated HepG2 cells produce less progeny fowl plague virus (FPV) 24 hours after FPV infection, proved IL-27 displays anti-viral activity on liver cells. IL-27 also induces interferon-regulated genes, like guanylate binding protein 2 (GBP2) in a way similar to IFN-γ (Bender, Wiesinger et al. 2009).

### 1.2.5 IL-27 with hepatitis

In a ConA model of T-cell mediated hepatitis, EBI3 and p28 expression levels were increased in APCs from the liver 6h after ConA injection. Serum IL-27 was significantly induced 4h after ConA administration indicating a potential regulatory role of IL-27 in T cell-mediated live injury. Significant elevated liver enzymes and obvious necrosis were observed in liver section; while in ConA treated IL-27/EBI3 deficient mice, no marked signs of inflammation and necrosis were observed in liver section. IL-27 induces STAT1 and T-bet activation in T-cell mediated hepatitis, while ConA administrated IL-27/EBI3 deficient mice witnessed a decreased hepatic IFN-γ expression, along with suppressed STAT1 and T-bet activation. All these findings together demonstrated that IL-27 plays an important regulatory function in T-cell mediated hepatitis (Siebler, Wirtz et al. 2008).

## 1.3 Single-nucleotide Polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs), refers to DNA sequence changes when a single nucleotide in the genome is altered, in short SNP is a single base change in single position; SNPs are the most common type of genetic variation. For a genetic variation to be considered as a SNP, it should be accounted for at least 1% in the population.

Since the completion of Human Genome Project in 2003, human genetic sequence

variation study has became the keystones of biomedical research, as SNPs, the inherited differences in DNA sequence not only contributed to phenotypic variation, it also influenced an individual's anthropometric characteristics, susceptibility to certain diseases and respond to environmental challenges (Sachidanandam, Weissman et al. 2001). In a human genome map, it described 1.42 million SNPs throughout the genome, with SNPs density of 1 SNP per 1.9 kilobases. It also estimated that 60,000 SNPs located in exon, these coding and untranslated regions; and 85% of exons in the human genome are within 5 kb of the nearest SNP (Sachidanandam, Weissman et al. 2001).

SNPs have a low rate of mutation, which provides reliable information of human evolution history. SNPs reflect historic mutations that were mostly unique events, and individuals sharing the same allele are believed to originated from the same evolutionary heritage. The human genome and SNP database, along with molecular tools, have bring anthropology into molecular anthropology which explains phenotypes and their roles in human evolution process (Stoneking 2001). By using SNP map, we can study the contributions of single gene on disease which was resulted from multiple gene changes. SNPs, the variation of genome sequences, determine different susceptibilities to and protection from all kinds of diseases; when to onset and how sever of certain illness; how effective our bodies responded to treatment (Chakravarti 2001).

### 1.3.1 Types of SNPs

SNPs, the genetic variations of genome, can generally be classified into exon SNPs, intron SNPs and intergenic region SNPs according to their relative locations to genes.

Exon SNPs refer to SNPs in nucleic acid sequence coding for amino acid and conjunctive untranslated regions. SNPs in amino acid coding regions can further be divided into synonymous SNPs and non-synonymous SNPs according to their effects

on amino acid substitution. Synonymous SNPs are SNPs which produce same sequence as of redundant genetic code usage; non-synonymous SNPs induce amino acid changes in translated peptide. Non-synonymous SNPs can either be missense or nonsense; missense SNPs result amino acid substitution, while nonsense SNPs introduce premature stop codon and prematurely stops translation. As non-synonymous SNPs code for different protein sequence, they are believed to be more relevant to different gene functions. SNPs in 5'/3' untranslated regions can exert their functions by regulating gene transcription and translation through affecting transcriptional factors binding.

Intron SNPs are SNPs located in non-translated regions of genes. As intron SNPs are not translated into protein, they may exert functions through influencing gene translation regulation.

Intergenic region SNPs are SNPs distributed in regions between clusters of genes which contain low density of or no genes. Intergenic region SNPs may influence transcription regulation.

### 1.3.2 Functions of SNPs

SNPs at different locations may exert different biological functions; all of them are involved in the Central Dogma by regulating gene transcription and translation.

SNPs have been widely used for uncovering susceptibility genes underlying the heritability of complex or common diseases in genetic association studies as its abundance and easy scoring. A variant gene alters an immunological properties can bridge the gap between genome sequence and immune response. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), the only systemic autoimmune disease described, was found to be directly associated with five mutations in AIRE gene, in which a nonsense SNP 889C/T caused premature stop, and resulted in APECED (Aaltonen, Bjorses et al. 1997). Genetic variations in

chemokines and their receptors pose impact on HIV transmission and progression to AIDS. Chemokine receptor CCR2, which also served as HIV co-receptor for HIV infection, was demonstrated to harbor a SNP and was related to AIDS progression (Smith, Dean et al. 1997); the CCR2 190G/A SNP, which substituted CCR2 64 amino acid valine into isoleucine, was designated as CCR2-64I. Cohort study has proved that HIV infected patients harboring CCR2-+/64I and CCR2-64I/64I genotypes have a consistent 2 to 3 years delay in median time to AIDS outcome compared to individuals with homozygous CCR2 +/+ genotype (Smith, Dean et al. 1997). Stromal-derived factor (SDF-1), the natural ligand of CXCR4, has been demonstrated to effectively inhibit HIV replication (Bleul, Farzan et al. 1996; Oberlin, Amara et al. 1996), also contain a SNP with delayed onset of AIDS (Winkler, Modi et al. 1998). The SDF1-3'UTR-801G/A SNP, which abbreviated as SDF1-3'A, is a G to A transition SNP on 3'UTR of SDF1 gene. Cohort study of HIV-infected persons later proved that individuals with SDF1-3'A/3'A genotype had a remarkable slow progression to AIDS when compared to individuals harbor SDF-+/+ and SDF1-+/3'A genotypes. The role of SNPs on complex diseases was further uncovered by the application of genome-wide association studies (GWAS). A GWAS with lung cancer cases revealed that a locus in 15q25 region was strongly associated with lung cancer; inside the locus, a non-synonymous SNP of CHRNA5 gene was most strongly associated with lung cancer (Hung, McKay et al. 2008).

Beside its roles in disease susceptibility, SNPs also related with treatment responses. In a Korea advanced non-small-cell lung cancer (NSCLC) study, patients were differently responded to irinotecan and cisplatin treatment, and these differences were introduced by SNPs of uridine diphosphate-glucuronosyltransferanse 1A1 and UGT1A9. UGT1A1*6 (UGT1A1 211G/A) and UGT1A9*22 (UGT1A9 -118$(T)_{10}$/$(T)_9$) genotypes were associated with irinotecan-related severe toxicity (Han, Lim et al. 2006). Another study about NSCLC patients also revealed that P-glycoprotein (ABCB1) and canalicular multi-specific organic anion transporter (ABCC2) gene

polymorphisms are related to irinotecan-pharmacokinetics and clinical outcome of NSCLC patients (Han, Lim et al. 2007).

## 1.4 Objectives of the study

HIV infection in human is considered as pandemic by World Health Organization (WHO). Since its discovery in 1980s, massive progress has been achieved. Although the drug cocktail used in HAART can significantly slow the progression of HIV-infected persons advance to AIDS, HIV/AIDS is still one of the leading causes of death, caused 1.8 million deaths worldwide in year 2009 (UNAIDS 2010). HBV and HCV infections are also severe global health challenges. As HIV, HBV and HCV share routes of transmission, HIV/HBV, HIV/HCV co-infection, even HIV/HBV/HCV triple-infection has emerged as new health problems with severe clinical consequences.

In order to understand the seroprevalence of HBV, HCV co-infections in HIV positive population in Shenzhen, an immigrant city in Southern China, we performed a cohort study in Shenzhen. Recruited HIV positive participants were tested for HBsAg and anti-HCV antibodies. Age, gender and HIV transmission risk behaviors were analyzed for association of co-infections.

IL-27 has been demonstrated to inhibit HIV replication *in vitro* (Fakruddin, Lempicki et al. 2007), while it has not yet been demonstrated whether HIV infection affects IL-27 expression, and how HBV, HCV co-infection affect IL-27 titer in HIV positive person. To determine the correlation of HIV infection and IL-27 expression *in vivo*, HBV, HCV co-infection with IL-27 titer; HIV mono-infected, HIV/HBV co-infected, HIV/HCV co-infected and HIV/HBV/HCV triple-infeted individuals were recruited in the study. Enzyme-linked immunosorbent assay was used to detect the plasma IL-27 expression levels in HIV infected persons and healthy individuals, $CD4^+$ T cell counts also quantified and analyzed with IL-27 expression level.

As HIV, HCV share routes of transmission, the prevalence of co-infection with HCV in HIV-infected individuals is high. IL-27 has been demonstrated to inhibit HIV and HCV replication *in vitro* (Frank, Zhang et al. 2010); while how HIV, HCV viral loads affect IL-27 expression level is mystery. We recruited HIV mono-infected and HIV/HCV co-infected people, quatify HIV, HCV viral loads and analyze the correlation among HIV, HCV viral loads, IL-27 titer and $CD4^+$ T cell counts.

Genetic polymorphisms influence diseases susceptibility and IL-27 p28 SNPs have been shown to be associated with the susceptibility to asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD) and chronic HBV infection (Chae, Li et al. 2007; Huang, Liu et al. 2008; Wang, Zhu et al. 2009). To test the possible association of IL-27 p28 polymorphisms with individual susceptibility to HIV infection, we use TaqMan® Allelic Discrimination Assay to compare IL-27 p28 -964A/G, 2905T/G and 4603G/A genotype distributions and allele frequencies between HIV-infected MSM individuals and healthy MSM controls in a Chinese population. LD coefficients and haplotype analysis were applied to analyze the risk of HIV susceptablity.

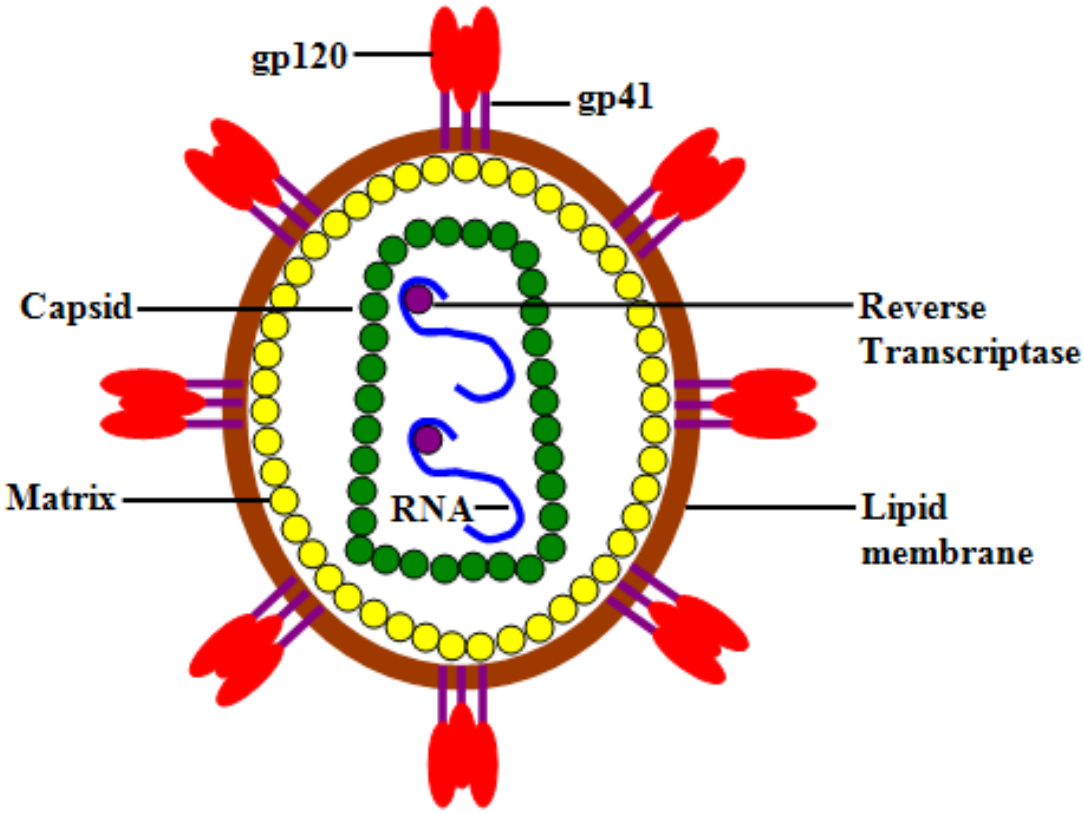



**Fig. 1-1. Diagram of mature HIV virion.** The HIV virion was surrounded by bilayer lipid membrane with gp120 and gp41 anchor to virion envelop; two copies of positive sense, single-stranded RNA was protected by cone-shaped nucleocapsid (core).

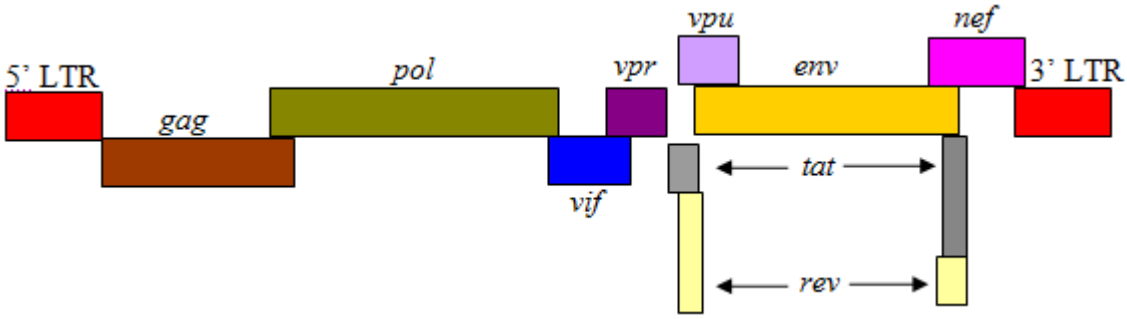



**Fig. 1-2. Schematic structure of HIV genome.** The 9.2 kb positive, single-stranded HIV RNA contains 9 ORFs by overlapping parts of the genome.

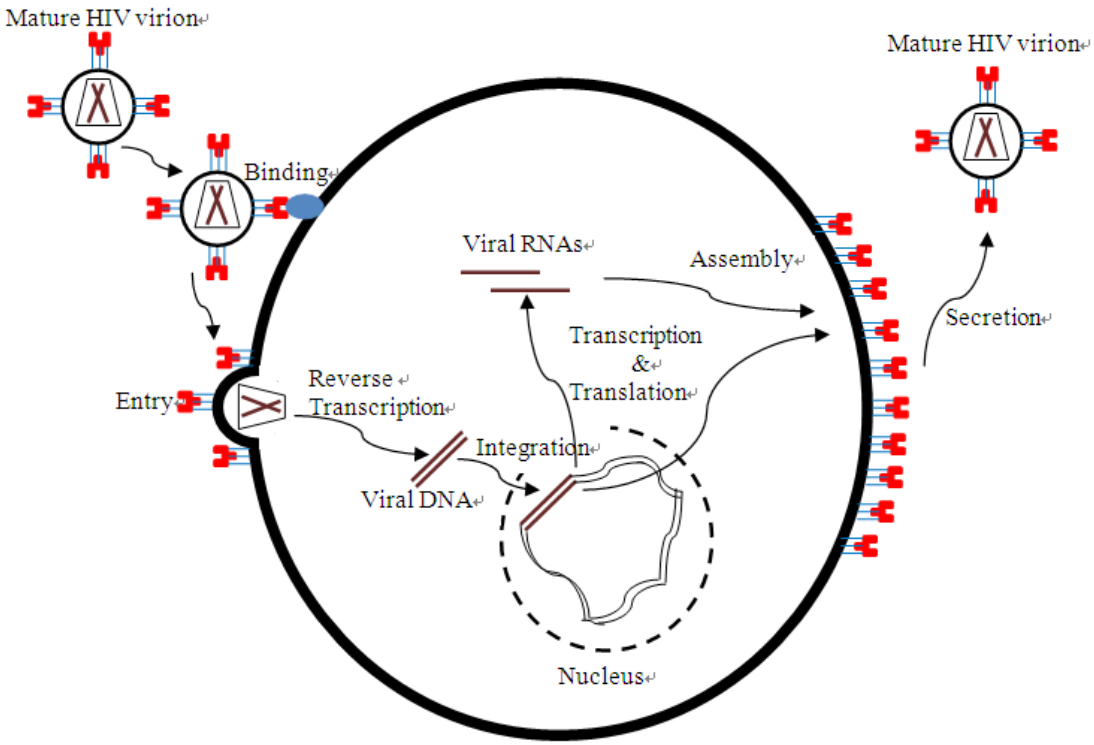



**Fig. 1-3. Schematic representation of HIV life cycle.** The HIV life cycle can be divided as binding and entry, reverse transcription and integration, transcription and translation, assembly and budding and maturation.

**Fig. 1-4. A global view of estimated number of people lives with HIV in 2009: 33.3 million.** (Source: UNAIDS)

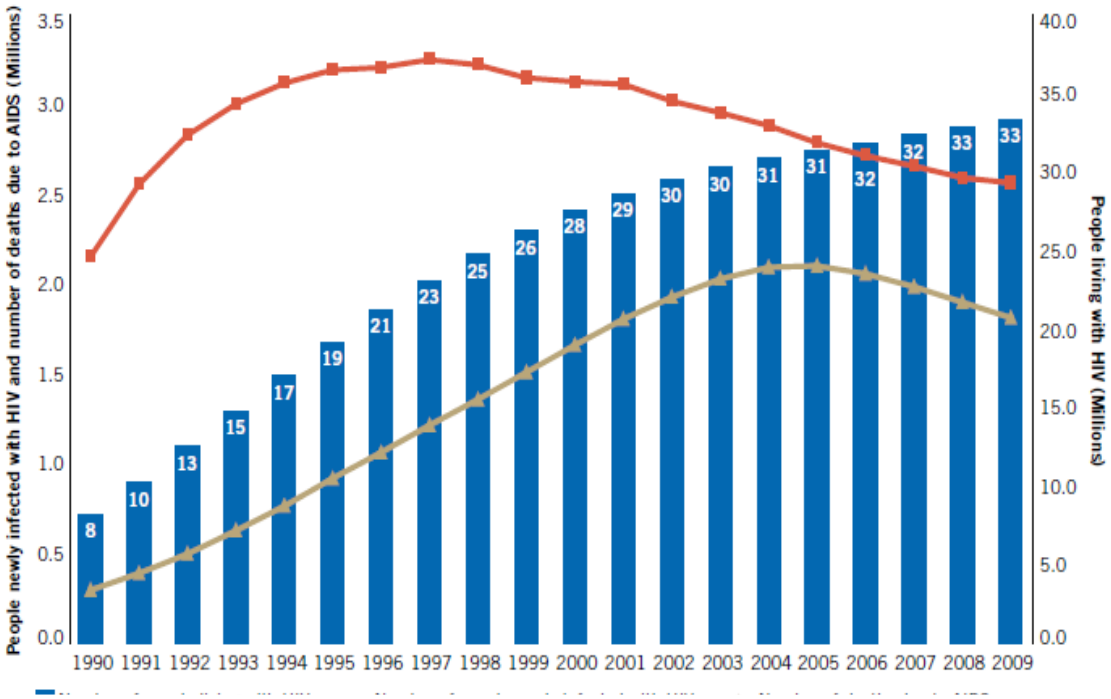



**Fig. 1-5. Numbers of people living with HIV, newly infected with HIV and AIDS deaths, 1990-2009.** (Source: UN (2011). The Millennium Development Goals Report 2011)

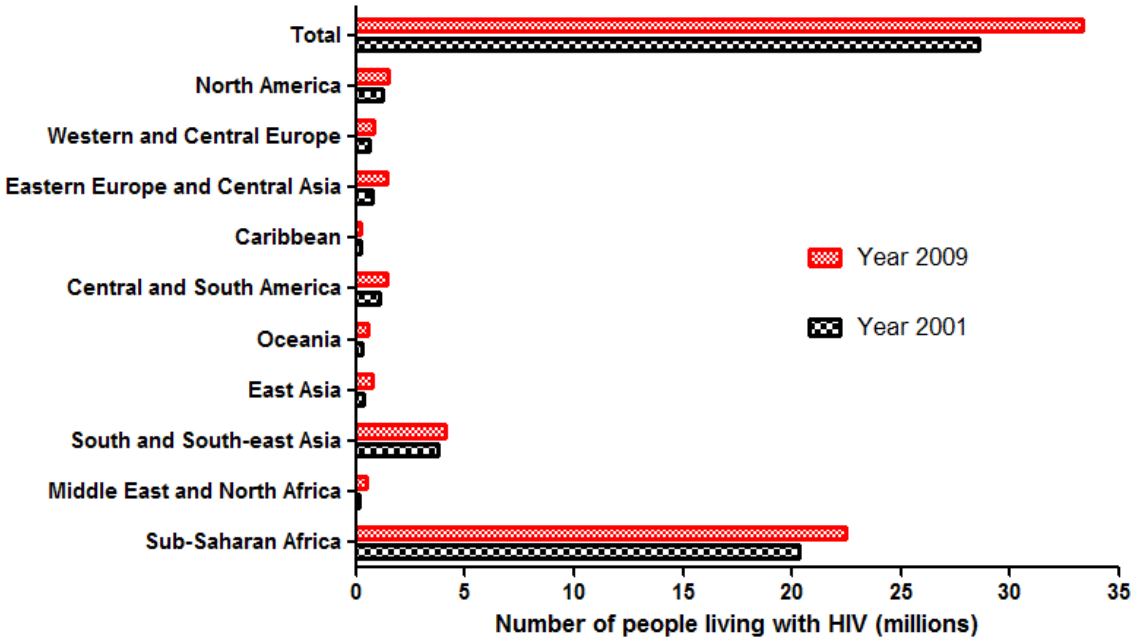



**Fig.1-6. Regional HIV and AIDS statistics, 2001 and 2009.** (Source: UNAIDS, 2010)

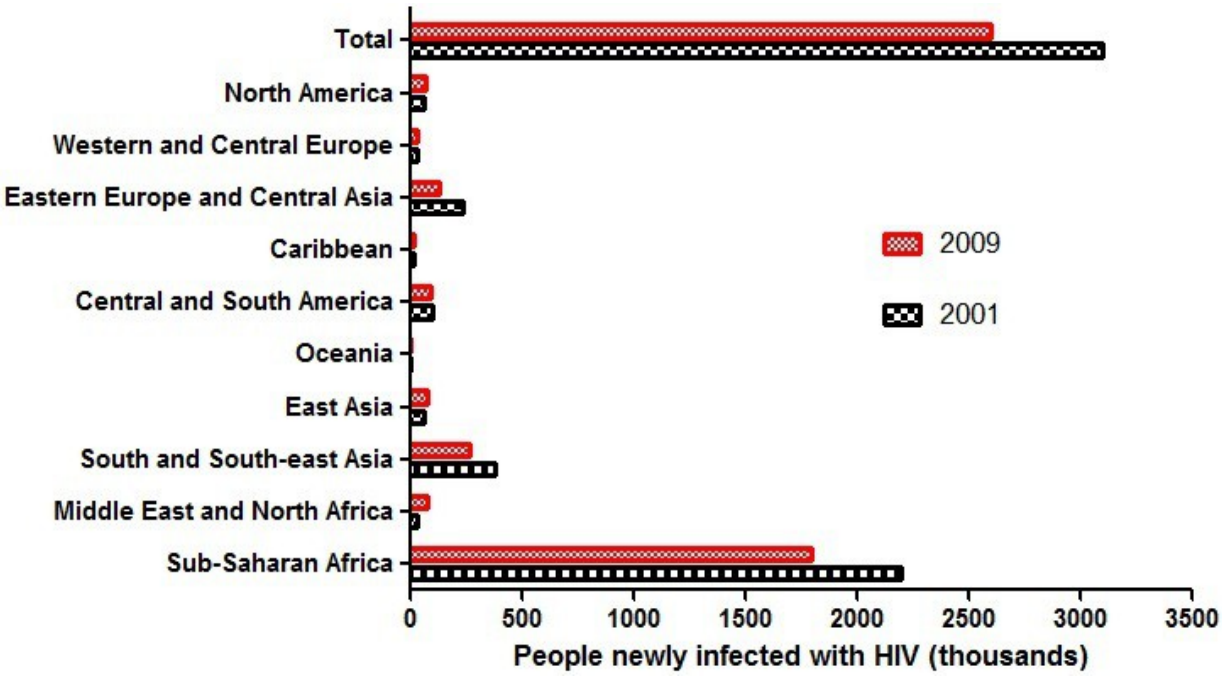



**Fig.1-7. Regional HIV newly infection statistics, 2001 and 2009.** (Source: UNAIDS, 2010)

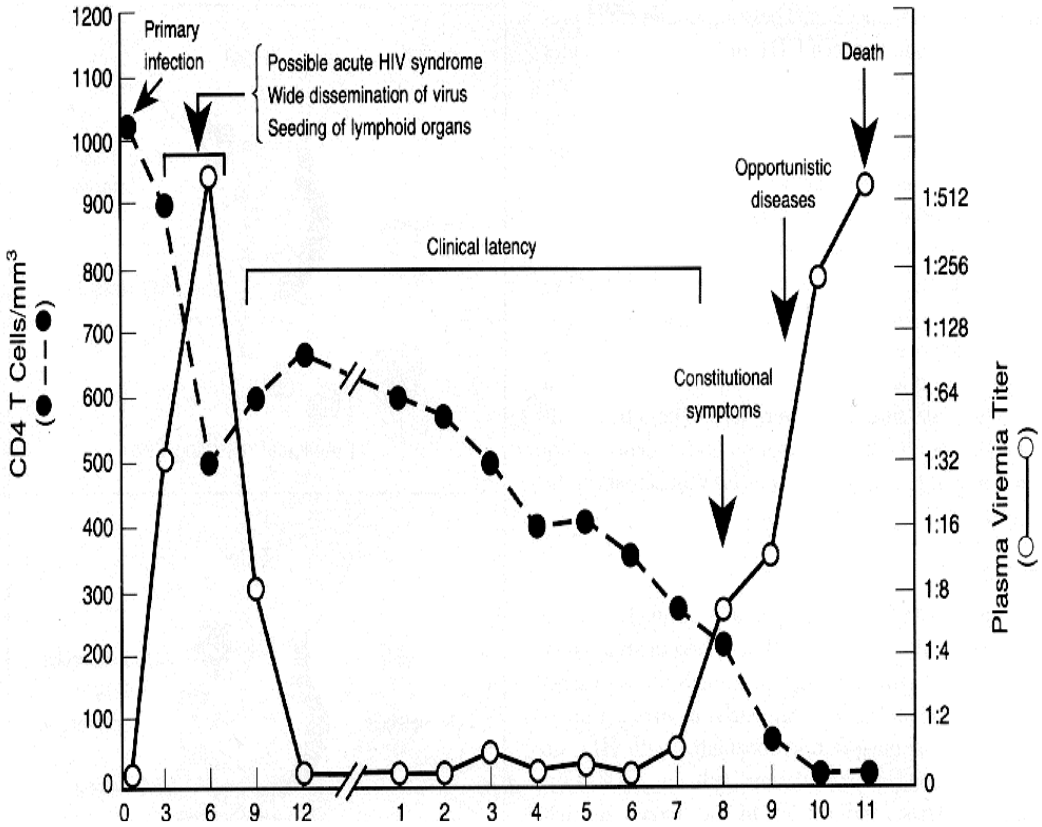



**Fig. 1-8. Typical course of HIV infection.** During the early period after primary infection there is widespread dissemination of virus and a sharp decrease in the number of CD4 T cells in peripheral blood. An immune response to HIV ensues, with a decrease in detectable viremia followed by a prolonged period of clinical latency. The CD4 T-cell count continues to decrease during the following years, until it reaches a critical level below which there is a substantial risk of opportunistic diseases (Pantaleo, 1993).

# Chapter 2

# Seroprevalence of HBV, HCV infection among HIV positive individuals in Shenzhen

## 2.1 Introduction

Since the first report of AIDS case in year 1981 and uncover of casual agent HIV in 1983 (Gottlieb, Schroff et al. 1981; Barre-Sinoussi, Chermann et al. 1983; Gallo, Sarin et al. 1983), HIV infection has become an pandemic and spread worldwide. Although antiretroviral therapy has been effective in controlling HIV replication, as lack of resources, an estimated 1.8 millions deaths were attributed to HIV/AIDS in year 2009, accumulate the total number of HIV/AIDS-related deaths to 28.8 millions worldwide (UNAIDS 2010).

In China, although significant efforts have been made to understand the situation of HIV/AIDS and comprehensive responses have been undertaken (State Council AIDS Working Committee Office and UN Theme Group on AIDS in China 2007; Wu, Sullivan et al. 2007), the HIV/AIDS infection is still a challenge. In 2011, the estimated number of residents living with HIV/AIDS was 780 000. 64% of them were transmitted through sexual activities, 46.8% of them were infected through heterosexual activities, 26.6% were via blood and blood products, 18.8% were IDUs. Among 48 000 new HIV infections, 52.2% were infected via heterosexual intercourse, 29.4% were transmitted through homosexual activities (Ministry of Health of China, UNAIDS et al. 2011). Since 2003, ambitious treatment, prevention, control programs and resources to combat HIV/AIDS have been implemented and resulted in increasing government capacity and infrastructure, many success have been achieved since then (Wu and Wang 2010). However, previously identified problems continue to evolve, such as infected blood donors in rural areas in mide-1990s (Zhang, Detels et al. 2008). New challenges were emerging. The HIV/AIDS epidemic has transferred from previously blood-driven to nowadays sex-driven epidemic (Lu, Jia et al. 2008). The expanding epidemic including HIV/AIDS among MSM, increasing drug resistance to antiretroviral therapy among new infections, the

evolving risk factors of high-risk populations and merge of HIV infection and tuberculosis (TB) (Li, Ha et al. 2010; Liao, Xing et al. 2010; Wang, Liu et al. 2010). HIV/AIDS epidemic among MSM has been the gravest challenge as a large portion of MSM have unprotected sex with women, made them a bridging group to heterosexual women who were presumed to have low risk to HIV infection (Xiao, Sun et al. 2010; Zou, Wu et al. 2010). Most new HIV infections nowadays are IDUs, MSM and sex workers due to the changes of social values and sexual behaviors, and also the population shift driven by migration (Yang, Li et al. 2005).

HIV, HBV and HCV are blood-borne pathogens. As they share routes of transmission, HIV/HBV co-infection, HIV/HCV co-infection and HIV/HBV/HCV triple-infection are common. Worldwide, chronic HBV infection affects 7% - 10% HIV-infected individuals (Lacombe, Bottero et al. 2010). The prevalence of HIV/HBV co-infection varies among different HBV endemic regions. In low HBV endemicity region, the HIV/HBV co-infection prevalence is around 5% - 7% (Alter 2006), while in regions with intermediate and high HBV endemicity, studies show a prevalence of about 10% - 20% (Diop-Ndiaye, Toure-Kane et al. 2008; Lee, Ko et al. 2008; Nyirenda, Beadsworth et al. 2008). Globally, seroprevalence of HCV in HIV-infected persons varies between 15% and 30% depending on population characteristics (Sherman, Rouster et al. 2002). HIV/HCV co-infection is common in high risk populations, like injection drug users (IDUs). In IDUs population, the prevalence of HIV/HCV co-infection can be as high as 85.1% (Sulkowski and Thomas 2003). In China, a systematic review summarized the overall prevalence of HIV/HCV co-infection in IDUs was 6.45%, ranging from 2.48% to 15.23% among different provinces (Bao and Liu 2009).

Although co-infection with HBV has no significant impact on progression to AIDS (Nikolopoulos, Paraskevis et al. 2009), individuals co-infected with HIV and HBV, especially those with $CD4^+$ nadir counts are at increased risk of liver-related mortality; the risk of liver-related mortality was doubled after highly active

antiretroviral therapy was introduced (Thio, Seaberg et al. 2002). Although HCV sero-status has not affected the overall virological and immunologic responses to HAART and the disease progression of HIV infection, the liver-related mortality was markedly elevated (Rockstroh, Mocroft et al. 2005).

Shenzhen, a prosperous city in southern Guangdong province, is located at north of Hong Kong. As an internal migrant city, Shenzhen holds totally 12 millions immigrants, which accounts for 87.0% of the Shenzhen population (Cai, Zhao et al. 2010). Because of its special migrant population and bound to Hong Kong, the HIV epidemic of Shenzhen was mainly contributed by high risk populations, IDUs, commercial sex workers and their clients, and MSM. Beginning from IDUs, HIV extended to commercial sex workers who are also IDUs, their clients and finally their regular partners (Yang, Li et al. 2005). In Shenzhen, 35.4% of the reported HIV cases were IDUs during 1988-1998 (Feng, Shao et al. 2000). The prevalence of HIV infection in MSM of Shenzhen was elevating from 0.9% in 2002, to 2.7% in 2005, then 3.7% in 2008 (Zeng, Qin et al. 2006; Hong, Zhou et al. 2009). The prevalence of HIV infection in male sex workers was reported as 5.3% in Shenzhen (Cai, Zhao et al. 2010). Seldom data has been available in Shenzhen about the seroprevalence of HBV, HCV infection among HIV positive individuals.

To understand the seroprevalence of HBV, HCV co-infections among HIV positive individuals in Shenzhen, we performed this cohort study.

## 2.2 Materials and Methods

### 2.2.1 Study participants

The study participants were recruited from an ongoing HIV/AIDS cohort study in Shenzhen, from September 2007 to July 2009.

The participants were recruited from follow-ups of HIV infected individuals. Informed consent was obtained from each participant. Age, gender and self-reported

risk behavior of HIV transmission were collected. For HIV transmission risk factors, we classified into five categories: blood (including former blood/plasma donors, transfusion recipients and hemophiliacs, needle-stick and other injuries), injection drug users (IDUs) with syringe and needle-sharing behaviors, heterosexual activities, homosexual activities, mother-to-child transmission. Homosexual activities mainly refer to male who had unprotected anal or oral intercourse with male. If participant have two or more risk behaviors, he/she will be allocated to only one category according to the relative risky of his/her behaviors. HBV, HCV co-infected HIV positive participants were referred to local hospitals for treatments. All infections were recorded following the requirements of reportable infectious diseases.

The study protocol was approved by Shenzhen Center for Disease Control and Prevention (Shenzhen CDC) and The Chinese University of Hong Kong.

### 2.2.2 Measure of HBV and HCV seroprevalence

Whole blood specimens were collected from all subjects with anticoagulant and centrifuged at 1000 rpm for 10 minutes, plasma were then gathered and stored at -80°C until use. Plasma was then screened for HBV infection and HCV infection.

(1). HBV infection was demonstrated by HBsAg positivity, and determined by ELISA kit (Beijing Wantai Biological Pharmacy Enterprise CO., LTD, Beijing) according to manufacturer's protocol.

HBsAg ELISA protocol:

1. Reagents preparation: Allow the reagents and samples to reach room temperature (18-30°C) for at least 15-30 minutes. Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37°C until crystals dissolve. Dilute the stock Wash Buffer 1 to 20 with distilled or deionized water.

2. Numbering Wells: Set the strips needed in strip-holder. Then number sufficient number of wells including three for the Negative control, two for the Positive control and one Blank.

3. Adding Sample and HRP-Conjugate: Add 50 µl of Positive control, Negative control, and specimen into their respective wells. Note: Use a separate disposal pipette tip for each specimen, Negative and Positive Control as to avoid cross-contamination. Add 50 µl HRP-Conjugate to each well except the Blank, and mix by tapping the plate gently.

4. Incubating: Cover the plate with the plate cover and incubate for 60 minutes at 37°C.

5. Washing: At the end of the incubation, remove and discard the plate cover. Wash each well 5 times with diluted Wash buffer. Each time, allow the microwells to soak for 30-60 seconds. After the final washing cycle, turn the plate down onto blotting paper or clean towel, and tap it to remove any remainders.

6. Coloring: Dispense 50 µl of Chromogen A and 50 µl Chromogen B solution into each well including the Blank, and mix by tapping the plate gently. Incubate the plate at 37°C for 15 minutes by avoiding light. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in Positive control and HBsAg positive sample wells.

7. Stopping Reaction: Using a multichannel pipette or manually, add 50 µl Stop Solution into each well and mix gently. Intensive yellow color develops in Positive control and HBsAg positive sample wells.

8. Measuring the Absorbance: Calibrate the plate reader with the Blank well and read the absorbance at 450 nm, then Calculate the Cut-off value with the following formula and evaluate the results (Note: read the absorbance within 5minutes after

stopping the reaction).

Calculation of Cut-off value:

Cut-off value (C.O.) = *Nc × 2.1

*Nc = the mean absorbance value for three negative controls.

Important: If the mean OD value of the negative control is lower than 0.05, take it as 0.05.

Interpretation of results:

(S = the individual optical density (OD) of each specimen)

Negative Results (S/C.O. <1): samples giving an optical density less than the Cut-off value are considered negative, which indicates that no hepatitis B virus surface antigen has been detected with Wantai HBsAg ELISA, therefore the participant is probably not infected with hepatitis B virus.

Positive Results (S/C.O.≥1): samples giving an optical density greater than or equal to the Cut-off value are considered positive, which indicates that HBV surfaces antigen has probably been detected with Wantai HBsAg ELISA.

Borderline: Samples with absorbance to Cut-off ratio between 0.9 and 1.00 are considered borderline samples and retesting is recommended. Repeatedly positive samples can be considered positive for HBsAg.

Clinical Specificity: This HBsAg ELISA kit reported 99.87% specificity in healthy blood donors and 99.63% specificity in undiagnosed hospital patients.

Clinical Sensitivity: This HBsAg ELISA kit claimed 100% sensitivity in acute hepatitis B patients, 99.75% sensitivity in chronic hepatitis B patients, and 100% sensitivity in recovered hepatitis B infected patients.

(2). HCV infection was showed by anti-HCV antibodies. HCV antibodies were tested by ELISA kit (Beijing Wantai Biological Pharmacy Enterprise CO., LTD, Beijing) as indicated by manufacturer's protocol.

HCV antibody ELISA protocol:

1. Reagents preparation: Allow the reagents and samples to reach room temperature (18-30°C) for at least 15-30 minutes. Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37°C until crystals dissolve. Dilute the stock Wash Buffer 1 to 20 with distilled or deionized water.

2. Numbering Wells: Set the strips needed in strip-holder and number sufficient number of wells including three for the Negative control, two for the Positive control and one Blank.

3. Adding Diluent: Add 100 µl Specimen Diluent into each well except the blank.

4. Adding Sample: Add 10 µl of Positive control, Negative control, and Specimen into their respective wells. Note: Use a separate disposal pipette tip for each specimen, Negative and Positive Control as to avoid cross-contamination. Mix by tapping the plate gently.

5. Incubating (1): Cover the plate with the plate cover and incubate for 30 minutes at 37°C.

6. Washing (1): At the end of the incubation, remove and discard the plate cover. Wash each well 5 times with diluted Wash buffer. Each time, allow the microwells to soak for 30-60 seconds. After the final wash cycle, turn the strips plate onto blotting

paper or clean towel, and tap it to remove any remainders.

7. Adding HRP-Conjugate: Add 100 µl HRP-Conjugate to each well except the Blank.

8. HRP-Conjugate Incubating (2): Cover the plate with the plate cover and incubate for 30 minutes at 37°C.

9. Washing (2): At the end of incubation, remove and discard the plate cover. Wash each well 5 times with diluted Wash buffer as in Step 6.

10. Coloring: Dispense 50 µl of Chromogen A and 50 µl Chromogen B solution into each well including the Blank and mix by tapping the plate gently. Incubate the plate at 37°C for 15 minutes by avoiding light. The enzymatic reaction between the Chromogen A/B solutions produces blue color in Positive control and anti-HCV positive sample wells.

11. Stopping Reaction: Using a multichannel pipette or manually, add 50 µl Stop Solution into each well and mix by tapping the plate gently. Intensive yellow color develops in Positive control and anti-HCV positive sample wells.

12. Measuring the Absorbance: Calibrate the plate reader with the Blank well and read the absorbance at 450 nm. Calculate the Cut-off value as illustrated below and evaluate the results (Note: read the absorbance within 5minutes after stopping the reaction).

Calculation of Cut-off value (C.O.):

C.O. = *Nc + 0.12

*Nc = the mean absorbance value for three negative controls.

Important: If the mean OD value of the negative control is lower than 0.02, take it as 0.02.

Interpretation of the results:
(S = the individual absorbance (OD) of each specimen)

Negative Results (S/C.O. <1): samples giving an optical density less than the Cut-off value are negative for this assay, which indicates that no antibodies to hepatitis C virus have been detected with Wantai anti-HCV ELISA kit. Therefore, the participant is probably not infected with HCV.

Positive Results (S/C.O.≥1): samples giving an optical density greater than, or equal to the Cut-off value are considered initially reactive, which indicates that antibodies to hepatitis C virus have probably been detected using Wantai anti-HCV ELISA kit.

Borderline: Samples with optical density O.D.≤ Cut-off × 2 are considered borderline and retesting of those samples in duplicates is recommended. Repeatedly positive samples could be considered positive for hepatitis C virus infection.

Clinical Specificity: This anti-HCV ELISA kit was reported a specificity of 99.55%.

Clinical Sensitivity: This anti-HCV ELISA kit claimed a 99.79% sensitivity in RIBA 3.0 confirmed hepatitis C patients.

### 2.2.3 Statistical analysis

Descriptive analyses were carried out to examine participants' demographic characteristics and infection status. Histogram was applied to test the distribution of data. Chi-square test and *T* test were performed to compare proportions and age mean of HBV, HCV infection among HIV positive population. Logistic regression (univariate) was applied to identify factors potentially associated with HIV, HBV,

HCV co-infections; variables with *P* value < 0.1 in univariate logistic regression were further used as candidate variables in multivariate stepwise logistic regression to assess risk factors while eliminating confounding factors. *P* value < 0.05 was regarded as statistically significant. All tests were analyzed by SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

## 2.3 Results

### 2.3.1 HIV infection in Shenzhen

From September 2007 to July 2009, totally 914 HIV positive individuals were successfully recruited in the cohort study. Among them, 677 (74.1%) were male and 237 (25.9%) were female (Table 2-1).

The mean age of the HIV positive population was 31 and ranges from 11 to 73. In terms of HIV distribution with age, we further equally divided them into three age subgroups: subgroup with age below 27 years, subgroup with age among 27 to 32 and subgroup with age above 32 years. Among them, 278 (30.4%) were aged below 27; 322 (35.2%) persons were aged between 27 to 32; and 314 (34.4%) were aged above 32 years old.

For HIV infection, we recorded four self-reported HIV risk behaviors, injection drug users (IDUs), heterosexual transmission, homosexual transmission, blood and blood products transmission. Among 914 HIV positive individuals, 152 (16.6%) were transmitted through IDUs, 470 (51.4%) were infected by heterosexual way, 269 (29.5%) were homosexual participants, and 23 (2.5%) were reported as infected by blood and blood products.

### 2.3.2 Seroprevalence of HBV, HCV infection among HIV positive individuals in Shenzhen

Among 914 HIV positive people, after serological tests for HBV and HCV infections, totally 267 individuals were determined as serologically co-infected with HBV, HCV or HBV and HCV (Table 2-1). Among 267 co-infected individuals, 100

(10.9%) were HIV/HBV co-infected; 133 (14.6%) were HIV/HCV co-infected; and 34 (3.7%) were HIV/HBV/HCV co-infected.

In 100 HIV/HBV co-infected people, 78 of them were male and 22 were female; in 133 HIV/HCV co-infection group, there were 107 male and 26 female; while there were 31 male and 3 female in HIV/HBV/HCV triple-infection group. Chi-square test was applied to test the the difference of gender distribution between HIV positive population and HIV, HBV, HCV co-infection populations, respectively. Results showed that the gender ratio in HIV/HBV and HIV/HCV co-infection groups were no significant different from HIV infected population (HIV/HBV: $P$ = 0.392; HIV/HCV: $P$ = 0.113); while in HIV/HBV/HCV co-infection group, there was a significant association between gender ratio and triple-infection ($P$ = 0.024).

The means age for HIV/HBV, HIV/HCV and HIV/HBV/HCV co-infection groups were 30, 31 and 32 years old, respectively. $T$ test was applied to test the the difference of mean age between HIV positive population and HIV, HBV, HCV co-infection populations, respectively. Results showed that the means of age were not related to status of co-infection (HIV/HBV: $P$ = 0.131; HIV/HCV: $P$ = 0.462; HIV/HBV/HCV: $P$ = 0.592).

Based on the same age classification standard as HIV positive population, in HIV/HBV co-infection group, 32 subjects were aged below 27 years, 40 subjects aged between 27 to 32, and 28 subjects aged above 32; In HIV/HCV and HIV/HBV/HCV co-infection groups, the numbers were 30, 57, 46 and 4, 16, 14, respectively. After grouping co-infection persons into different groups according to age, Chi-square test indicate that age has no correlation with HIV/HBV, HIV/HCV and HIV/HBV/HCV co-infection status (HIV/HBV: $P$ = 0.422; HIV/HCV: $P$ = 0.116; HIV/HBV/HCV: $P$ = 0.062).

For risk behavior consideration, the numbers of heterosexual, homosexual, IDUs and

blood in HIV/HBV co-infection group were 57, 38, 5 and 0, respectively; for HIV/HCV co-infection group, they were 30, 4, 89 and 10; for HIV/HBV/HCV co-infection group, the numbers were 5, 1, 28 and 0. Chi-square test was applied to test distribiution of risk behavior between HIV positive group and HIV, HBV, HCV co-infection groups. Results showed that for three co-infection groups, risk behavior was significantly correlated with conditions of co-infection (HIV/HBV: $P = 0.002$; HIV/HCV: $P < 0.001$; HIV/HBV/HCV: $P < 0.001$).

As gender and risk behavior were separately linked with HIV, HBV, HCV co-infection status, logistic regression (univariate) was applied to identify factors potentially associated with HIV, HBV, HCV co-infections; based on univariate logistic regression result, multivariate logistic regression was further applied to assess risk factors while controlling for confounding factors.

For HIV/HBV co-infection group, as stated in table 2-2 (Table 2-2), gender and age has no significant impact on individuals' susceptibility to HIV/HBV co-infection (Gender: Female Vs Male: OR = 0.79, 95% CI: 0.49 – 1.29, $P = 0.343$; Age: aged below 27 Vs aged among 27 – 32: OR: 0.92, 95% CI: 0.56 – 1.51, $P = 0.732$; aged more than 32 Vs aged among 27 – 32: OR: 0.69, 95% CI: 0.41 – 1.15, $P = 0.154$). Among four risk behaviors, homosexual transmission as reference, individuals with heterosexual behavior has no significant difference in terms of getting HIV/HBV co-infection (Heterosexual: OR = 0.84, 95% CI: 0.54 – 1.30, $P = 0.435$); while IDUs has a significant lower risk of getting HIV/HBV co-infection (IDUs: OR = 0.21, 95% CI: 0.08 – 0.54, $P = 0.001$); No subject in HIV/HBV co-infection group was transmitted HIV through blood and blood products.

For HIV/HCV co-infection group, as stated in Table 2-3, gender has no obvious function on HIV/HCV co-infection (Female Vs Male: OR = 0.66, 95% CI: 0.42 – 1.04, $P = 0.071$). For age subgroups, persons aged younger than 27 has significantly

lower risk of HIV/HCV co-infection compared to individuals aged between 27 and 32 years (OR = 0.56, 95% CI: 0.35 – 0.90, $P$ = 0.018) in univariate logistic regression; after adjusting for risk behavior in multivariate logistic regression, the association was not significant anymore (OR = 0.77, 95% CI: 0.43 – 1.39, $P$ = 0.386). People aged older than 32 has no significant difference of HIV/HCV co-infection comparing to people aged between 27 and 32 (OR = 0.90, 95% CI: 0.53 – 1.53, $P$ = 0.688). Among four risk behavior, IDUs as reference, people with heterosexual behavior and homosexual behavior have significantly lowered risky of HIV/HCV co-infection (Heterosexual: OR = 0.05, 95% CI: 0.03 – 0.09, $P < 0.001$; Homosexual: OR = 0.01, 95% CI: 0.004 – 0.03, $P < 0.001$). While for people got HIV via blood and blood products, the risky of HIV/HCV co-infection is not differ from IDUs (OR = 0.56, 95% CI: 0.22 – 1.39, $P = 0.208$).

In HIV/HBV/HCV co-infection group, as stated in Table 2-4, female has lower possibility of being infected with HIV/HBV/HCV when compared to male (OR = 0.27, 95% CI: 0.08 – 0.88, $P$ = 0.030) in univariate logistic regression. After controlling for confounding factors in multivariate logistic regression, the difference for co-infection with HIV/HBV/HCV between two genders was not significant in the study (OR = 0.46, 95% CI: 0.13 – 1.63, $P = 0.227$). For age, people aged more than 32 has no obvious difference to people aged between 27 to 32 in terms of HIV/HBV/HCV co-infection (OR = 1.17, 95% CI: 0.52 – 2.61, $P = 0.704$); while for people aged younger than 27, they have a significant lower risk of HIV/HBV/HCV co-infection (OR = 0.28, 95% CI: 0.09 – 0.85, $P$ = 0.024), only the significance disappeared after multivariate logistic regression (OR = 0.37, 95% CI: 0.12 – 1.18, $P$ = 0.092). For risk behavior, IDUs as reference, people transmitted HIV via heterosexual behavior and homosexual behavior have significantly lower risky of HIV/HBV/HCV co-infection even after controlling for confounding factors (Heterosexual: OR = 0.06, 95% CI: 0.02 – 0.16, $P < 0.001$; Homosexual: OR = 0.02, 95% CI: 0.002 – 0.12, $P < 0.001$). Most (28/34) HIV/HBV/HCV triple-infections

were IDUs; no person co-infected with HIV/HBV/HCV was mediated HIV through blood and blood products.

As HIV risk behavior was significantly associated with HIV/HBV, HIV/HCV and HIV/HBV/HCV co-infections, we analyzed the possible association of risk behavior with age and gender in this HIV, HBV, and HCV co-infected population. As stated in Table 2-5, gender has a significant association with HIV risk behavior ($P < 0.001$); while age group has no significant relationship with HIV risk behavior ($P = 0.164$).

To test the relationship between HIV risk behavior and gender, odds ratio was applied to test the strength and significance. As stated in Table 2-6, compared to IDUs, male heterosexual behaviors was significantly less susceptible to HIV, HBV, HCV co-infections (OR = 0.23, 95% CI: 0.12 – 0.47, $P < 0.001$); male transmitted HIV through blood and blood products were less likely to be HIV, HBV, HCV co-infected (OR = 0.19, 95% CI: 0.05 – 0.78, $P = 0.031$).

## 2.4 Discussion

### 2.4.1 HIV infection in Shenzhen

In Shenzhen, the first HIV infection among IDUs was reported in 1995 (Feng, Li et al. 2000). From 1988 to 1998, 35.4% of the reported HIV infections were IDUs (Feng, Li et al. 2000). Since 2000, IDUs has been the main route of HIV transmission in Shenzhen with an average 55% percentage of yearly HIV/AIDS infection number (Zhang, Zhao et al. 2009). In another report about the HIV/AIDS epidemic in Shenzhen from 2005 to 2007, among all reported HIV/AIDS in Shenzhen center for disease control and prevention, 48.9% were IDUs, range from 53.5% in 2005 to 40.8% in 2007 (Chen, Wang et al. 2009). In 2008, the proportion of IDUs among HIV/AIDS population has declined to 27.6%, indicated IDUs has not been the main route of HIV transmission in Shenzhen (Zhang, Zhao et al. 2009). In this study, among 914 HIV positive individuals, 16.6% (152/914) were reported as transmitted through injection drug use, significantly smaller than previous reported

data, which further confirmed that the proportion of IDUs among HIV/AIDS population has been dramatically decreased, IDUs has not been the predominant transmission route in Shenzhen. This change may be explained by implementation of government-run detoxification and rehabilitation measures and improved cautiousness of HIV spreading among needle sharing in IDUs.

During late 1980s and early 1990s, in rural areas of Henan, Anhui, Hubei, Shanxi and Shandong provinces, illegal commercial plasma and blood collection was popular (Wu, Rou et al. 2001). This activity has caused rapid spread of blood-borne diseases, including HIV. In 2011, among 780 000 people living with HIV/AIDS, 6.6% of them were transmitted via blood and blood products (Ministry of Health of China, UNAIDS et al. 2011). In our study, out of 914 HIV positive individuals, only 23 (2.5%) confirmed HIV infection was resulted from blood and blood products, including commercial blood/plasma donation and blood transfusion. Seldom HIV/AIDS case was resulted from blood and blood products in this study, because these activities were most happened in rural area of inner provinces, while not common in coastal industrialized Shenzhen.

HIV transmission via sexual contact has grown recently, either through heterosexual contact with female sex workers or male to male sexual transmission. Commercial sex appeared since the start of the Reform and Opening-up Policy and free market economy in 1978. With rapid industrial modernization and economic development in China, large population movement from rural China to cities has caused concerns for potential risk of HIV infection through sexual transmission. As being poorly educated and afraid of arrest, condom use was seldom and commercial sex workers were vulnerable to HIV infection. HIV infected commercial sex workers will endanger their clients if without proper protections. In Shenzhen, a report stated that 30.9% of diagnosed HIV infections during 2005 to 2007 were transmitted through heterosexual behaviors (Chen, Wang et al. 2009). In this study, 470 (51.4%) HIV

infections were resulted from heterosexual behaviors. This relative high proportion of HIV infection transmitted through heterosexual behaviors proved that heterosexual transmission still the key for controlling HIV epidemic. The high rate of HIV transmission via heterosexual activities in Shenzhen may be explained by cross-border sex-networking between Hong Kong and Shenzhen, relative open atmosphere in developed cities, changes of social values and sexual behaviors, and also the huge population immigrated into Shenzhen.

Homosexual behavior, specifically men who have sex with men (MSM), was risky for sexual transmitted diseases, including HIV. MSM was at high risk of HIV infection mainly because of multiple partners and unprotected anal intercourse (Liu, Yang et al. 2005). In our study, 269 (29.4%) were mediated through homosexual behaviors, which was consistent with the national statistic. In Shenzhen, from 2005 to 2007, HIV transmitted through homosexual/bisexual has been risen from 3.9% in 2005, to 15.7% in 2007; the prevalence of HIV infection among male sex workers in Shenzhen of year 2008 was 5.3% (Chen, Wang et al. 2009; Cai, Zhao et al. 2010). In Nanjing, it reported a 5.12% per year HIV sero-conversion among MSM population in 2007 (Yang, Hao et al. 2010). In Chengdu, it reported a 9.1% HIV prevalence in MSM population in 2007 (Feng, Wu et al. 2010). Beijing reported a 4.8% HIV prevalence in 2007 among MSM population (Ruan, Luo et al. 2009). Shenzhen, a special economic zone bordering to Hong Kong, together with its huge immigrant workers, expanded the MSM population (Liu, Liu et al. 2009). HIV prevalence in Shenzhen may be worsening as some MSM also sell commercial sex to female and have unprotected sex with their female partners.

In the study, 677 (74.1%) subjects were male and 237 (25.9%) subjects were female. Among female HIV positive individuals, most of them were commercial sex workers. For female in China, the HIV prevalence doubled in the past decades (State Council AIDS Working Committee Office and UN Theme Group on AIDS in China

2007). While for male in this study, most of them were homosexual behaviors, and most IDUs were male.

### 2.4.2 HIV, HBV, HCV co-infections in Shenzhen

Globally, 7% - 10% HIV positive people were affected by chronic HBV infection (Lacombe, Bottero et al. 2010). The prevalence of HIV/HBV co-infection varies among different HBV endemic regions. In low HBV endemicity region, the HIV/HBV co-infection prevalence was around 5% - 7% (Alter 2006), while in regions with intermediate and high HBV endemicity, studies showed a prevalence of about 10% - 20% (Diop-Ndiaye, Toure-Kane et al. 2008; Lee, Ko et al. 2008; Nyirenda, Beadsworth et al. 2008). In a study with children infected with HIV, it revealed a HIV/HBV co-infection rate of 4.9% (Zhou, Zhao et al. 2010). In China, most chronic HBV infections were occurred at perinatal and early childhood, prior to HIV infection. A national serosurvey stated a 7.2% prevalence of HBsAg and HBsAg risk in adults was associated with male (Liang, Bi et al. 2009). In a multicentre epidemiological survey in China, including Yunnan, Xinjiang, Sichuan, Guangdong, Beijing, Henan and Shanxi, it reported a 12.5% HBsAg seroprevalence (Yan, Gao et al. 2010). In our study, 100 out of 914 HIV positive persons were co-infected with chronic HBV infection (10.9%), which is significantly higher than the national prevalence of general population; while slightly lower than the multicentre epidemiological survey. Although risk behavior in HIV positive population also endangered them with chronic HBV infection, statistics revealed there was not significant impact of HIV risk behaviors on chronic HBV infection. This may mainly due to the successful implementation of HBV vaccine program in 1992, which decreased the prevalence since then.

In the study, among 100 HIV/HBV co-infected persons, 57 were infected HIV through heterosexual contact and 38 through homosexual contact; only 5 were IDUs and none was infected through blood and blood products. For HIV/HBV co-infection in this study, sexual routes, both heterosexual and homosexual contact, were the main

route of HIV transmission, significantly popular than IDUs (homosexual, $P = 0.001$). Studies from West Europe and American showed that chronic HBV infection was about 6% to 14% in HIV positive population, including 4-6% in heterosexual, 9-17% of MSM, 7-10% of IDUs (Alter 2006). The difference of HIV risk behavior among HIV/HBV co-infections between Western countries and our study is high-risk sexual activity (heterosexual and MSM) and IDUs accounts for a large portion of newly acquired hepatitis B in Western countries; while in China including Shenzhen, chronic HBV infection mainly occurred at perinatal and childhood.

In HIV-infected individuals, the prevalence of HCV infection varies from 15% to 30% among different characteristic populations (Sherman, Rouster et al. 2002). In China, a systematic review summarized the overall prevalence of HIV/HCV co-infection in IDUs was 6.45%, ranging from 2.48% to 15.23% among different provinces (Bao and Liu 2009). In a children HIV population in China, the prevalence of HIV/HCV co-infection was 9.6% (Zhou, Zhao et al. 2010). In the national multicenter epidemiological survey, it reported an overall 41.8% HCV prevalence in HIV population (Yan, Gao et al. 2010). In this study, among 914 HIV positive individuals, 133 (14.6%) were co-infected with HCV; this prevalence in this study is significantly lower than the multicentre epidemiological report, the difference may resulted from sampling as in the multicentre survey, they specifically choose several centers located in areas rich of IDUs and HIV/HCV co-infection is highly prevalent in IDUs among Yunnan, Xinjiang and Sichuan provinces.

HIV/HCV co-infection is common in high risk populations, like IDUs. In western countries, a report described a prevalence of 25% to 30% HCV infection in HIV positive persons overall; among them, 72 – 95% belong to IDUs, 1 – 12% of MSM and 9 – 27% of heterosexuals (Alter 2006). In Guangxi province, a report stated a 17.6% HIV/HCV co-infection rate in IDUs (Garten, Zhang et al. 2005). Another study reported a 21% HIV/HCV co-infection rate in IDUs in Guangxi (Tan, Wei et al.

2008). A report about IDUs in Yunnan stated a 99.3% of HCV co-infection among HIV positive IDUs and the HIV prevalence in female IDUs is significantly higher than male IDUs (Zhang, Yang et al. 2002). Among these 133 HIV/HCV co-infected persons in this study, most of them were IDUs (89/133). In our study, among total 152 IDUs, 89 were HIV/HCV co-infected and 28 were HIV/HBV/HCV co-infected, indicating a rather high HCV co-infection rate in IDUs (77.0%) in Shenzhen. The high prevalence of HIV/HCV co-infection among IDUs may mainly caused by rapid economic development and dramatic social value changes during industrial modernization, also as of needle and syringe-sharing during injection drug use.

HIV/HCV co-infection was also common among former blood/plasma donors. In a former plasma/blood donor population, among HIV positive persons, the HIV/HCV co-infection rate was 85% (Qian, Vermund et al. 2006). In our study, among total 23 HIV positive person infected by blood and blood products, 10 were HIV/HCV co-infected (43.5%). The high prevalence of HIV/HCV co-infection was attributed to recipients of contaminated blood or blood products, and commercial selling blood or plasma as re-infuse of red blood cells, reuse of needles, and unsterilized equipment made blood/plasma donors susceptible to blood-borne pathogens, both HIV and HCV (Qian, Vermund et al. 2005).

In a Iran HIV positive population, it reported a HIV/HBV/HCV co-infection prevalence of 7.9% (31 in 391) (Mohammadi, Talei et al. 2009). Housewives were more susceptible to triple-infection and have sex with IDUs was most risky for triple-infection. The prevalence of HIV/HBV/HCV was 1% in a Nigeria HIV-infected patients (Otegbayo, Taiwo et al. 2008). In the national multicenter epidemiological survey, it reported an overall 5.23% HBV/HCV co-infection prevalence in HIV population (Yan, Gao et al. 2010). In our study, among 914 HIV positive persons, 34 were HIV/HBV/HCV co-infected (3.7%). Male were easier to be infected by HIV/HBV/HCV (male/female: 31/3); IDUs was the main route of HIV transmission

(28/34). Although gender and age were risk factors for HIV/HBV/HCV triple-infection in univariate analysis, they were confirmed as confounders in multivariate alaysis.

When HIV transmission risk behavior was analyzed with age and gender, results showed that most IDUs were male (108/122), near half aged between 27 and 32 (56/122). IDUs have been a long-lasting HIV population. HCV was spread into China from Southeast Asia as mediated by IDUs. In Shenzhen, as most residents were migrated from other provinces and economic boosting, together with social value changes has made male aged between 27 and 32 a huge market for illicit drug consumption. The prevalence of HIV, HBV, HCV co-infections in Shenzhen was rather high and the situation was rather challenge, more resources and work were needed to solve the problem.

### 2.4.3 Limitations of the study

HIV, HBV and HCV can be transmitted through blood, behaviors related to blood and blood products can be potential risk factors for infection of the three pathogens, in succession or even simultaneously. There have been some reports about HBV or HCV prevalence among HIV positive individuals in specific high-risk populations, only a few reported the seroprevalence of HBV and HCV co-infections among HIV population around the world (Sherman, Rouster et al. 2002; Sulkowski and Thomas 2003; Alter 2006; Otegbayo, Taiwo et al. 2008; Bao and Liu 2009; Mohammadi, Talei et al. 2009; Lacombe, Bottero et al. 2010). In Shenzhen, there also reports about HIV prevalence in MSM, IDUs population (Feng, Li et al. 2000; Zeng, Qin et al. 2006; Chen, Wang et al. 2009; Cai, Zhao et al. 2010). There are no reports about the HBV, HCV seroprevalence among HIV positive individuals. To get a picture about the seroprevalence of HBV, HCV infections among HIV population in Shenzhen, we conducted the study. Due to restricted resources and other reasons; there are some limitations existed in the study.

1. In the study, we studied the seroprevalence of HBsAg positivity among HIV population in Shenzhen while not include hepatitis B surface antibody (anti-HBs) and hepatitis B core antibody (anti-HBc). The result of HBsAg positivity only indicates the situation of chronic HBV infection among HIV population, while neglecting information about previous acute HBV infection and HBV immunization. Even for chronic HBV infection, a lot of patients belong to inactive HBsAg carrier state which characterized by HBeAg negativity, HBeAb positivity, low or undetectable HBV DNA, normal ALT levels, minor changes of liver biopsy (Sharma, Saini et al. 2005). As the progression of inactive HBsAg carrier state is generally benign, the result based on HBsAg ELISA may be misleading. And for patients with chronic HBV infection, HBeAg, HBeAb and HBV DNA should be tested for viral replication for treatment decision-making (Gordon 2001).

2. In China, the majority of HBV infections occured during perinatal and early childhood as of direct contact with HBV contaminated blood, breast milk and saliva, etc. As most HBV infections in Chinese are prior to HIV infection, it's hard to differentiate patients got HBV via injection drug use and sexual activities from patients infected during perinatal and early childhood in the lack of HBV infection family history. Occult HBV infection is defined as infection of HBV with the detection of HBV DNA while lack of HBsAg. The reported occult HBV infection prevalence in HIV positive women is 2%, more than half of them were IDUs (Tsui, French et al. 2007). Another report stated an 89.5% prevalence of occult HBV infection among 57 HIV-infected individuals composed mainly by IDUs and Homosexual behaviors (Hofer, Joller-Jemelka et al. 1998). A study about sexual transmitted HIV population reported a 12.2% occult HBV prevalence (Rai, Mathur et al. 2007). Occult HBV infection may transmit HBV through blood transfusion and organ transplantation; it may also have acute reaction in condition of

immunosuppressive status and favors progression of liver fibrosis and development to HCC (Giovanni, Teresa et al. 2007). As no HBsAg can be detected in occult HBV infections, the study described here may underestimate the prevalence of HBV in HIV population in some extent. Also, as of lack HBV DNA data and liver disease markers alaine aminotransferase (ALT) and aspirate aminotransferase (AST) in the study, the result reported may not useful for prognosis and HBV infection treatment.

3. The anti-HCV assay is reliable only for patients exposure to HCV for more than 3 months as it can detects greater than 90% (Chen and Morgan 2006). As nearly 30% of patients will have negative result for anti-HCV at onset of their symptoms, making anti-HCV testing unreliable for the diagnosis of acute HCV infection. HCV RNA can be detected in serum 1 to 2 weeks after exposure, while anti-HCV antibodies only appears approximately 1 to 3 months after exposure, thus HCV RNA is useful for detection of early acute HCV infection (Gretch 1997). In HIV/HCV co-infections, even in the presence of HCV viraemia, the anti-HCV may not be detectable as patients may fail to mount or sustain the antibody response (Cribier, Rey et al. 1995). Anti-HCV antibody can also be negative in 10% to 15% HCV/HIV co-infected due to their immunocompromised state, thus HCV RNA should be tested in cases of unexplained transaminase elevation in patients with CD4 counts less than 200/$mm^3$ and among high HCV risk population, like IDUs (Matthews and Dore 2008).

4. Occult HCV infection is defined as HCV RNA exists in liver and peripheral blood mononuclear cells (PBMCs), and negative for anti-HCV and HCV RNA in serum. One study reported a 3.3% occult HCV infection rate in a population unselected for hepatic disease (De Marco, Gillio-Tos et al. 2009). Another report stated a relative high prevalence of occult HCV infection

among sexually transmitted HIV population, revealed a 10.3% occult HCV infection (Rai, Mathur et al. 2007). Occult HCV infection may associated with HCC development (Carreño 2006). For occult HCV infection, as HCV RNA only be detected in PBMC or liver tissue, anti-HCV ELISA test used in the study will exclude occult HCV infection cases. As approximately 85% of acute HCV infection will develop into chronic HCV infection and at risk for development of compensated and decompensated cirrhosis, and HCC, liver disease markers ALT and AST may have important applications in decision making for HIV/HCV, HIV/HBV/HCV co-infection treatment (Chen and Morgan 2006).

To summarize, the study may underestimate the prevalence of HBV, HCV infection in the selected HIV population, and further tests may needed for HBV, HCV, HIV co-infection treatment. From epidemiology angel, the study revealed a full perspective of the seroprevalence of HBV, HCV infection among HIV population in Shenzhen, which may provide essential reference for prevention and controlling of HIV, HBV, HCV co-infections in HIV high-risk population.

**Table 2-1. Demographics of HIV, HBV, HCV co-infections in Shenzhen.**

| Variables | N (%) | HIV/HBV | | HIV/HCV | | HIV/HBV/HC | |
|---|---|---|---|---|---|---|---|
| | | N (%) | *P* | N (%) | *P* | N (%) | |
| Total | 914 (100) | 100 (10.9) | | 133 (14.6) | | 34 (3.7) | |
| Gender | | | 0.392[a] | | 0.113[a] | | 0. |
| Male | 677 (74.1) | 78 (11.5) | | 107 (15.8) | | 31 (4.6) | |
| Female | 237 (25.9) | 22 (9.3) | | 26 (11.0) | | 3 (1.27) | |
| Age (years) | | | 0.131[b] | | 0.462[b] | | 0. |
| Mean | 31 | 30 | | 31 | | 32 | |
| SD | 8 | 7 | | 6 | | 6 | |
| Age group | | | 0.422[c] | | 0.116[c] | | 0. |
| ≤ 26 | 278 (30.4) | 32 (11.5) | | 30 (10.8) | | 4 (1.4) | |
| 27-32 | 322 (35.2) | 40 (12.4) | | 57 (17.7) | | 16 (5.0) | |
| ≥ 33 | 314 (34.4) | 28 (8.9) | | 46 (14.6) | | 14 (4.5) | |
| Risk behavior | | | 0.002[d] | | < 0.001[d] | | < 0 |
| Heterosexual | 470 (51.4) | 57 (12.1) | | 30 (6.4) | | 5 (1.1) | |
| Homosexual | 269 (29.5) | 38 (14.1) | | 4 (1.5) | | 1 (0.4) | |
| IDUs | 152 (16.6) | 5 (3.3) | | 89 (58.6) | | 28 (18.4) | |
| Blood | 23 (2.5) | 0 (0) | | 10 (43.5) | | 0 (0) | |

a: Chi-square test was used to test the distribution of gender between HIV psotive population and HIV, HBV, HCV co-infection groups.

b: *T* test was applied to compare the mean age of HIV, HBV, HCV co-infection groups and HIV positive group.

c: Chi-square test was applied to test the distribution of age between HIV psotive population and HIV, HBV, HCV co-infection groups.

d: Chi-square test was applied to test the distribution of risk behavior between HIV psotive population and HIV, HBV, HCV co-infection groups.

**Table 2-2. Risk factors for HIV/HBV co-infection in Shenzhen.**

| Variables | HBV/HIV | OR (95% CI) | *P* value |
|---|---|---|---|
| Gender | | | |
| Male | 78/677 | 1 | |
| Female | 22/237 | 0.79 (0.48 – 1.29) | 0.343 |
| Age (years) | | | |
| 27-32 | 40/322 | 1 | |
| ≤ 26 | 32/278 | 0.92 (0.56 – 1.51) | 0.732 |
| ≥ 33 | 28/314 | 0.69 (0.41 – 1.15) | 0.154 |
| Risk behavior | | | |
| Homosexual | 38/269 | 1 | |
| Heterosexual | 57/470 | 0.84 (0.54 – 1.30) | 0.435 |
| IDUs | 5/152 | 0.21 (0.08 – 0.54) | 0.001 |
| Blood | 0/23 | NA | |

NA: not available, as of null cell.

**Table 2-3. Risk factors for HIV/HCV co-infection in Shenzhen.**

| Variables | HCV/HIV | Univariate | | Multivariate | |
|---|---|---|---|---|---|
| | | OR (95% CI) | *P* value | OR (95% CI) | *P* value |
| Gender | | | | | |
| Male | 107/677 | 1 | | | |
| Female | 26/237 | 0.66 (0.42 – 1.04) | 0.071 | 0.87 (0.49 – 1.53) | 0.619 |
| Age (years) | | | | | |
| 27-32 | 57/322 | 1 | | | |
| ≤ 26 | 30/278 | 0.56 (0.35 – 0.90) | 0.018 | 0.77 (0.43 – 1.39) | 0.386 |
| ≥ 33 | 46/314 | 0.80 (0.52 – 1.22) | 0.297 | 0.90 (0.53 – 1.53) | 0.688 |
| Risk behavior | | | | | |
| IDUs | 89/152 | 1 | | | |
| Blood | 10/23 | 0.55 (0.23 – 1.32) | 0.178 | 0.56 (0.22 – 1.39) | 0.208 |

| | | | | | |
|---|---|---|---|---|---|
| Heterosexual | 30/470 | 0.05 (0.03 – 0.08) | < 0.001 | 0.05 (0.03 – 0.09) | < 0.001 |
| Homosexual | 4/269 | 0.01 (0.004 – 0.030) | < 0.001 | 0.01 (0.004 – 0.030) | < 0.001 |

**Table 2-4. Risk factors for HIV/HBV/HCV co-infection in Shenzhen.**

| Variables | (HBV, HCV)/HIV | Univariate | | Multivariate | |
|---|---|---|---|---|---|
| | | OR (95% CI) | *P* value | OR (95% CI) | *P* v |
| Gender | | | | | |
| Male | 31/677 | 1 | | | |
| Female | 3/237 | 0.27 (0.08 – 0.88) | 0.030 | 0.46 (0.13 – 1.63) | 0. |
| Age (years) | | | | | |
| 27-32 | 16/322 | 1 | | | |
| ≥ 33 | 14/314 | 0.89 (0.43 – 1.86) | 0.762 | 1.17 (0.52 – 2.61) | 0. |
| ≤ 26 | 4/278 | 0.28 (0.09 – 0.85) | 0.024 | 0.37 (0.12 – 1.18) | 0. |
| Risk behavior | | | | | |
| IDUs | 28/152 | 1 | | | |
| Heterosexual | 5/470 | 0.05 (0.02 – 0.13) | < 0.001 | 0.06 (0.02 – 0.16) | < 0 |
| Homosexual | 1/269 | 0.02 (0.002 – 0.123) | < 0.001 | 0.02 (0.002 – 0.12) | < 0 |
| Blood | 0/23 | NA | | NA | |

NA: not available, as of null cells.

**Table 2-5. HIV risk behavior by gender and age in HIV, HBV, HCV co-infected population.**

| Variables | HIV, HBV, HCV co-infection (%) | Risk behavior | | | | *P* valu |
|---|---|---|---|---|---|---|
| | | IDUs | Heterosexual | Homosexual | Blood | |
| Gender | | | | | | < 0.00 |
| Male | 216 (80.9) | 108 (50.0) | 59 (27.3) | 43 (19.9) | 6 (2.8) | |
| Female | 51 (19.1) | 14 (27.5) | 33 (64.7) | 0 (0) | 4 (7.8) | |
| Age (years) | | | | | | 0.164 |
| ≤ 26 | 66 (24.7) | 29 (43.9) | 24 (36.4) | 13 (19.7) | 0 (0) | |
| 27-32 | 113 (42.3) | 56 (49.6) | 33 (29.2) | 20 (17.7) | 4 (3.5) | |
| ≥ 33 | 88 (33.0) | 37 (42.1) | 35 (39.8) | 10 (11.4) | 6 (6.8) | |

**Table 2-6. Odds ratio of risk behavior on gender.**

| Risk behavior | Gender (Male/Female) | OR (95% CI) | *P* value |
|---|---|---|---|
| IDUs | 108/14 | 1 | |
| Heterosexual | 59/33 | 0.23 (0.12 – 0.47) | < 0.001 |
| Blood | 6/4 | 0.19 (0.05 – 0.78) | 0.031 |
| Homosexual | 43/0 | NA | |

NA: not available, as of null cells

# Chapter 3

# Upregulation of Interleukin-27 titer in HIV-infected persons

## 3.1 Introduction

Human Immunodeficiency Virus (HIV) is a member of the genus *Lentivirus*, causes progressive impairment of the immune system leading to Acquired Immunodeficiency Syndrome (AIDS). Although the drug cocktails used in the highly active antiretroviral therapy (HAART) can significantly slow the progression of HIV-infected people to AIDS, HIV/AIDS is still one of the leading causes of death. In 2009, there were totally 33.3 millions people living with HIV/AIDS worldwide, about 1.8 millions people died of AIDS-related diseases worldwide (UNAIDS 2010). Hepatitis B virus (HBV) and hepatitis C virus (HCV) are liver-tropic viruses and cause both acute and chronic infections. Worldwide, an estimated 350 millions people are chronically infected with HBV and 200 millions are infected by HCV. HIV, HBV and HCV infections are global public health challenges.

HIV, HBV and HCV are blood-borne pathogens and share routes of transmission, the prevalence of HBV, and HCV co-infection among HIV positive population is remarkably high and bring severe clinical consequences. Globally, chronic HBV infection affects 7% - 10% HIV-infected subjects (Lacombe, Bottero et al. 2010). It has been shown that HBV co-infection significantly increases the risk of liver-related diseases, the risk was doubled after highly active antiretroviral therapy was introduced (Thio, Seaberg et al. 2002). The worldwide seroprevalence of HCV in HIV-infected patients varies from 15% to 30% among different characteristic populations (Sherman, Rouster et al. 2002). Although HCV serostatus has not affected the overall virologic and immunologic responses to HAART and the disease progression of HIV infection, the liver-related mortality was markedly elevated (Rockstroh, Mocroft et al. 2005).

Upon HIV infection, human immune system responds to restrict, inhibit and destroy HIV through different mechanisms. Among them, cytokines and chemokines play pivotal roles. IFN-α, -β, and -γ inhibit HIV-1 replication in primary human macrophages by reducing viral DNA synthesis during the early life cycle of virus (Meylan, Guatelli et al. 1993); IFN-λ not only induces the expression of CC chemokines, but also upregulates type I IFNs and APOBEC3G/3F to inhibit HIV-1 infection and replication of monocyte-derived macrophages (Hou, Wang et al. 2009). Human CC chemokines RANTES, MIP-1 alpha, and MIP-1 beta are also HIV suppressors, they are produced by $CD8^+$ T Cells and inhibit different strains of HIV in a dose-dependent manner (Cocchi, DeVico et al. 1995). IL-16 has also been found to prohibit HIV replication at level of RNA transcription by repressing HIV promoter activity (Baier, Werner et al. 1995; Maciaszek, Parada et al. 1997; Zhou, Goldstein et al. 1997).

Interleukin-27 (IL-27) is a heterodimeric cytokine consists of EBI3 protein and p28 protein, belongs to IL-6/IL-12 family of type I cytokines. IL-27 is mainly secreted by antigen presenting cells (APCs) in response to inflammatory challenge. IL-27 not only induces naive $CD4^+$ T cells proliferation, but also promotes naive $CD4^+$ T cells polarize to Th1 cells and IFN-γ production, which are mediated through its functional signal-transducing receptor composed of gp130 and orphan cytokine receptor WSX-1/TCCR (Pflanz, Timans et al. 2002; Pflanz, Hibbert et al. 2004). Shimizu et al has demonstrated that IL-27 also possess antiangiogenic and antitumor activities via $CD8^+$ T cells (Shimizu, Shimamura et al. 2006). It has been also shown that IL-27 inhibits X4 and R5 HIV-1 replication in PBMCs, $CD4^+$ T cells and macrophages through the induction of type I interferon and activation of multiple interferon-inducible genes (Fakruddin, Lempicki et al. 2007; Imamichi, Yang et al. 2008; Greenwell-Wild, Vazquez et al. 2009). HBV infected patients showed significantly elevated IL-27 levels and serum IL-27 level was positively correlated with HBV e antigen (Zhu, Zhang et al. 2009). IL-27 has been shown to induce

STAT1 and STAT3 in Huh7.5 cell line, and inhibit HCV replication in a dose-dependent manner (Frank, Zhang et al. 2010). IL-27 was further demonstrated to be responsible for the pathogenic role in T cell-mediated liver injury during fulminant hepatitis (Siebler, Wirtz et al. 2008).

Till now, although IL-27 has been demonstrated to inhibit HIV *in vitro*, no data has been available about the expression levels of IL-27 in HIV-positive population, including HIV, HBV and HCV co-infection individuals. To solve this puzzle, we conduct this study to compare plasma IL-27 titer in healthy controls and HIV-infected individuals.

## 3.2 Materials and methods

### 3.2.1 Study participants

The study participants were recruited from voluntary-based HIV/AIDS screening program in Shenzhen from September 2007 to July 2009. Informed consent was obtained from each participant. Participants were blooded and tested for HIV infection, HBsAg positivity and anti-HCV antibodies. Confirmed HIV positive participants were referred to on-going HIV prevention, treatment, and care programs. HBsAg positivity and anti-HCV antibody positive participants were referred to local hospitals for treatments. All infections were recorded following the requirements of reportable infectious diseases.

The study protocol was approved by Shenzhen Center for Disease Control and Prevention (Shenzhen CDC) and The Chinese University of Hong Kong.

### 3.2.2 Measure of HIV, HBV and HCV infection

Whole blood specimen was collected from all subjects with anticoagulant and centrifuged at 1000 rpm for 10 minutes, plasma were then gathered and stored at -80°C until analysis.

Plasma was screened for HIV antibody according to standard procedures of Shenzhen CDC laboratory. In brief, samples were tested using ABBOTT PRISM HIV O Plus (Detect anti-HIV-1 group O and M, anti-HIV-2, Abbott Laboratories, IL) and ELISA (Beijing Wantai Biological Pharmacy Enterprise CO., LTD, Beijing) for screening, positive samples were then confirmed by HIV-1/-2 western blot assay (HIV Blot 2.2 WB; Genelabs Diagnostics, Singapore).

HBsAg positivity was determined by ELISA kit (Beijing Wantai Biological Pharmacy Enterprise CO., LTD, Beijing) according to manufacturer's protocol.

HCV antibody was tested using ELISA kit (GBI Biotech Co., Ltd., Beijing) as indicated.

**3.2.3 Detection of IL-27 in plasma**

IL-27 titer in plasma was measured with LEGEND MAX™ Human IL-27 ELISA Kit (BioLegend Inc., San Diego, CA) according to manufacturer's instructions. Standard curve was generated using serial diluted recombinant human IL-27 with known concentrations. Each plate runs an independent standard curve. Absorbance at 450 nm was recorded and calculated using standard curve for sample plasma IL-27 concentrations. The minimum detectable concentration of the ELISA kit was 11 pg/ml and has no cross-reaction with other 14 human cytokines.

Procedures were as follow:

Reagent and Sample Preparations:

1. Dilute the 20X Wash Buffer to 1X with deionized water.

2. Reconstitute the lyophilized Human IL-27 Standard by adding the volume of Assay Buffer A described on the vial label to make the 16 ng/ml standard stock solution, vortex. Allow the reconstituted standard to sit at room temperature for 15

minutes, vortex again to mix completely.

3. In general, serum and plasma samples are analyzed without dilutions. If dilutions are necessary, samples should be diluted in Assay Buffer A.

Assay Procedure:

1. Bring all reagents to room temperature prior to use. A standard curve is required for each assay.

2. Prepare 500 µl of the 16 ng/ml top standard by pipetting 500 µl of the standard stock solution into the top standard tube. Perform six two-fold serial dilutions of the 16 ng/ml top standard in separate tubes. Thus, the human IL-27 standard concentrations in the tubes are 16 ng/ml, 8 ng/ml, 4 ng/ml, 2 ng/ml, 1 ng/ml, 500 pg/ml, and 250 pg/ml, respectively. Assay Buffer A serves as the zero standard (0 pg/ml).

3. Wash the plate 4 times. For each wash dispense 300 µl of 1X Wash Buffer per well, soak for 30 seconds, dump the plate contents in a sink, then blot the remaining wash buffer by firmly tapping the plate upside down on a stack of absorbent paper. All subsequent washes steps should be performed similarly.

4. Add 50 µl of Assay Buffer A to each well that will contain either standards or samples.

5. Add 50 µl/well of standard dilutions or samples to the respective wells.

6. Seal the plate with a Plate Sealer provided in the kit and then incubate at room temperature for 2 hours with shaking.

7. Dump the plate contents in a sink then wash the plate 4 times with 1X Wash Buffer as in step 3.

8. Add 100 µl of the IL-27 Detection Antibody Solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.

9. Dump the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 3.

10. Add 100 µl of Avidin-HRP A Solution to each well, seal the plate and incubate at room temperature for 30 minutes in the dark with shaking.

11. Dump the contents of the plate into a sink then wash the plate 5 times with 1X Wash Buffer as in step 3. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

12. Add 100 µl of Substrate Solution F to each well and incubate for 15 minutes in the dark. Wells with higher concentrations of human IL-27 should turn a blue color. It is not necessary to seal the plate during this step.

13. Stop the reaction by adding 100 µl of Stop Solution to each well. The blue color should change to yellow color.

14. Read absorbance at 450 nm within 30 minutes.

Calculation of Results:

Standard curve was generated according to serial diluted recombinant IL-27 with known concentrations. The IL-27 titer for each sample was then calculated using absorbance 450 nm value.

### 3.2.4 CD4 counting

$CD4^+$ T cell counts of HIV-positive individual were assessed by three-color fluorescence kit for CD4/CD8/CD3 (BD Biosciences, San Jose, CA) counting according to kit manual. Fresh whole blood was analyzed on FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) within 8 hours. Multiset software was used for CD4/CD8/CD3 analysis.

### 3.2.5 Statistical analysis

Data were analyzed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Histogram was applied to assess the distribution of data. Base on data distribution, parametric and nonparametric tests were applied for analysis. Mann-Whitney *U* test was used for comparison of plasma IL-27 titer, independent-samples *T* test was used for comparison of $CD4^+$ T cell counts among HIV positive groups. Pearson's correlation coefficient was used to evaluate the correlation between IL-27 titers and $CD4^+$ T cell counts in HIV positive groups. $P$ value $< 0.05$ was considered as statistically significant.

## 3.3 Results

### 3.3.1 Demographics of study participants

After tested for HIV infection, HBsAg and anti-HCV positivity, totally 414 antiretroviral therapy-naïve Chinese were recruited in the study. Among them, 80 were HIV, HBV and HCV negative healthy controls; 143 were HIV mono-infected; 79 were HIV/HBV co-infected; 84 were HIV/HCV co-infected and 28 were HIV/HBV/HCV co-infected. The means of age were 28, 30, 30, 31 and 32 years for control, HIV mono-infection, HIV/HBV co-infection, HIV/HCV co-infection and HIV/HBV/HCV co-infection groups, respectively (Table 3-1). The gender ratios (male/female) were 62/18, 104/39, 64/15, 69/15 and 25/3 for control, HIV mono-infection, HIV/HBV co-infection, HIV/HCV co-infection and HIV/HBV/HCV co-infection groups, respectively.

### 3.3.2 Upregulation of IL-27 levels in HIV-infected persons

IL-27 protein levels were measured by ELISA. After testing for distribution, as the non-normal distribution of IL-27 titer, median and standard deviation (SD) were applied to describe the central tendency, Mann-Whitney *U* test was used for comparison of IL-27 titer. The medians of IL-27 levels in control, HIV mono-infection, HIV/HBV co-infection, HIV/HCV co-infection and HIV/HBV/HCV co-infection groups were 387, 488, 569, 516 and 415 pg/ml; the IL-27 titer ranges between 27 pg/ml to 1993 pg/ml in the study (Table 3-1). As shown in Figure 3-1, compared to healthy controls, the IL-27 level was significantly elevated in HIV mono-infected persons (488 ± 345 versus 387 ± 226 pg/ml, expressed as median ± SD; $P = 0.001$), HIV/HBV co-infection group (569 ± 310 versus 387 ± 226 pg/ml; $P < 0.001$) and HIV/HCV co-infected individuals (516 ± 282 versus 387 ± 226 pg/ml; $P < 0.001$); while the IL-27 level in HIV/HBV/HCV triple-infected individuals was not significantly differ from healthy controls (415 ± 465 versus 387 ± 226 pg/ml; $P = 0.274$).

Plasma IL-27 titer also compared among HIV-infected groups by using Mann-Whitney *U* test. As shown in Fig.3-2, the IL-27 titers were not significantly varied between HIV mono-infection group and HIV/HBV co-infection group (488 ± 345 versus 569 ± 310 pg/ml; $P = 0.153$), HIV mono-infection group and HIV/HCV co-infection group (488 ± 345 versus 516 ± 282 pg/ml; $P = 0.144$), HIV mono-infection group and HIV/HBV/HCV triple-infection group (488 ± 345 versus 415 ± 465 pg/ml; $P = 0.248$), HIV/HBV co-infection group and HIV/HCV co-infection group (569 ± 310 versus 516 ± 282 pg/ml; $P = 0.993$), HIV/HBV co-infection group and HIV/HBV/HCV triple-infection group (569 ± 310 versus 415 ± 465 pg/ml; $P = 0.072$); while the IL-27 concentration between HIV/HCV co-infection and HIV/HBV/HCV triple-infection was significantly different (516 ± 282 versus 415 ± 465 pg/ml; $P = 0.034$).

### 3.3.3 Correlation of plasma IL-27 titer with $CD4^+$ T cell counts

$CD4^+$ T cell counts in HIV positive individuals was determined by flow cytometer and stated in Table 3-1, as the distribution of $CD4^+$ T cell counts were normal; mean was applied to describe the central tendency. Independent-samples *T* test was applied to compare $CD4^+$ T cell counts, results showed that significant difference were observed between HIV mono-infection and HIV/HBV co-infection group (389 ± 225 versus 314 ± 193 cells/μl, $P = 0.013$), HIV/HBV co-infection and HIV/HCV co-infection group (314 ± 193 versus 413 ± 213 cells/μl, $P = 0.002$), and HIV/HCV co-infection and HIV/HBV/HCV triple-infection group (413 ± 213 versus 298 ± 231 cells/μl, $P = 0.017$); while lack of difference for HIV mono-infection and HIV/HCV co-infection group (389 ± 225 versus 413 ± 213 cells/μl, $P = 0.432$), HIV mono-infection and HIV/HBV/HCV triple-infection group (389 ± 225 versus 298 ± 231 cells/μl, $P = 0.053$), HIV/HBV co-infection and HIV/HBV/HCV triple-infection (314 ± 193 versus 298 ± 231 cells/μl, $P = 0.727$).

$CD4^+$ T cell counts in HIV positive individuals was further analyzed with plasma IL-27 titer by Pearson's correlation coefficient. As illustrated in Figure 3-3, a significant positive correlation between $CD4^+$ T cell counts and plasma IL-27 titers was observed within HIV mono-infected people ($r = 0.177$, $P = 0.034$, Pearson's Correlation); while other HIV positive groups were lack of the correlation between $CD4^+$ T cell counts and plasma IL-27 titers (HIV/HBV co-infection: $r = - 0.034$, $P = 0.763$; HIV/HCV co-infection group: $r = - 0.076$, $P = 0.492$; HIV/HBV/HCV triple-infection group: $r = 0.065$, $P = 0.742$).

## 3.4 Discussion

In the past two decades, a remarkable increase of HIV infection has been observed in China along with other sexually transmitted diseases (STDs) due to the changes of social values and sexual behaviors, and also the population shift by migration (Yang, Li et al. 2005). In 2011, the estimated number of people living with HIV/AIDS in China was 780,000, and of those, more than 64% got infection through sexual transmission (Ministry of Health of China, UNAIDS et al. 2011).

IL-27 is secreted by antigen presenting cells (APCs) upon immunity challenge. Before antiretroviral therapy is applied, HIV will continue its replication and infection of susceptible cells. The persistent infection of HIV will induce APCs to secret IL-27, making IL-27 a detectable cytokine in HIV-infected plasma. As IL-27 has been demonstrated to inhibit HIV replication *in vitro*, it is worthwhile to study IL-27 titer in clinical samples. As HIV, HBV and HCV share routes of transmission, HIV/HBV and HIV/HCV co-infections are common in patients. Both HIV/HBV and HIV/HCV co-infections significantly elevate liver-related mortality (Thio, Seaberg et al. 2002; Rockstroh, Mocroft et al. 2005). It has been further demonstrated that the expression of hepatic inflammatory cytokines was suppressed in patients with HBV/HIV or HCV/HIV co-infections (Gonzalez, Zhang et al. 2008; Roe and Hall 2008). HBV infection would enhance IL-27 expression both *in vitro and in vivo*; HBV infected patients have elevated serum IL-27 concentration (Zhu, Zhang et al. 2009). HCV co-infection with HIV suppresses IL-27 expression (Guzzo, Hopman et al. 2010). To investigate the expression levels of human IL-27 among HIV-positive individuals, and possible impact of HBV and HCV co-infections on IL-27 expression level, we divided research participants into HIV mono-infection group, HIV/HBV co-infection group, HIV//HCV co-infection group and HIV/HBV/HCV co-infection group.

HIV infection changes the cytokine profile both in T cells infected with HIV *in vitro* and HIV-infected individuals *in vivo*. IL-1, IL-4, IL-6, IL-8, IL-10, IFN-α, IFN-β, M-CSF, TNF-α, TNF-β, SDF-1, MIP-1α, MIP-1β and RANTES are elevated by HIV infection both *in vitro* and/or *in vivo*; whereas IL-2, IL-12, IL-13, IFN-γ, and GM-CSF are downregulated (Kedzierska and Crowe 2001). IL-32, a pro-inflammatory cytokine produced by T-lymphocytes, NK cells and blood monocytes, is also significantly elevated in serum of HIV patients. As IL-32 suppresses HIV replication *in vitro*, it is possible that the upregulation of IL-32 in serum after HIV infection may

inhibit HIV replication *in vivo* (Rasool et al., 2008). IL-27 inhibits HIV replication in PBMCs, $CD4^+$ T cells and MDM *in vitro*. Fakruddin and colleagues described that IL-27, released by PBMCs and macrophages on human papilloma virus (HPV)-like particles (VLPs) treatment, inhibited HIV-1 replication. IL-27 hampered the replication of both X4 and R5 HIV without affecting the expression of cell surface markers-CD4, CXCR4 and CCR5. The inhibitory effect of IL-27 on HIV replication was dose-dependent. The underlying mechanism was activation of multiple genes encoding antiviral proteins (Fakruddin, Lempicki et al. 2007). DNA microarray experiments revealed that IL-27 activated multiple IFN-inducible genes (IFIG) in MDM (Imamichi, Yang et al. 2008). Further study on macrophages and $CD4^+$ T cells uncovered that IL-27 induced the expression of type I interferons which drove the intracellular antiretroviral cytidine deaminases (APOBECs) expression to counterattack HIV (Greenwell-Wild, Vazquez et al. 2009). In our study, we showed that IL-27 was significantly increased in HIV mono-infected individuals, indicating that IL-27 was indeed responds to HIV infection and may be an important factor for combating HIV infection.

Acute HIV infection leads an intense cytokine storm prior to viremia peak, while it has little perturbation in plasma cytokine/chemokine levels, and only witness a delayed and more intermediate magnitude of cytokine/chemokine changes (Stacey, Norris et al. 2009), indicating that HIV is stronger in immune activation than HBV and HCV. We hypothesized that HIV might play a major role in inducing immune responses during HIV, HBV and HCV co-infection circumstances. In this study, we found significant elevation of plasma IL-27 in HIV/HBV, HIV/HCV co-infection groups, but no significant difference between HIV mono-infection, HIV/HBV co-infection and HIV/HCV co-infection group, indicating that HIV was the main target of immune responses in HIV co-infected with HBV, HCV individuals. We observed no significant change of plasma IL-27 in HIV/HBV/HCV co-infection individuals in comparing with healthy control, while the plasma IL-27 in HIV/HBV/HCV co-

infection persons was significantly lower than HIV/HCV co-infection group. The difference of IL-27 may be explained by the relative lower $CD4^+$ T cell counts in HIV/HBV/HCV triple-infection group ($P$ = 0.017). IL-27 is secreted by antigen-presenting cells, induces naïve $CD4^+$ T cell proliferation and enhances antigen presentation by APCs (Pflanz, Timans et al. 2002; Feng, Chen et al. 2007; Feng, Liu et al. 2008). In HIV infection circumstance, immune system counterattacks HIV by ensuring its integrity to slow down disease progression. By responding to HIV infection, secreted IL-27 can in turn enhance antigen presentation to produce more pro-inflammatory cytokines including IL-27, and induces naïve $CD4^+$ T cell proliferation to maintain the integrity of immune system. However, when HIV infection progress into AIDS, the integrity of immune system may be harmed to some extent. It will result impaired IL-27 secretion and dramatically decline of $CD4^+$ T cell counts.

HIV infection kills $CD4^+$ T cells in three pathways: direct cytopathic effect, apoptosis and $CD8^+$ CTLs. HIV infection induced IL-27 prompted rapid clonal expansion of naïve $CD4^+$ T cell to recover its status. In this study, we observed positive correlation between $CD4^+$ T cell counts and plasma IL-27 titer among HIV mono-infected individuals (r = 0.177, $P$ = 0.034), suggesting IL-27 indeed prompt expansion of naïve $CD4^+$ T cell *in vivo*. However, the correlation was not observed among HIV, HBV and HCV co-infected groups. For HIV/HBV co-infected group, the $CD4^+$ T cell counts were lower than HIV mono-infected (Thio 2009). Poor immune function secondary to HBV specific $CD8^+$ cells related immune dysregulation may affect IL-27 expression or $CD4^+$ T cell proliferation, resulting to a complex relationship between $CD4^+$ T cell counts and plasma IL-27 titer for HIV/HBV co-infection individuals. Reports showed HCV co-infection with HIV has no obvious impact on $CD4^+$ T cell decrease, while it significantly elevated AIDS-defining illnesses (ADIs) probability when compared with HIV mono-infection, and the suggested mechanisms include inhibited $CD4^+$ T cell proliferation, impaired

immune recovery, and promotion of AIDS onset by HCV-induced apoptosis (Miller, Haley et al. 2005; d'Arminio, Cozzi-Lepri et al. 2009; Kondo, Machida et al. 2009). In HIV and HCV co-infected people, HCV-induced inhibition of $CD4^+$ T cell proliferation may result in a lack of correlation between $CD4^+$ T cell counts and plasma IL-27 titer.

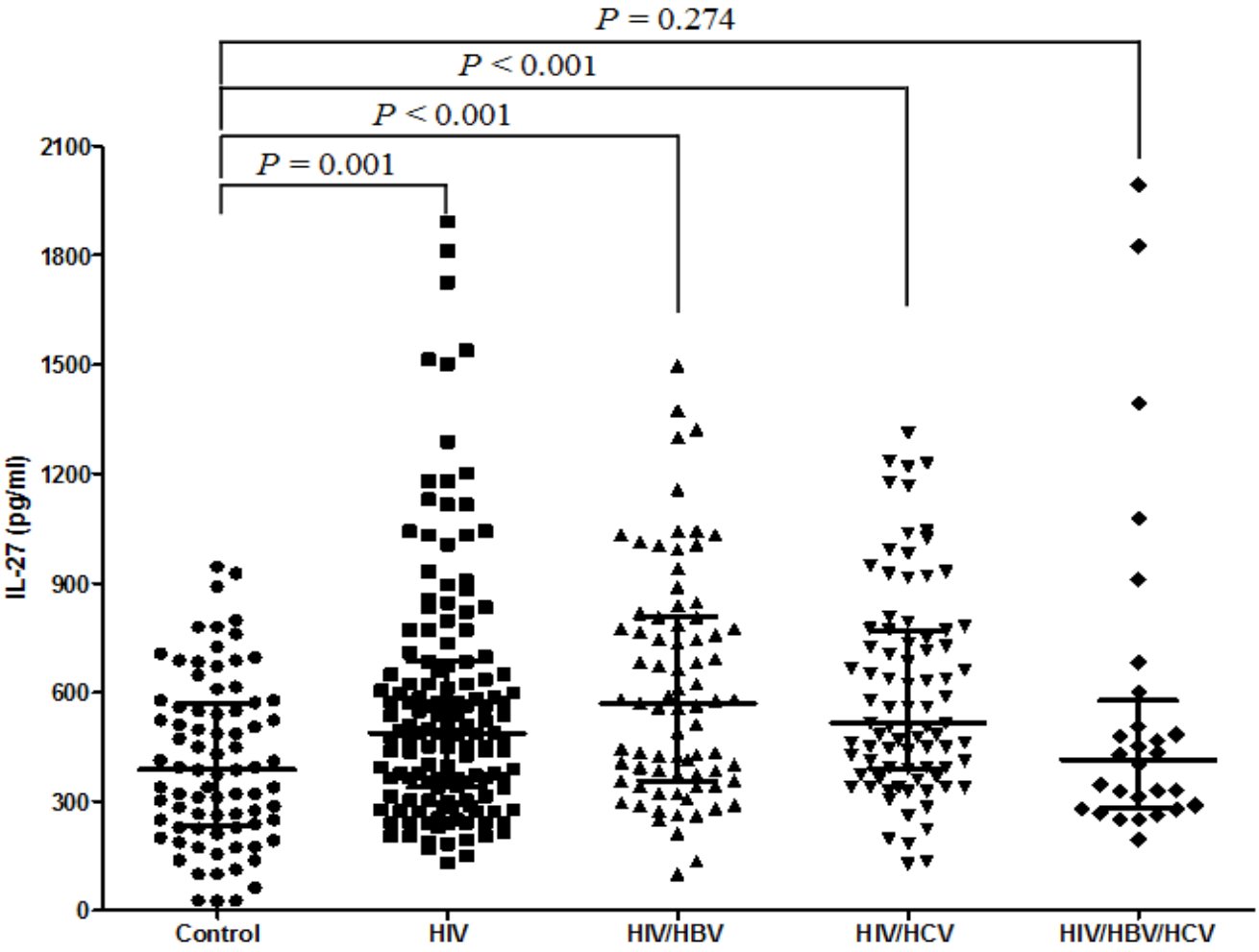
Co-infection with HBV and HCV in HIV-infected individuals has been shown an elevated liver-related mortality (Monga, Rodriguez-Barradas et al. 2001; Thio, Seaberg et al. 2002). It also reported that IL-27 plays an important pathogenic role in T cell-mediated hepatitis (Siebler, Wirtz et al. 2008). In China, most chronic HBV infections are due to perinatal and early childhood infection, which is prior to HIV infection. HCV transmission is mainly due to injection drug use. HCV infection may occur simultaneously or successively with HIV infection. In HIV, HBV and HCV co-infection settings, high level of IL-27 produced by APCs upon HIV infection may directly cause live injuries, worsen hepatic microenvironment and may partly be responsible for elevated liver-related mortality in co-infected people. As IL-27 exhibits both pro-inflammatory and anti-inflammatory properties (Pflanz, Timans et al. 2002), it may also modulate hepatic cytokine environment and be partially responsible for the elevated liver-related mortality in HIV, HBV, and HCV co-infected individuals. The situation may become even worse as there is increasing evidence that HIV can directly infect hepatic stellate cells and hepatocytes and cause direct cytopatic effect on liver tissue (Babu, Suwansrinon et al. 2009; Tuyama AC 2010).

**Table 3-1. Characteristics and IL-27 concentration of study participants**

| Characteristics | Control | HIV mono-infection | HIV/HBV co-infection | HIV/HCV co-infection | HIV/HBV/HCV co-infection |
|---|---|---|---|---|---|
| Number | 80 | 143 | 79 | 84 | 28 |
| Age (years) | | | | | |
| mean | 28 | 30 | 30 | 31 | 32 |
| SD | 6 | 6 | 7 | 7 | 6 |
| Gender | | | | | |
| male | 62 | 104 | 64 | 69 | 25 |
| female | 18 | 39 | 15 | 15 | 3 |
| IL-27 (pg/ml)[a] | 387 ± 226 | 488 ± 345 | 569 ± 310 | 516 ± 282 | 415 ± 465 |
| CD4 count (cells/µl)[b] | NA | 389 ± 225 | 314 ± 193 | 413 ± 213 | 298 ± 231 |

a: IL-27 titer was expressed as Median ± SD;
b: CD4 count was expressed as Mean ± SD;
NA: not applicable as of null cell.

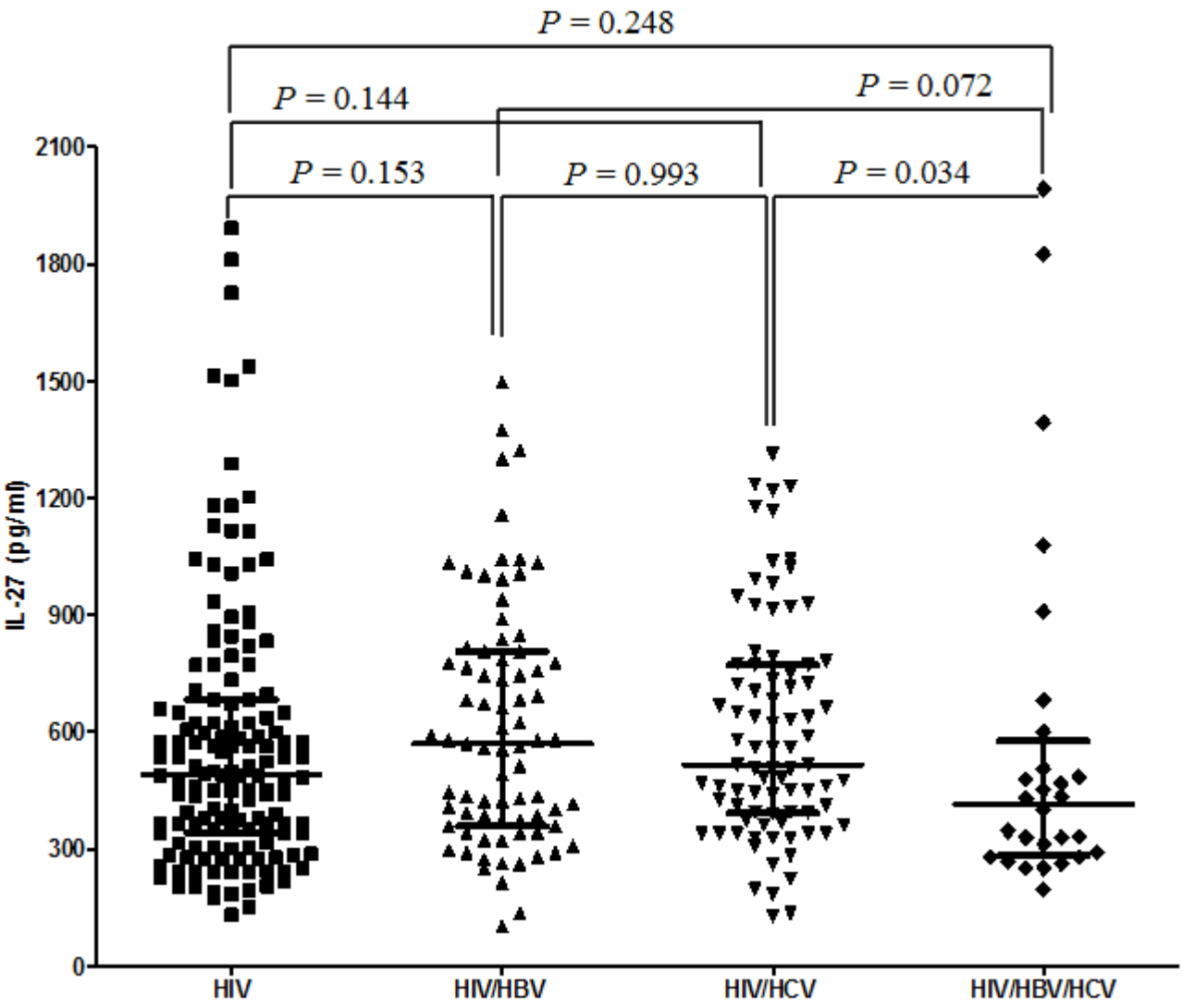
**Figure 3-1. Comparison of plasma IL-27 titer in the study**




Plasma IL-27 concentration was measured by ELISA and compared among 143 HIV mono-infected person, 79 HIV/HBV co-infected people, 84 HIV/HCV co-infected person, 28 HIV/HBV/HCV triple-infected individuals and 80 healthy controls by Mann-Whitney *U* test. Median with 25% and 75% percentile was illustrated.

**Fig.3-2 Comparison of plasma IL-27 titer between HIV, HBV, HCV co-infection**

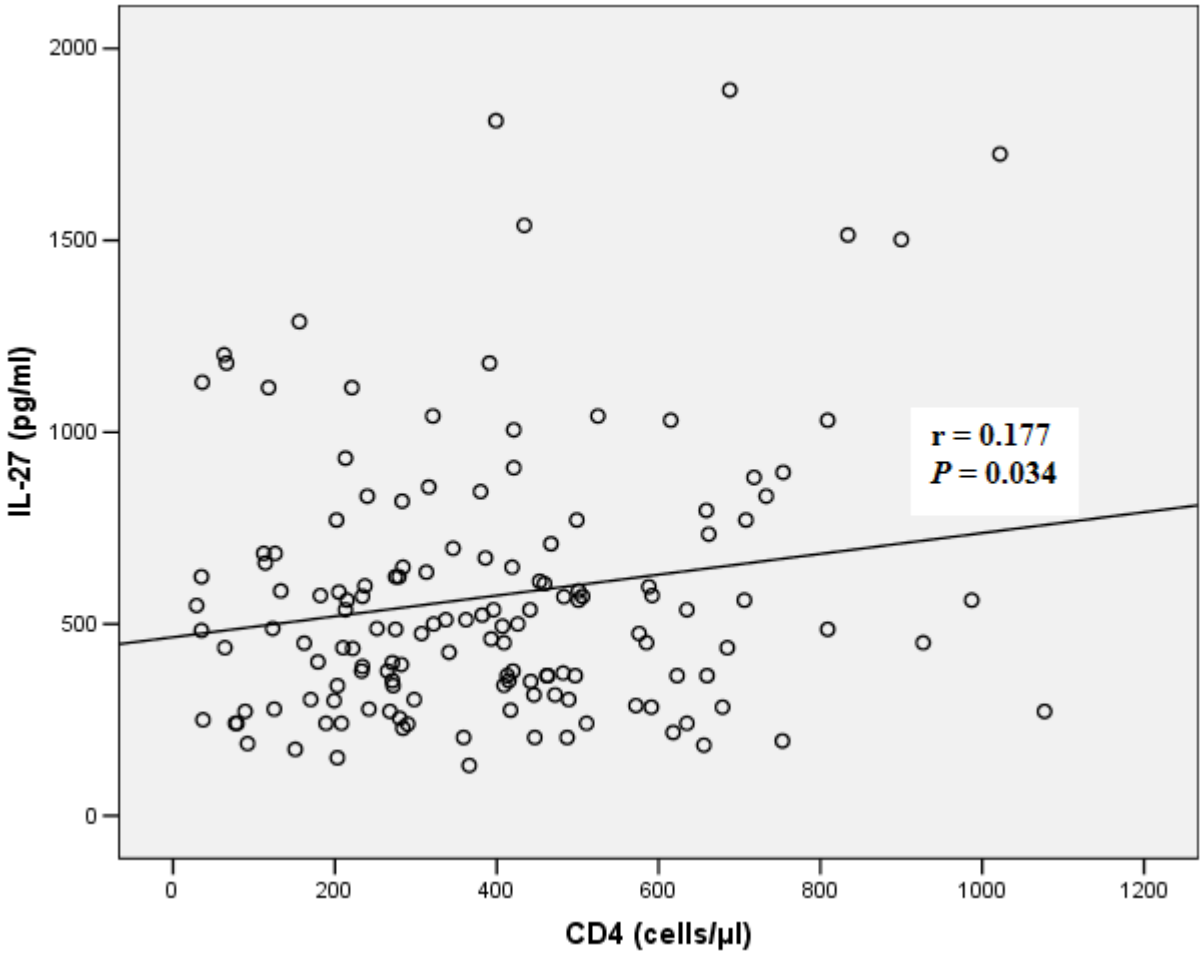
**groups**




Plasma IL-27 titer was compared among 143 HIV mono-infected person, 79 HIV/HBV co-infected people, 84 HIV/HCV co-infected person and 28 HIV/HBV/HCV triple-infected individuals by Mann-Whitney *U* test. Median with 25% and 75% percentile was illustrated.

**Fig. 3-3. Correlation of IL-27 titer with $CD4^+$ T cell counts in HIV mono-**

**infected people**




Plasma IL-27 titer and $CD4^+$ T cell counts were analyzed with Pearson's correlation coefficient in HIV mono-infected individuals.

# Chapter 4

# Impact of HIV, HCV viral loads on Interleukin-27 titer among Antiretroviral Therapy-Naïve HIV positive Chinese

## 4.1 Introduction

Human Immunodeficiency Virus (HIV) causes progressive failure of the immune system which eventually leads to Acquired Immunodeficiency Syndrome (AIDS) characterized by susceptibility to opportunistic infections and tumors. Since the first report of AIDS in 1981 and the discovery of HIV in 1983 (Gottlieb, Schroff et al. 1981; Barre-Sinoussi, Chermann et al. 1983; Gallo, Sarin et al. 1983), HIV/AIDS has caused nearly 30 million deaths globally (UNAIDS 2010). Although drug cocktails used in highly active antiretroviral therapy (HAART) significantly slows the progression of HIV-infected individuals to AIDS, HIV/AIDS is still one of the leading causes of death. In 2009, an estimated 33.3 million people lived with HIV/AIDS and about 1.8 million people died of AIDS-related diseases worldwide (UNAIDS 2010). Along with the implementation of the Reforming and Open-up Policy, HIV/AIDS severity in China grows. The rapidly increased HIV prevalence in China has shifted from high-risk populations such as injection drug users (IDUs) and former blood donors into men who have sex with men and female sex workers (Lu, Jia et al. 2008). HIV/HCV co-infection worsens the HIV/AIDS epidemic as HCV co-infection facilitates HIV disease progression and increase morbidity and mortality of AIDS patients (Monga, Rodriguez-Barradas et al. 2001). Globally and locally, the HIV/AIDS epidemic deserves more attention.

Upon HIV infection, human immune system responds to restrict, inhibit and destroy HIV through different mechanisms. Among the system, Interlekin-27 (IL-27) is a newly described cytokine with important implications in HIV/AIDS epidemic. Composed by EBI3 protein and p28 protein, IL-27 plays pivotal roles in both pro-

inflammatory and anti-inflammatory responses (Pflanz, Timans et al. 2002; Stumhofer, Laurence et al. 2006; Batten and Ghilardi 2007). In vitro experiments demonstrated that IL-27 inhibits X4 and R5 HIV replication in peripheral blood mononuclear cells (PBMC), $CD4^+$ T cells and monocyte-derived macrophages (MDMs) through the induction of type I interferon and activation of multiple interferon-inducible genes (Fakruddin, Lempicki et al. 2007; Imamichi, Yang et al. 2008; Greenwell-Wild, Vazquez et al. 2009). In cultured cell system, IL-27 induces IFN-γ-like signals and induction of antiviral responses in hepatoma cells, hepatocytes and hepatic stellate cells (Bender, Wiesinger et al. 2009; Schoenherr, Weiskirchen et al. 2010). Frank et al further demonstrate that IL-27 inhibits HCV in a dose-dependant manner, indicating that IL-27 may be a potential therapeutic cytokine for HCV and HIV/HCV co-infection (Frank, Zhang et al. 2010).

In clinical settings, serum IL-27 was reported negatively correlated with HIV viral load; HCV co-infection with HIV decreased serum IL-27 titer. $CD4^+$ T cell counts affected IL-27 expression level; while HAART has no significant implication in serum IL-27 level (Guzzo, Hopman et al. 2010). In our previous study, compared to healthy control, plasma IL-27 titer was significantly elevated in HIV mono-infected, HIV/HBV co-infected and HIV/HCV co-infected Chinese, and IL-27 titer was positively correlated with $CD4^+$ T cell count among HIV mono-infected, antiretroviral therapy-naïve Chinese (He, Zhao et al. 2011). As IL-27 was secreted upon HIV infection, and HCV was simultaneously or successively infect with HIV for most Chinese, how HIV, HCV viral loads affect serum IL-27 titer among antiretroviral treatment-naïve Chinese worthy further elucidation.

In the study, we assessed plasma IL-27 titers among HIV mono-infected and HIV/HCV co-infected antiretroviral therapy-naïve Chinese, and evaluated how HIV, HCV viral loads correlated with plasma IL-27 expression levels.

## 4.2 Materials and methods

### 4.2.1 Study participants

A Chinese population was recruited from an ongoing voluntary-based HIV/AIDS

surveillance study in Shenzhen, an immigrant city in Guangdong province, from September 2009 to December 2010. Written consents were obtained from participants and interviews were conducted by experienced research staff. Participants were screened for HIV, HBV and HCV infections. Confirmed HIV positive individuals were referred to HIV prevention, treatment, and care programs. HBV and HCV positive participants were referred to local hospitals for treatments. All infections were recorded following the requirements of reportable infectious diseases.

The study protocol was approved by Shenzhen Center for Disease Control and Prevention (Shenzhen CDC) and The Chinese University of Hong Kong.

### 4.2.2 HIV, HBV and HCV Serological assays:

Whole blood specimen was collected with EDTA anticoagulant and centrifuged at 1000 rpm for 10 minutes; plasma was then gathered and stored at -80°C until analysis. Plasma was screened by ABBOTT PRISM HIV O Plus (Abbott Laboratories, IL) and ELISA (Beijing Wantai Biological Pharmacy Enterprise CO., LTD, Beijing) for HIV infection, positive sample was further confirmed by HIV-1/2 western blot assay (HIV Blot 2.2 WB; Genelabs Diagnostics, Singapore).

HBV and HCV infections were determined by HBsAg ELISA kit (Beijing Wantai Biological Pharmacy Enterprise CO., LTD, Beijing) and antibody to hepatitis C virus (HCV) ELISA kit (Beijing Wantai Biological Pharmacy Enterprise CO., LTD, Beijing), according to manufacturer's protocols, respectively.

### 4.2.3 CD4 counting:

$CD4^+$ T cell counts in fresh whole blood were assessed by three-color fluorescence kit for CD4/CD8/CD3 (BD Biosciences, San Jose, CA) on FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) within 8 hours. Multiset software was used for CD4/CD8/CD3 counts analysis.

### 4.2.4 Detection of plasma IL-27

Plasma IL-27 level was measured with pre-coated LEGEND MAX™ Human IL-27 ELISA Kit (BioLegend Inc., San Diego, CA) according to manufacturer's instructions. Standard curve was generated by using serial diluted standards. Each plate ran an independent standard curve for reference. Plasma IL-27 concentration was calculated by generated standard curve. The minimum detectable level of the ELISA kit was 11 pg/ml and the kit has no cross-reaction with other 14 human cytokines.

### 4.2.5 Quantification of HIV, HCV viral loads

HIV and HCV viral loads were measured among HIV mono-infected individuals and HIV/HCV co-infected individuals.

Plasma HIV RNA levels were measured with quantitative reverse polymerase chain reaction (COBAS AMPLICOR HIV-1 Monitor Test version 1.5, Roche Molecular Systems, Branchburg, NJ, USA) following manufacture's instructions as follow:

COBAS AMPLICOR HIV-1 Monitor procedures:

1. Pre-warm HIV-1 Monitor Lysis Buffer at room temperature to complete dissolve any crystal formed.

2. Prepare Working Lysis Buffer: put 100 µl vortexed HIV-1 Monitor Quantitation Standard (HIV-1 QS) into a pre-warmed HIV-1 Monitor Lysis Buffer tube.

3. Freshly prepared 70% ethanol: add 6 ml deionized water into 14 ml ethyl alcohol and vortex.

4. Add 600 µl Working Lysis Buffer into each sample tube.

5. Add 200 µl Cobas Amplicor Normal Human Plasma (NHP) into each HIV-1(-) control, HIV-1 L (+) control HIV-1 H (+) control tubes and vortex.

6. Add 200 µl vortexed human plasma samples into each sample tube and vortex.

7. Add 50 n µl vortexed HIV-1 (-) C, HIV-1 L (+) C and HIV-1 H (+) C into each control tuber and vortex.

8. Incubate each tube for 10 minutes in room temperature.

9. Add 800 µl isopropano into each tube and vortex.

10. Centrifuge tubes at 13 000 – 16 000 g, room temperature for 15 minutes.

11. Remove upper liquid without disturbing sediment.

12. Add 1 ml fleshly prepared 70% ethanol to each tube and vortex.

13. Centrifuge tubes at 13 000 – 16 000 g, room temperature for 5 minutes.

14. Remove upper liquid without disturbing sediment.

15. Add 400 µl HIV-1 Monitor Specimen Diluent into each tube.

16. Vortex for 10 seconds, and adds 50 µl prepared samples into reaction tube.

17. Prepare PCR reaction mix: vortex HIV Monitor $Mn^{2+}$, add 100 µl vortexed $Mn^{2+}$ into HIV Monitor Master Mix and mix upside down, vortex is prohibited.

18. Add 50 PCR reaction mix into each reaction tube, gently mix and centrifuge.

19. Prepare IM probe: vortex IM PS1 for 10 seconds, add 2.5 ml IM PS1 into IM4, mix by pipetting.

20. Prepare IQ reference Probe: vortex IQ PS1 for 10 seconds. Add 2.5 ml IQ PS1 into IQ4 and pipetting.

21. Prepare substrate SB: add 5 ml SB into SB3 to prepare substrate, mix by pipetting.

22. Amplify HIV specific fragment by COBAS@ TaqMan@ analyzer.

23. Batch validation: Check AMPLILINK software results to ensure the batch is valid.

24. Interpretation of results: if the calculated HIV titer is below the limit of detection, report as ”HIV RNA detected, less than 48 HIV RNA copies/ml”; if the calculated titer is within detection range, report the calculated data; if the calculated HIV titer above the detection range, dilute and repeat the test.

The kit can detect HIV-1 RNA ranges over 48 to 10, 000, 000 copies/ml.

Plasma HCV RNA was quantified by using a commercial quantitative RT-PCR kit (Shenzhen PG Biotech Co., Ltd. Shenzhen, China) according to instructions as follow:

1. Add 150 μl Lysis buffer into each tube.

2. Add 50 μl EDTA-coagulated human plasma samples, negative control,

positive control and cut-off positive control into each tube, mix by pipetting.

3. Add 50 µl Chloroform into each tube, vortex for 5 seconds or mix by inverting for 15 times.

4. Centrifuge at 13 000 rpm for 15 minutes.

5. Add 100 µl isopropanol into new tubes, and move the upper liquid produced in step 4 into the new tubes, mix by inverting.

6. Centrifuge at 13 000 rpm for 15 minutes, gently remove upper liquid and reversely lay onto tissue to absorb liquid.

7. Add 300 µl freshly prepared 75% ethanol, mix by inverting.

8. Centrifuge at 13 000 rpm for 15 minutes, gently remove upper liquid and reversely lay onto tissue to absorb liquid.

9. Centrifuge at 2000 rpm for 5 seconds, remove liquid by pipetting, and dry in room temperature for 1 – 5 minutes.

10. Dissolve sediment with 15 µl RNase free water, gently mix and centrifuge for 5 seconds and keep the extracted RNA in icy water.

11. Add 34.1 µl RT-PCR reaction buffer, 0.5 µl RT-PCR enzyme, 0.4 µl PCR Enhancer and 15 µl extracted RNA samples into reaction tube.

12. RT-PCR: 42°C: 30 min; 95°C: 3 min;
    95°C: 10sec; 55°C: 30sec; 72°C: 60sec; 5 cycles;
    95°C: 5 sec; 60°C: 30sec; 42 cycles.

The reaction volume is 50 μl, collect fluorescent signal at 60°C.

13. The RT-PCR was carried out in ABI-7300 system Real-Time PCR system.

14. Quality control: use quantitative standards to evaluate the batch validation.

15. Use negative, cut-off positive and positive controls to evaluate the batch validation.

16. Interpretation of results: if the calculated HCV titer is among the detection range, report the data; if the calculated data is below the detection range, reported as less than the lower detection limit and for reference only; if the calculated data is over the upper detection limit, dilute and repeat the test.

The HCV RNA kit displayed linear range of $10^3$ – $10^7$ IU/ml. Dilution was conducted when necessary.

### 4.2.6 Statistical analysis

Data was analyzed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Histogram was applied to assess the distribution of data. Independent-samples *T* test was used for comparison of plasma IL-27 between HIV-infected samples and healthy controls. Pearson's correlation coefficient was applied to test the correlation between plasma IL-27 titer and $CD4^+$ T cell counts in HIV-positive groups. Spearman's rank correlation was used to test the associations between IL-27 titer and HIV viral load, HCV viral load, and $CD4^+$ T cell counts. *P* value $< 0.05$ was considered as statistically significant.

## 4.3 Results

### 4.3.1 Demographics of study participants

In the study, after excluding HBsAg positive individuals, totally 155 antiretroviral therapy-naïve Chinese were recruited. Among them, 80 were HIV, HBV and HCV

negative healthy controls and 45 participants were HIV mono-infected, 30 were HIV/HCV co-infected. The averages of age were 28 years, 30 years and 32 years for healthy control group, HIV mono-infection group and HIV/HCV co-infection group, respectively (Table 4-1). In HIV mono-infection and HIV/HCV co-infection groups, 84.4% and 90.0% individuals were male, while 75.5% were male in healthy control group.

### 4.3.2 Plasma IL-27 was elevated in HIV-positive persons

Plasma IL-27 levels were measured by ELISA. The IL-27 levels were between 27 pg and 890 pg per ml in the control group, whereas the IL-27 levels were ranged from 188 pg to 1539 pg per ml in HIV mono-infected group, 135 pg to 1229 pg per ml for HIV/HCV co-infected persons. As shown in Figure 4-1, compared to healthy controls, IL-27 titer was significantly upregulated in HIV-infected individuals (HIV mono-infection Vs control: 675 ± 334 versus 410 ± 226 pg/ml, mean ± SD, $P < 0.001$; HIV/HCV co-infection Vs Control: 678 ± 300 versus 410 ± 226 pg/ml, $P < 0.001$). While, there was no significant difference between HIV mono-infection and HIV/HCV co-infection individuals (675 ± 334 versus 678 ± 200 pg/ml, $P = 0.969$).

### 4.3.3 Correlation of IL-27 titer and $CD4^+$ T cell counts

As plasma IL-27 titers and $CD4^+$ T cell counts were normally distributed in target populations, Pearson's correlation coefficient was applied to test the correlation between IL-27 and $CD4^+$ T cell counts in HIV mono-infected group and HIV/HCV co-infected group. As illustrated in Figure 4-2a, a moderate positive correlation between $CD4^+$ T cell counts and plasma IL-27 titers was observed among HIV mono-infected individuals ($r = 0.171$, $P = 0.261$; Fig. 4-2a); while for HIV/HCV co-infected people, there was a moderate negative correlation between IL-27 titer and $CD4^+$ T cell counts ($r = - 0.198$ , $P = 0.294$; Fig. 4-2b).

### 4.3.4 Correlation of HIV viral load and IL-27 titer

As HIV viral load distribution was skewed, Spearman's rank correlation was applied

in assessing the associations between HIV quantity, plasma IL-27 titer and $CD4^+$ T cell counts. As shown in Figure 4-3a, a noteworthy negative clinical trend was observed between HIV viral load and $CD4^+$ T cell counts among HIV positive Chinese (r = - 0.205, $P$ = 0.078). As HIV viral load distribution in common logarithm in both HIV mono-infection and HV/HCV co-infection groups were normal, independent-samples $T$ test showed that HIV titer between HIV mono-infection group (a mean of 4.50 $\log_{10}$ copies/ml) and HIV/HCV co-infection group (a mean of 4.37 $\log_{10}$ copies/ml) was not significantly varied ($P$ = 0.773, Fig. 4-3b).

HIV viral loads were further plotted with IL-27 titers; no significant correlation between HIV quantity and IL-27 was found among HIV mono-infected individuals (r = - 0.063, $P$ = 0.679; Fig.4-3c); while a significant positive correlation was observed between HIV viral load and IL-27 titer in HIV/HCV co-infected individuals (r = 0.362, $P$ = 0.049; Fig.4-3d).

### 4.3.5 Correlation of HCV viral load and IL-27 titer

HCV viral load in HIV/HCV co-infection individuals were skewed distributed; we apply Spearman's rank correlation to assess the associations between HCV viral load, HIV viral load, plasma IL-27 titer and $CD4^+$ T cell counts.

As stated in Fig. 4-4a, although there is lacking of linear correlation between HIV and HCV viral loads (r = - 0.072, $P$ = 0.704); individuals in HIV/HCV co-infection group can be divided into three subgroups, (a) people with high HIV viral load and low HCV viral load subgroup, (b) people with low HIV and HCV viral loads, and (c) people with high HCV viral load and low HIV viral load subgroup. IL-27 titer in high HIV and low HCV viral loads subgroup was significantly higher than in high HCV and low HIV viral loads subgroup (High HIV and low HCV Vs High HCV low HIV: 900 ± 214 versus 576 ± 219 pg/ml, mean ± SD, $P$ = 0.014). HCV viral load was also analyzed with IL-27 titer among HIV/HCV co-infected people, no significant correlation was observed among HCV quantity and IL-27 titer (r = - 0.119, $P$ =

0.530; Fig.4-4b). When $CD4^+$ T cell counts was plotted with HCV viral load, no significant correlation was observed (r = - 0.116, *P* = 0.541; Fig.4-4c).

## 4.4 Discussion

Along with other sexual transmitted diseases (STDs), the HIV/AIDS epidemic in China continuous worsen in the past 25 years. (Yang, Li et al. 2005). In 2011, the estimated number of people lived with HIV/AIDS in China was 780,000, and AIDS-related death was 28, 000 (Ministry of Health of China, UNAIDS et al. 2011). As HIV, HCV share routes of transmission, the prevalence of HIV/HCV co-infection is remarkably high, especially among IDUs (Tan, Wei et al. 2008). Previously, we reported plasma IL-27 titer was significantly elevated in HIV mono-infected antiretroviral therapy-naïve Chinese, and IL-27 titer was positively correlated with $CD4^+$ T cell counts (He, Zhao et al. 2011). In this report, we further recruited 45 HIV mono-infected and 30 HIV/HCV co-infected antiretroviral therapy-naïve Chinese to elucidate how HIV, HCV viral loads affect IL-27 expression.

As HBV infection enhances IL-27 expression both *in vitro* and *in vivo* (Zhu, Zhang et al. 2009), HBsAg positive individuals were excluded from the study. Compared to healthy controls, plasma IL-27 titer in HIV mono-infected and HIV/HCV co-infected individuals were evidently elevated, while HCV co-infection has no significant impact on IL-27 expression level among HIV positive people. Upon immune activation, IL-27 is produced by antigen-presenting cells (Pflanz, Timans et al. 2002); plasma IL-27 expression was also elevated in HIV positive patients (Guzzo, Hopman et al. 2010; He, Zhao et al. 2011). Guzzo reported HCV co-infection suppresses IL-27 expression in HIV positive individuals (Guzzo, Hopman et al. 2010). In our study, no difference was observed between HIV mono-infection and HIV/HCV co-infection. This may be due to different sample characteristics, as our HIV/HCV co-infection individuals were antiretroviral therapy-naïve and with $CD4^+$ T cell count range between 122 – 1057 cells/μl. Stacey's study also proved HIV is stronger than HBV and HCV in inducing immune activation, which may indicate

HIV plays the main role in immune responses under HIV/HCV co-infection circumstances (Stacey, Norris et al. 2009).

As IL-27 promotes rapid clonal expansion of naïve $CD4^+$ T cells, $CD4^+$ T cell counts were plotted with IL-27 titer in the study (Pflanz, Timans et al. 2002). In HIV mono-infected group, although the statistical power is not as strong as previous result due to decreased sample size, there is a moderate positive clinical relevance between IL-27 and $CD4^+$ T cell counts which further proves that IL-27 counterattack HIV infection by promoting naïve $CD4^+$ T cell proliferation (He, Zhao et al. 2011). For HIV/HCV co-infection group, there is a moderate negative clinical relevance between IL-27 and $CD4^+$ T cell counts. The $CD4^+$ T cell counts in HIV mono-infected group (ranged from 35 to 927 cells/μl with a mean of 412 cells/μl) and HIV/HCV co-infected group (ranged from 122 to 1057 cells/μl, with a mean of 400 cells/μl) was not significantly varied (Student's *t* test, $P = 0.815$). Reports showed HCV co-infection with HIV had no obvious impact on $CD4^+$ T cell decrease, while it significantly elevated AIDS-defining illnesses (ADIs) probability when compared to HIV mono-infection, and the suggested mechanisms include the inhibited $CD4^+$ T cell proliferation, impaired immune recovery, and promotion of AIDS onset by HCV-induced apoptosis (Miller, Haley et al. 2005; d'Arminio, Cozzi-Lepri et al. 2009; Kondo, Machida et al. 2009). In our study, although the $CD4^+$ T cell counts is similar in the two groups, HCV-induced inhibition of $CD4^+$ T cell proliferation may exist and the immune impairment may severe for HIV/HCV co-infection group, which will induce low $CD4^+$ T cell counts, high HIV viral load and high IL-27 titer phenomena.

In our study, we found that HIV viral load inversely correlated with $CD4^+$ T cell counts among HIV positive antiretroviral therapy-naïve Chinese, which further proved HIV is the primary driving force for immune dysfunction (Zhang, Xu et al. 2008). As our study groups were in relatively complete immune status, HIV viral loads in HIV mono-infection group did not significantly differ from HIV/HCV co-

infection individuals.

HIV infection sensitizes antigen-presenting cells, which secretes IL-27 to combat invaded HIV depending on its anti-inflammatory properties, and restore intact immune system by boosting naïve $CD4^+$ T cells (Pflanz, Timans et al. 2002; Fakruddin, Lempicki et al. 2007; Imamichi, Yang et al. 2008; Greenwell-Wild, Vazquez et al. 2009). In this anti-HIV feedback arc set, IL-27 acted as mediator therefore it is increased when HIV replicates and decreased when HIV replication was prohibited. This feedback arc set explains the lack of linear correlation between HIV viral load and IL-27 titer in HIV mono-infected individuals as they have comparatively intact immune system. While for HIV/HCV co-infected people, this anti-HIV feedback arc set was interrupted. Although secreted IL-27 promotes rapid clonal expansion of naïve $CD4^+$ T cells, HCV co-infection impairs $CD4^+$ T cell restoration and affects the integrity of immune system, which then results in uncontrolled or less effective inhibition of HIV replication (Miller, Haley et al. 2005; Stebbing, Waters et al. 2005; d'Arminio, Cozzi-Lepri et al. 2009; Kondo, Machida et al. 2009). So, in the interrupted anti-HIV feedback arc set, high HIV viral load means high IL-27 titer in plasma with low existence of $CD4^+$ T cell counts. This interprets the positive correlation among HIV viral load and IL-27 level in HIV/HCV co-infection group. As IL-27 plays key pathogenic role in T-cell mediated hepatitis, high IL-27 with high HIV viral load in HIV/HCV co-infection individuals may elevate liver-related morbidity and mortality (Monga, Rodriguez-Barradas et al. 2001; Rockstroh, Mocroft et al. 2005; Siebler, Wirtz et al. 2008). In the study, based on antiretroviral therapy-naïve Chinese, we clearly stated the relationship between HIV viral load and plasma IL-27 titer in HIV mono-infection and HIV/HCV co-infection individuals. Our results were different from Guzzo's report which may resulted from different sample characteristics including antiretroviral therapy, HCV co-infection and sample size (Guzzo, Hopman et al. 2010).

When plotted with HIV viral load, HCV viral load was not linearly correlated in HIV/HCV co-infected individuals. Among HIV/HCV co-infection individuals, the three subgroups may represent three separate circumstances. In high HIV and low HCV viral load subgroup, high titer IL-27 elicited by high HIV viral load may in turn inhibit HCV replication, making HIV the main factor for disease progression and resulting in AIDS-defining illnesses (Stebbing, Waters et al. 2005; Bender, Wiesinger et al. 2009; d'Arminio, Cozzi-Lepri et al. 2009; Frank, Zhang et al. 2010; Schoenherr, Weiskirchen et al. 2010). In high HCV viral load and low HIV viral load people, high HCV viral load may eventually increase liver-related morbidity and mortality while not affecting HIV disease progression as low level of IL-27 is produced (Monga, Rodriguez-Barradas et al. 2001; Chen, Ding et al. 2009). And for low HIV and HCV viral loads subgroup, individuals may be at early HIV/HCV infection status with relatively complete immune system, elicited IL-27 in turn inhibits HIV and HCV (Fakruddin, Lempicki et al. 2007; Imamichi, Yang et al. 2008; Bender, Wiesinger et al. 2009; Greenwell-Wild, Vazquez et al. 2009; Frank, Zhang et al. 2010; Schoenherr, Weiskirchen et al. 2010). These subgroups may well explain the controversial results regarding the effect of HCV co-infection in HIV diseases progression and the morbidity and mortality of AIDS patients (Monga, Rodriguez-Barradas et al. 2001; Miller, Haley et al. 2005; Rockstroh, Mocroft et al. 2005; Stebbing, Waters et al. 2005; Zhang, Xu et al. 2008; Chen, Ding et al. 2009; d'Arminio, Cozzi-Lepri et al. 2009). In our study, it showed lack of correlation between HCV viral load and plasma IL-27 titer which further suggested that HIV is stronger in inducing immune activation than HCV (Stacey, Norris et al. 2009).

The correlations between IL-27 and $CD4^+$ T cell counts were not statistically significant in our study, this may partly attributed to small sample size. A large sample size may be needed for further elucidation. In the case of HIV/HCV co-infection individuals, although we quantified HIV and HCV viral loads, liver enzyme alanine aminotransferase and aspartate aminotransferase levels were not evaluated,

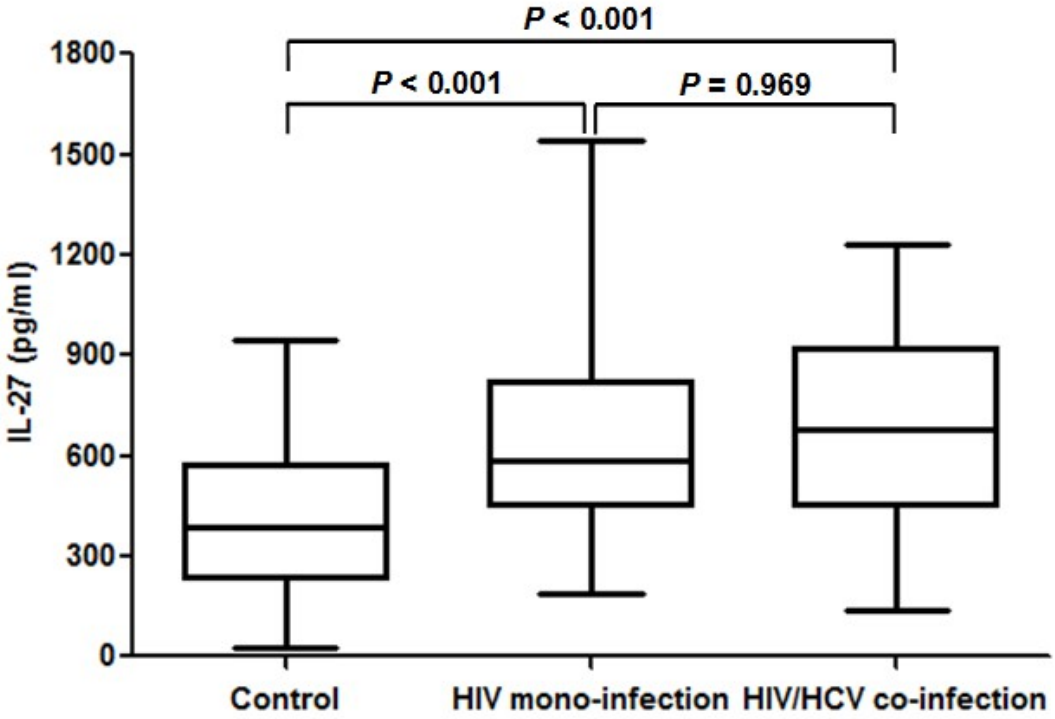
leaving liver damage status uncharacterized. The lack of liver damage characteristics hinders our further analysis of HIV viral load and IL-27 titer on liver damage progression. As we only take a spot look of HIV, HCV viral loads and IL-27 titer in the study, HIV and HCV disease progressions were not analyzed; a follow-up study with larger sample size is needed to further confirm our findings that HCV co-infection significantly altered HIV infection characteristics.

We further proved that plasma IL-27 was elevated in HIV/HCV co-infected people. HCV co-infection altered the correlation between HIV viral load, IL-27 titer and $CD4^+$ T cell counts. We also observed three obvious subgroups with distinct HIV and HCV viral loads. In conclusion, our results suggest IL-27 differs in HIV mono-infection and HIV/HCV co-infection clinical settings, which could be a critical point to be considered in HIV infection and HIV/HCV co-infection treatment with the cytokine.

**Table 4-1. Demographic characteristics of participants**

| Characteristics | Control | HIV mono-infection | HIV/HCV co-infection |
|---|---|---|---|
| Number | 80 | 45 | 30 |
| Age (Mean ± SD, years) | 28 ± 6 | 30 ± 5 | 32 ± 8 |
| Gender (Male/Female) | 62/18 | 38/7 | 27/3 |
| IL-27 (Mean ± SD, pg/ml) | 410 ± 226 | 675 ± 334 | 678 ± 300 |
| CD4 count (Mean ± SD, cells/µl) | | 412 ± 229 | 400 ± 208 |
| HIV viral load (copies/ml) | | | |
| Median | | 25800 | 38100 |
| Range | | 338 - 578000 | 260 - 446000 |

**Fig. 4-1. IL-27 titer in HIV positive individuals and healthy controls.**




Plasma IL-27 concentration was measured in 80 healthy controls, 45 HIV mono-infected persons and 30 HIV/HCV co-infection persons by ELISA. Box-plot illustrated the medians with 25% and 75%, error bars indicate 5% and 95% percentiles.

**Fig. 4-2. Correlation of IL-27 and $CD4^+$ T cell counts.**

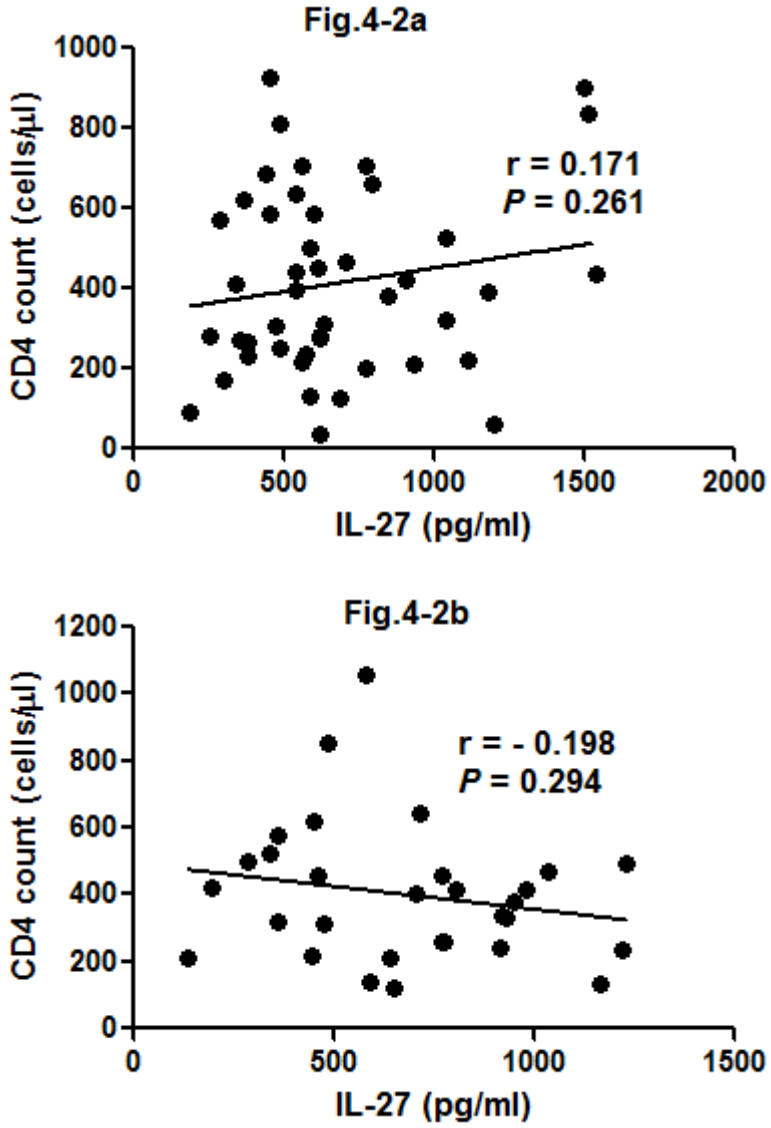
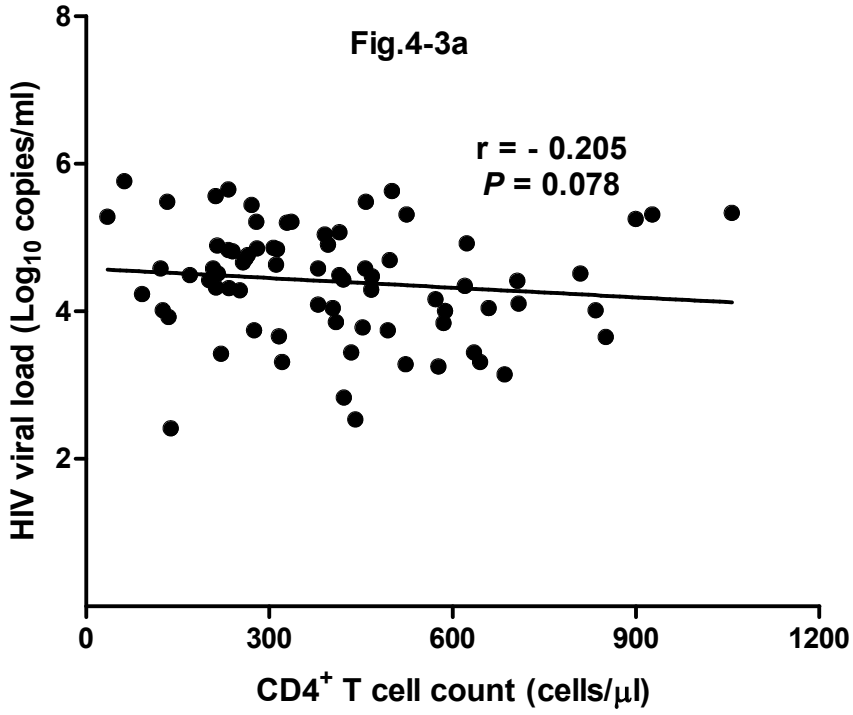



Fig.4-2a, the association between IL-27 and $CD4^+$ T cell counts in HIV mono-infected individuals. Fig.4-2b, the association between IL-27 and $CD4^+$ T cell counts in HIV/HCV co-infected individuals. X axis indicates the plasma IL-27 titer (pg/ml); Y axis indicates the $CD4^+$ T cell counts (cells/μl).

**Fig. 4-3. Correlation of HIV viral load and IL-27 titers.**



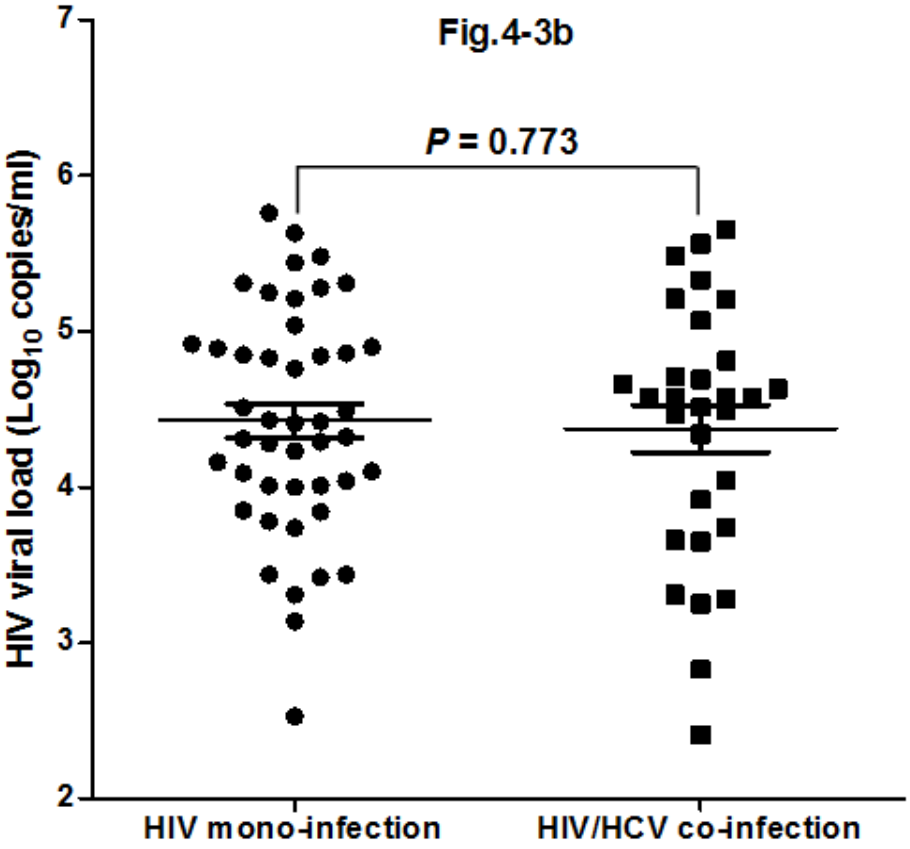


Fig.4-3b
P = 0.773
HIV viral load (Log10 copies/ml)
7
6
5
4
3
2
HIV mono-infection
HIV/HCV co-infection

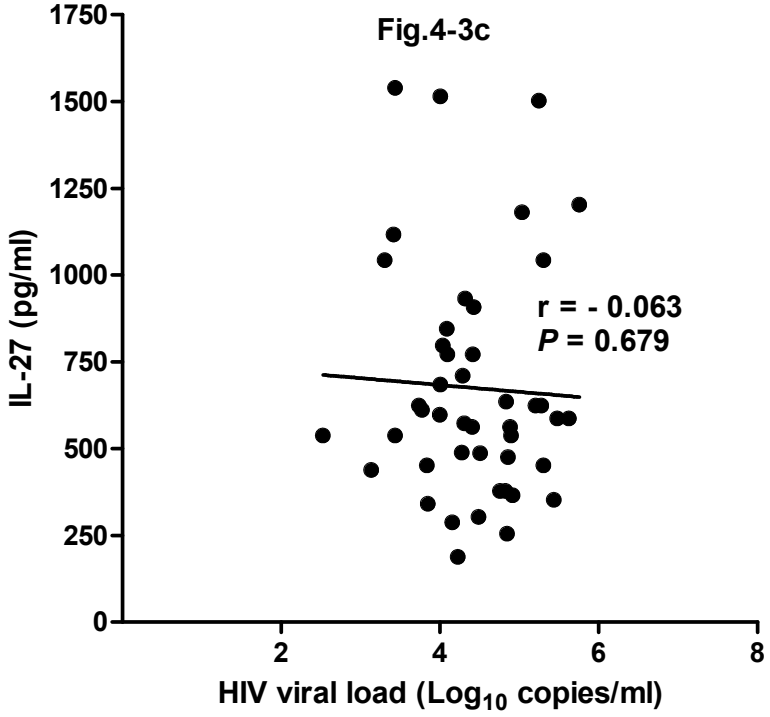


Fig.4-3c
IL-27 (pg/ml)
1750
1500
1250
1000
750
500
250
0
r = - 0.063
P = 0.679
2
4
6
8
HIV viral load (Log10 copies/ml)

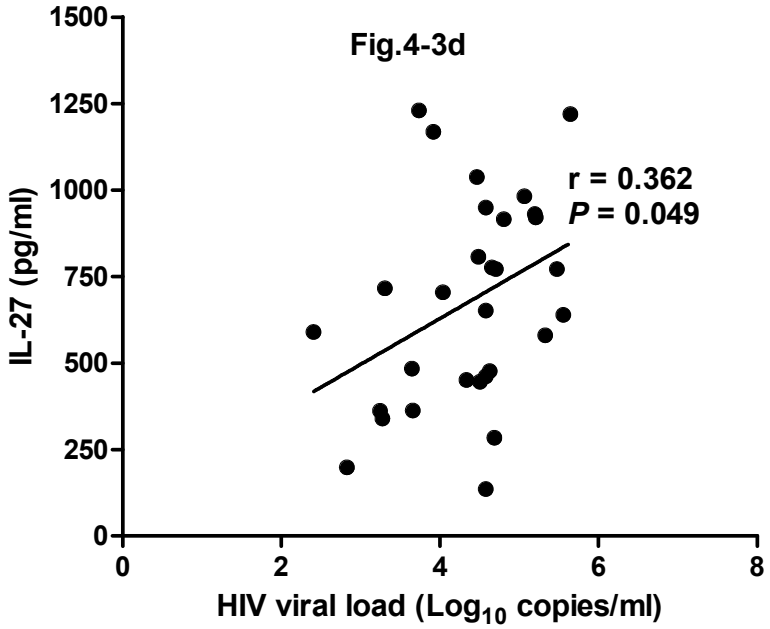
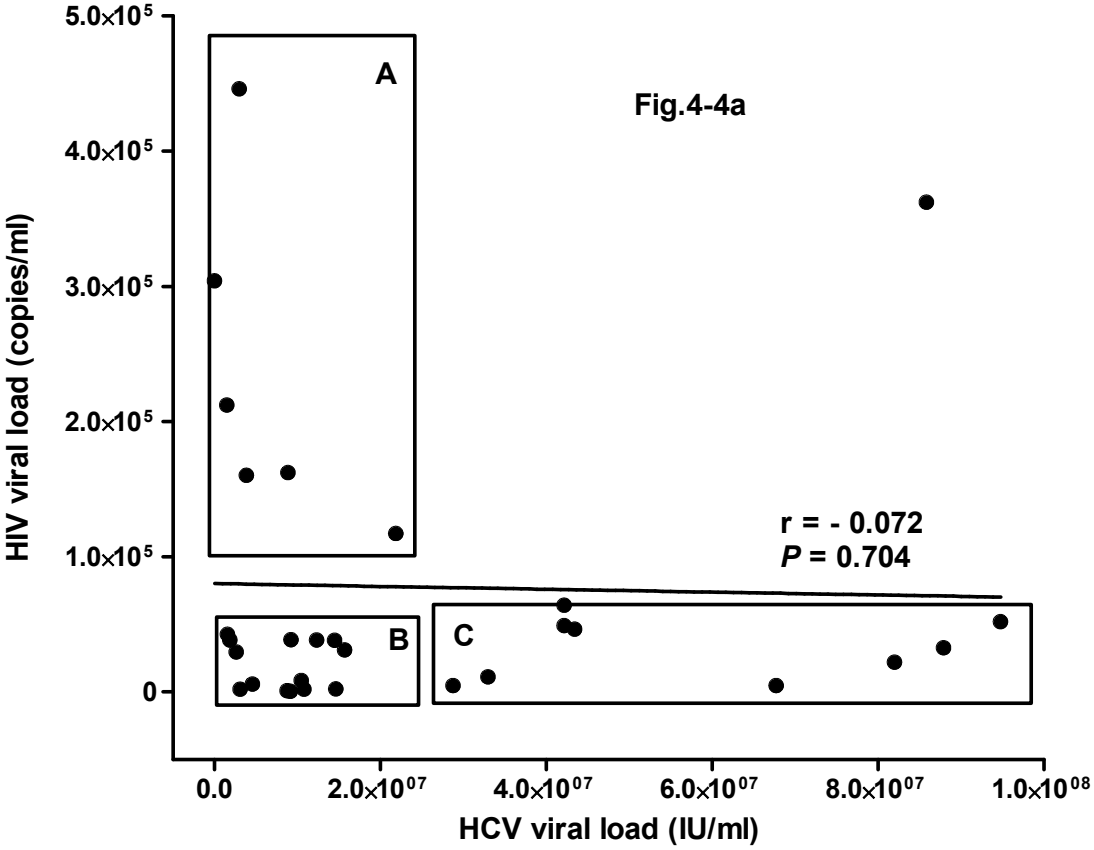
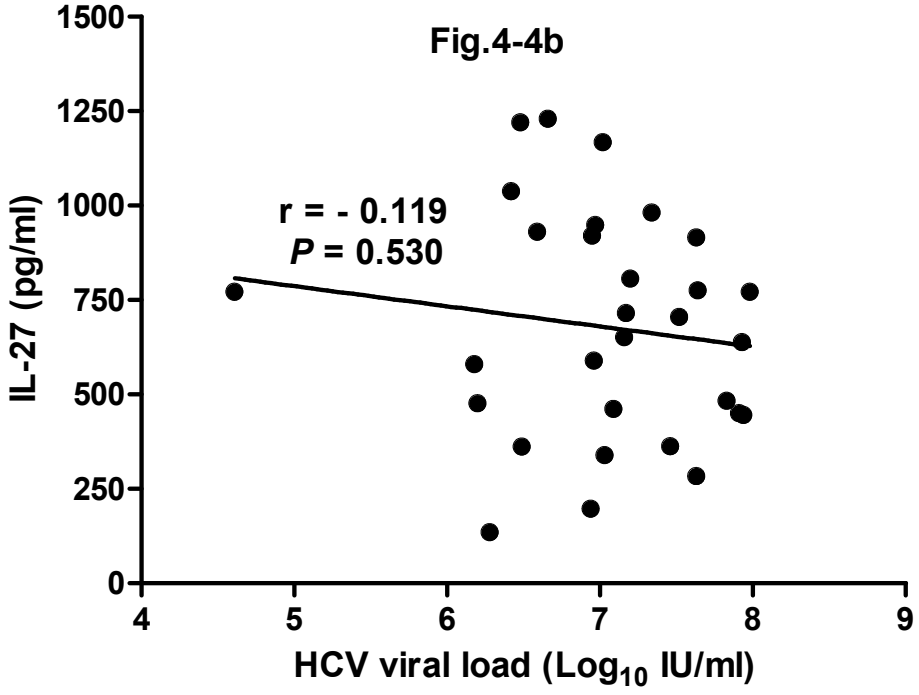



Fig.4-3a, the association of HIV viral load and $CD4^+$ T cell counts among HIV positive antiretroviral therapy-naïve Chinese. X axis indicates the $CD4^+$ T cell counts (cells/μl), Y axis indicates the HIV viral load ($Log_{10}$ copies/ml). Fig.4-3b, the comparison of HIV viral loads between HIV mono-infection and HIV/HCV co-infection group. Y axis indicates the HIV viral loads ($Log_{10}$ copies/ml). Fig.4-3c and Fig.4-3d, the correlations of HIV viral loads and plasma IL-27 titers in HIV mono-infection and HIV/HCV co-infection people. X axis indicate the HIV viral load ($Log_{10}$ copies/ml), Y axis indicate the IL-27 titer (pg/ml).

**Fig. 4-4. Correlation of HCV viral load and IL-27 titers.**



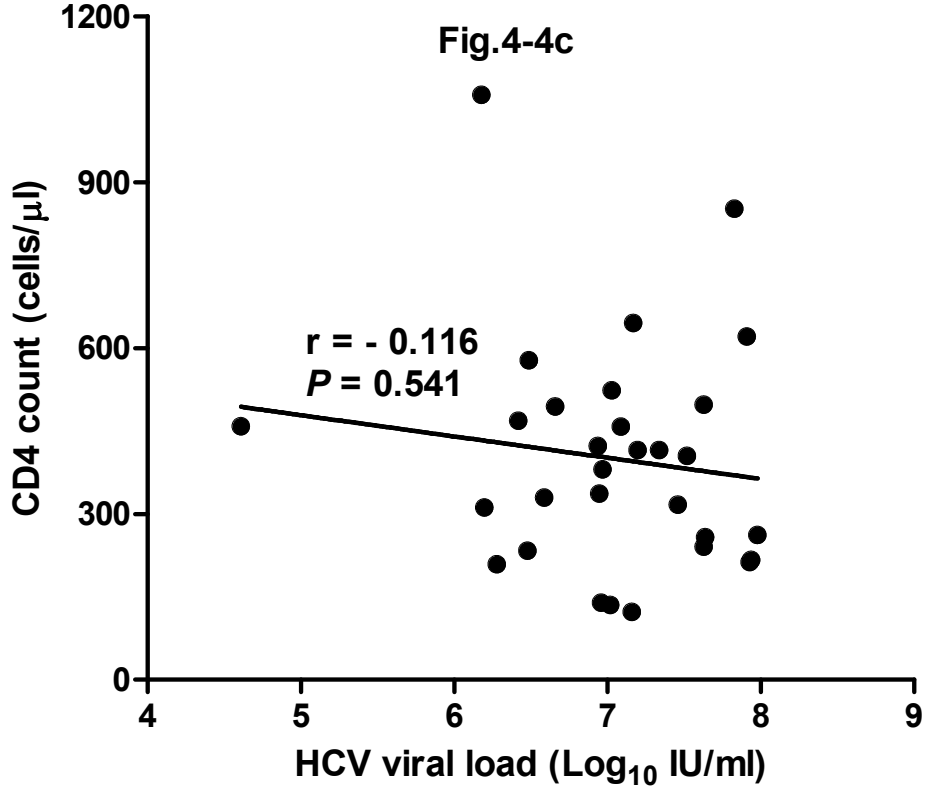



Fig.4-4a, the correlation of HCV viral load and HIV viral load. X axis indicates the HCV viral load (IU/ml).Y axis indicates the HIV viral load (copies/ml). Three boxes A, B and C represent three subgroups, high HIV and low HCV viral loads subgroup, low HIV and HCV viral loads subgroup, high HCV and low HIV viral loads subgroup, respectively. Fig.4-4b, the relationship between HCV viral loads and IL-27 expression levels. X axis indicates the HCV viral load ($Log_{10}$ IU/ml).Y axis indicates the IL-27 titer (pg/ml). Fig.4-4c, the relationship between HCV viral load and $CD4^+$ T cell count in HIV/HCV co-infected individuals. X axis indicates the HCV viral load ($Log_{10}$ IU/ml).Y axis indicates the $CD4^+$ T cell count (cells/μl).

# Chapter 5

# Association of Interleukin-27 polymorphisms with the susceptibility to HIV infection in a Chinese men who sex with men population

## 5.1 Introduction

Human Immunodeficiency Virus (HIV), which belongs to the family *Retroviridae,* is the causative agent of Acquired Immunodeficiency Syndrome (AIDS) (Sierra, Kupfer et al. 2005). HIV infection is characterized by progressive destruction of $CD4^+$ T lymphocytes and dysregulation of cytokine production (Altfeld, Addo et al. 2000; Douek, Brenchley et al. 2002). In 2009, an estimated 33.3 million people were lived with HIV and 1.8 million were died of AIDS-related diseases globally (UNAIDS 2010). The HIV/AIDS epidemic has been a global public health challenge.

Susceptibility to HIV infection is partially determined by host genetic factors. Innate immunity gene polymorphisms, HIV receptors and coreceptors genetic variants, and endogenous antiretroviral gene single nucleotide polymorphisms (SNPs) were associated with HIV susceptibility as these genes participate in the early elimination and control of HIV infection (Soriano, Lozano et al. 2005; Fellay, Shianna et al. 2007; Naicker, Werner et al. 2009; Oyugi, Vouriot et al. 2009). Interleukin-27 (IL-27) is a heterodimeric cytokine composed of Epstein-Barr virus-induced gene 3 (EBI3) protein and p28 protein and belongs to IL-6/IL-12 cytokine family (Pflanz, Timans et al. 2002). IL-27 function as an immunomodulator posses both pro-inflammatory and anti-inflammatory properties (Villarino, Huang et al. 2004). IL-27 inhibits X4 and R5

HIV replication in peripheral blood mononuclear cells, $CD4^+$ T cells and macrophages (Fakruddin, Lempicki et al. 2007). Three SNPs, -964A/G (rs153109), 2905T/G (rs17855750), and 4603G/A (rs181207) have been identified in IL-27 p28 gene and reported to be associated with the susceptibility to asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD) and chronic hepatitis B virus infection (Chae, Li et al. 2007; Huang, Liu et al. 2008; Li, Zhang et al. 2009; Wang, Zhu et al. 2009).

Homosexual behavior, specifically men who have sex with men (MSM), was risky for sexual transmitted diseases, including HIV. MSM was at high risk of HIV infection mainly because of multiple partners and unprotected anal intercourse (Liu, Yang et al. 2005). HIV/AIDS epidemic among MSM has been the gravest challenge as a large portion of MSM have unprotected sex with women, made them as a bridging group to heterosexual women who were presumed to have low risk of HIV infection (Xiao, Sun et al. 2010; Zou, Wu et al. 2010). Globally, MSM accounts for about 5% to 10% of all HIV/AIDS infections (Joint United Nations Program on HIV/AIDS (UNAIDS) 2004). In China, the proportion of HIV cases among MSM was increased eight folds from 0.4% in 2005 to 3.3% in 2007 (State Council AIDS Working Committee Office and UN Theme Group on AIDS in China 2007); percentage of homosexual transmitted HIV in new infections was 32.5% in year 2009 and 29.4% in 2011 (Ministry of Health of China, UNAIDS et al. 2011). In our previous study, 269 (29.4%) HIV positive person were mediated through homosexual behaviors, which was consistent with the national statistic. Shenzhen, a special economic zone bordering to Hong Kong, together with its huge immigrant workers, expanded the MSM population (Liu, Liu et al. 2009). In Shenzhen, from 2005 to 2007, HIV transmitted through homosexual/bisexual has been risen from 3.9% in 2005, to 15.7% in 2007; the prevalence of HIV infection among male sex workers in Shenzhen of year 2008 was 5.3% (Chen, Wang et al. 2009; Cai, Zhao et al. 2010). HIV prevalence in Shenzhen may be worsening as some MSM also sell commercial sex to female and have unprotected sex with their female partners.

To our knowledge, no studies to date have investigated the possible correlation of IL-27 p28 polymorphisms with individual susceptibility to HIV infection. As Chinese

MSM is 45-fold high of HIV susceptibility than other males in the general population, MSM is a suitable population for HIV susceptibility study (Baral et al., 2007).

The primary aim of this study was to determine the possible association of IL-27 p28 polymorphisms with the susceptibility to HIV infection in a Chinese MSM population.

## 5.2 Materials and Methods

### 5.2.1 Study participants

The study participants were recruited from the time-location MSM sampling scheme from April 2008 to July 2008 and May 2009 to November 2009 in Shenzhen. The criteria for subject selection including biologically male, aged 18 years or above; have homosexual contact in Shenzhen during the past six months before the survey and agree to join the scheme by completing the informed consent. Blood was drawn by experienced research staff for HIV screening. Confirmed HIV positive participants were referred to HIV treatment programs. All HIV infection cases were recorded according to the national requirement of reportable infectious diseases.

The study protocol was approved by Shenzhen Center for Disease Control and Prevention and The Chinese University of Hong Kong.

### 5.2.2 HIV screening

Participants were blooded with anticoagulant and centrifuged at 1000 rpm for 10 minutes; plasma and whole blood cells were gathered and stored at -80°C for further use. HIV screening was carried following the standard procedures of Shenzhen Center for Disease Control and Prevention. Plasma was screened by ABBOTT PRISM HIV O Plus (Abbott Laboratories, IL) and ELISA (Beijing Wantai Biological Pharmacy Enterprise CO., LTD, Beijing) for HIV infection; HIV positive sample was further confirmed by HIV-1/2 western blot assay (HIV Blot 2.2 WB; Genelabs Diagnostics, Singapore).

### 5.2.3 Genomic DNA extraction

Genomic DNA was isolated from whole blood cells using QIAGEN QIAamp DNA Mini Blood Kit (Hilden, Germany) according to the following procedures:

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.

2. Add 200 µl sample to the microcentrifuge tube. Use up to 200 µl whole blood, plasma, serum, buffy coat, or body fluids, or up to 5 x $10^6$ lymphocytes in 200 µl PBS.

3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.

4. Incubate at 56°C for 10 min.

5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove rops from the inside of the lid.

7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1

without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

### 5.2.4 IL-27 p28 -964A/G, 2905T/G and 4603G/A genotyping

IL-27 p28 -964A/G, 2905T/G and 4603G/A genotypes were analyzed with TaqMan® Allelic Discrimination Assay. Each assay contains two allele-specific TaqMan® probes (labeled with either VIC® or 6-FAM™ dye) and a primer pair to detect the specific SNP target. Each TaqMan® probe incorporates the minor groove binder (MGB) group on the 3' end that allows for superior allelic discrimination. The MGB molecule binds to the minor groove of the DNA helix, improving hybridization-based assays by stabilizing the MGB-probe/template complex.

Primers and TaqMan MGB probes used for allelic discrimination assays in this study were as follows:

1. IL-27 p28 -964A/G:

Forward: GACCTGGTTGAGCCCTGAT

Reverse: AGCTCAGTCAGTGACCAGGAT

Wild-type: VIC-ACTCAACTCAAGCCTG-MGB

Mutant: FAM-ACTCAACTCGAGCCTG - MGB.

2. IL-27 p28 2905T/G:

Forward: TTCACAGTCAGCCTGCATCTC

Reverse: GCGCCAGCTGCATTAGG

Wild-type: VIC- CCTCGGAGAGCAGC –MGB

Mutant: FAM- CTCGGCGAGCAGC - MGB.

3. IL-27 p28 4603G/A:

Forward: AGGCTGGAGCGTGGAATG

Reverse: GCCAAGGATGGCCATATCACA

Wild-type: VIC- AAGGAGACCCGGATAGG –MGB

Mutant: FAM- AGGAGACCCAGATAGG - MGB.

PCR reaction was carried out using an ABI 7500 Real Time PCR System with the following conditions: 50 ºC for 2 min, 95 ºC for 10 min, then 40 cycles of 95 ºC for 15 s and 60 ºC for 60 s. The SNP was analyzed by ABI 7500 Real Time SDS software.

### 5.2.5 Statistical analysis

Data was analyzed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Histogram was applied to assess the distribution of data. Population age was analyzed by independent *T* test. Genotype and allele frequencies of subjects were examined by using Pearson $\chi^2$ test or Fisher's exact *T* test when feasible, odds ratio (OR) and 95% confidence intervals (CIs) were calculated to asses the relative risk of a particular genotype and allele. Hardy-Weinberg analysis was performed by comparing the observed and expected genotype frequencies using Pearson $\chi^2$ test or

Fisher's exact *T* test as appropriate. The linkage disequilibrium (LD) among the three polymorphisms, haplotypes and their frequencies were estimated by using SHEsis software (Li et al., 2009). A *P* value less than 0.05 was considered to be statistically significant, Bonferroni correction was applied where necessary.

## 5.3 Results

### 5.3.1 Demographics of study participants

In this study, 210 HIV positive and 240 healthy MSM were finally recruited from the sampling scheme (Table 5-1). The mean age for HIV-infected MSM and healthy controls were 30 years. No significant difference in mean age was observed between HIV positive MSM group and healthy MSM controls.

### 5.3.2 IL-27 genotypes and allele frequencies in HIV MSM and healthy MSM controls

Genotypes and allele frequencies of three IL-27 p28 polymorphisms -964A/G, 2905T/G, 4603G/A in HIV positive MSMs and healthy MSM controls were listed in Table 5-2.

As stated in Table 5-3, the genotype distributions of the three polymorphisms between HIV positive MSMs and healthy MSM controls were in Hardy-Weinberg Equilibrium.

For IL-27 p28 -964A/G, AA, AG and GG genotype proportions in HIV positive MSM group were 40.9%, 43.8% and 15.3%, respectively; in healthy MSM controls, genotype proportions were 37.1%, 49.6%, 13.3%, respectively. AA, AG and GG genotype proportions in HIV positive MSM group and healthy MSM controls were not statistically different (AG Vs AA: OR = 0.80, 95% CI: 0.54 – 1.20, $P = 0.277$; GG Vs AA: OR = 1.04, 95% CI: 0.58 – 1.84, $P = 0.907$). Allele A and G of IL-27 p28 -964A/G in HIV positive MSM person were 62.9% and 37.1%; allele A and G of IL-27 p28 -964A/G in HIV negative MSM person were 61.9% and 38.1%; they were not significantly different (OR = 0.96, 95% CI: 0.73 – 1.26, $P = 0.762$).

For IL-27 p28 3905T/G, TT, TG and GG genotype proportions in HIV positive MSM group were 90.0%, 10.0% and 0, respectively; in healthy MSM controls, genotype proportions were 75.8%, 23.3%, 0.8%, respectively. Allele T and G of IL-27 p28 2905T/G in HIV positive MSM person were 95.0% and 5.0%; allele T and G of IL-27 p28 2905T/G in HIV negative MSM person were 87.5% and 12.5%. A significant IL-27 p28 2905T/G genotype and allele frequencies differences were observed among HIV positive MSMs and healthy MSM controls. As illustrated in Table 5-2, when compared to healthy MSM control group, subjects with the TG genotype of IL-27 p28 2905T/G had a 2.77-fold decreased risk of HIV susceptibility (OR = 0.36; 95% CI: 0.21 – 0.62; $P < 0.001$). Consistantly, subjects with G allele of IL-27 p28 2905T/G had a 2.72-fold decreased risk of HIV susceptibility compared to healthy MSM controls (OR = 0.34: 95% CI: 0.22 – 0.62; $P < 0.001$).

For IL-27 p28 4603G/A, GG, GA and AA genotype proportions in HIV positive MSM group were 80.5%, 16.2% and 3.3%, respectively; in healthy MSM controls, genotype proportions were 82.1%, 17.1%, 0.8%, respectively. GG, GA and AA genotype proportions in HIV positive MSM group and healthy MSM controls were not statistically different (GA Vs GG: OR = 0.97, 95% CI: 0.59 – 1.59, $P = 0.894$; AA Vs GG: OR = 4.08, 95% CI: 0.84 – 19.90, $P = 0.089$). Allele G and A of IL-27 p28 4603G/A in HIV positive MSM person were 88.6% and 11.4%; allele G and A of IL-27 p28 4603G/A in HIV negative MSM person were 90.6% and 9.4%; they were not significantly different (OR = 1.25, 95% CI: 0.81 – 1.92, $P = 0.313$).

### 5.3.3 LD analysis and haplotype analysis

LD coefficients (|D'|) between all SNP pairs were calculated, and the LD was found between -964A/G and 2905T/G (|D'| = 0.942), and -964A/G and 4603G/A (|D'| = 0.930). Haplotype analyses were performed and the possible eight haplotype frequencies were shown in Table 5-4. In both HIV positive MSM and healthy MSM control groups, major ATG haplotype accounted for 62.0% and 61.2% among these haplotypes and no frequency difference was observed (OR = 1.03; 95% CI: 0.79 –

1.35; $P$ = 0.805); while the frequency of haplotype GGG (4.1% and 12.1%, respectively) was significantly different among the two groups (OR = 0.31; 95% CI: 0.18 – 0.55; $P < 0.001$), the difference remains significant even after Bonferroni correction.

## 5.4 Discussion

Individual susceptibility to HIV infection is genetically and immunogically dependant. Host genetic variants play crucial role in individual susceptibility to HIV cell entry and modulate immune responses (Kaslow, Dorak et al. 2005). As IL-27 inhibits HIV replication *in vitro* (Fakruddin, Lempicki et al. 2007; Imamichi, Yang et al. 2008; Greenwell-Wild, Vazquez et al. 2009), we hypothesized that IL-27 p28 polymorphisms may participate in restricting and controlling of HIV infection and affect the susceptibility to HIV infection. Due to penile-anal intercourse and increased unprotected high-risk sexual behaviors, the HIV susceptibility of Chinese MSM is 45-fold higher than that of other males in general population (Baral et al., 2007). From this high HIV susceptibility population, we successfully recruited 210 HIV positive MSMs and 240 HIV negative MSM controls with similar age to test the association of IL-27 p28 polymorphisms with susceptibility to HIV infection.

IL-27 is an early product of activated antigen-presenting cells, regulates early differentiation of T helper type I immune response and drives rapid clonal expansion of naive $CD4^+$ T cells (Pflanz, Timans et al. 2002). Recently, the immunomodulatory cytokine IL-27 was identified to play a key role in suppressing HIV replication in peripheral blood mononuclear cells, $CD4^+$ T cells and macrophages through the induction of type I interferon and activation of multiple interferon-inducible genes (Fakruddin, Lempicki et al. 2007; Imamichi, Yang et al. 2008; Greenwell-Wild, Vazquez et al. 2009). Studies from repeated exposion to HIV but non-infected commercial sex workers and HIV infected long-term non-progressors demonstrate that these HIV resistant individuals exhibit a biased T helper type I (Th1) immune responses thus lowering the susceptibility to HIV infection (Fowke, Nagelkerke et al.

1996; Easterbrook 1999; Ball, Ji et al. 2007). In the early HIV infection stage, APCs produced IL-27 may induce biased Th1 immune responses to eradicate HIV and to inhibit HIV replication *in vivo*. In this study, we analyzed the genotype and allele frequencies of three SNPs of IL-27 p28 gene between HIV positive MSM and healthy MSM control. We found that the genotype and allele frequency of IL-27 p28 2905T/G polymorphism in HIV positive MSM group was significantly different from healthy MSM controls. To induce biased Th1 immune response during early microbial infection, secreted IL-27 need to be combined with its heterodimeric receptor complex gp130 and WSX-1 to induce functional signal-transduction (Pflanz, Hibbert et al. 2004). IL-27 p28 2905T/G polymorphism is locates in exon 2 and the single nucleotide transition from T to G resultes in an amino acid transition of pSer59Ala, thus probably influences the affinity of IL-27 to its functional receptors and resultes in varied early HIV restriction and elimination results. This result suggests that IL-27 p28 2905T/G polymorphism might be associated with the HIV susceptibility. Although the frequency of major haplotype (ATG) was not significantly different between HIV positive MSM and healthy MSM controls, our findings suggest that people with GGG haplotype would be more resistant to HIV infection.

In the present study, we assessed the impact of IL-27 p28 polymorphisms on HIV susceptibility in Chinese MSM population, and observed significant differences of 2905T/G polymorphism genotype and allele frequencies, together with significant haplotype frequency difference between HIV positive MSM and healthy MSM controls. However, our study has some limitations. First, this is a preliminary study with relatively small sample size; larger sample size is requireded for further study. Second, IL-27 titer was not measured, so we can’t exclude the possibility that different IL-27 quantity may leads to the differences we observed. Third, further functional study was needed to explain the association of IL-27 p28 polymorphism with the affinity of IL-27 cytokine to its receptors.

In summary, to our knowledge, this is the first study to assess the possible association of IL-27 polymorphisms with HIV susceptibility. Our results prove that IL-27 p28 2905T/G polymorphism is associated with individual susceptibility to HIV infection and GGG haplotype has a protective role against HIV infection in MSM population.

**Table 5-1. Demographics of study participants**

| Characteristics | HIV | Control | *P* value |
|---|---|---|---|
| Number | 210 | 240 | |
| Age ( Mean ± SD) | 30 ± 8 | 30 ± 7 | 0.253 |

**Table 5-2. IL-27 p28 genotype and allele frequencies in study populations**

| Polymorphisms | HIV n = 210 (%) | Control n = 240 (%) | OR (95% CI) | *P* value |
|---|---|---|---|---|
| IL-27 -964A/G | | | | |
| Genotypes | | | | |
| AA | 86 (41.0) | 89 (37.1) | 1.00 (Ref) | |
| AG | 92 (43.8) | 119 (49.6) | 0.80 (0.54 – 1.20) | 0.277 |
| GG | 32 (15.2) | 32 (13.3) | 1.04 (0.58 – 1.84) | 0.907 |
| Alleles | | | | |
| A | 264 (62.9) | 297 (61.9) | 1.00 (Ref) | |
| G | 156 (37.1) | 183 (38.1) | 0.96 (0.73 – 1.26) | 0.762 |
| IL-27 2905T/G | | | | |
| Genotypes | | | | |
| TT | 189 (90.0) | 182 (75.8) | 1.00 (Ref) | |
| TG | 21 (10.0) | 56 (23.4) | 0.36 (0.21 – 0.62) | 0.001 |
| GG | 0 (0) | 2 (0.8) | NA | NA |
| Alleles | | | | |
| T | 399 (95.0) | 420 (87.5) | 1.00 (Ref) | |
| G | 21 (5.0) | 60 (12.5) | 0.37 (0.22 – 0.62) | 0.001 |
| IL-27 4603G/A | | | | |
| Genotypes | | | | |
| GG | 169 (80.5) | 197 (82.1) | 1.00 (Ref) | |
| GA | 34 (16.2) | 41 (17.1) | 0.97 (0.59 – 1.59) | 0.894 |
| AA | 7 (3.3) | 2 (0.8) | 4.08 (0.84 – 19.90) | 0.089 |
| Alleles | | | | |
| G | 372 (88.6) | 435 (90.7) | 1.00 (Ref) | |
| A | 48 (11.4) | 45 (9.3) | 1.25 (0.81 – 1.92) | 0.313 |

NA: not available as of null cell.

**Table 5-3. Hardy-Weinberg test result for genotype distribution of the study**

| SNP | Genotype | Observed (%) | Expected (%) | OR (95% CI) | *P* valu |
|---|---|---|---|---|---|
| HIV -964A/G | AA | 86 (41.0) | 83 (39.5) | 1.00 (Ref) | |
| | AG | 92 (43.8) | 98 (46.7) | 1.11 (0.73 – 1.67) | 0.641 |
| | GG | 32 (15.2) | 29 (13.8) | 0.94 (0.52 – 1.69) | 0.833 |
| Control -964A/G | AA | 89 (37.1) | 92 (38.3) | 1.00 (Ref) | |
| | AG | 119 (49.6) | 113 (47.2) | 0.92 (0.62 – 1.36) | 0.669 |
| | GG | 32 (13.3) | 35 (14.5) | 1.06 (0.60 – 1.85) | 0.844 |
| HIV 2905T/G | TT | 189 (90.0) | 189 (90.3) | 1.00 (Ref) | |
| | TG | 21 (10.0) | 20 (9.5) | 0.95 (0.50 – 1.82) | 0.882 |
| | GG | 0 (0) | 1 (0.3) | NA | NA |
| Control 2905T/G | TT | 182 (75.8) | 183 (76.6) | 1.00 (Ref) | |
| | TG | 56 (23.4) | 53 (21.9) | 0.94 (0.61 – 1.44) | 0.782 |
| | GG | 2 (0.8) | 4 (1.5) | 2.00 (0.36 – 11.00) | 0.685 |
| HIV 4603G/A | GG | 169 (80.5) | 164 (78.4) | 1.00 (Ref) | |
| | GA | 34 (16.2) | 43 (20.3) | 1.31 (0.79 – 2.15) | 0.297 |
| | AA | 7 (3.3) | 3 (1.3) | 0.44 (0.11 – 1.74) | 0.338 |
| Control 4603G/A | GG | 197 (82.1) | 197 (82.1) | 1.00 (Ref) | |
| | GA | 41 (17.1) | 41 (17.0) | 1.00 (0.62 – 1.61) | 1.000 |
| | AA | 2 (0.8) | 2 (0.9) | 1.00 (0.14 – 7.17) | 1.000 |

NA: not available as of null cell.

**Table 5-4. The IL-27 p28 haplotype frequencies in the study populations**

| IL-27 haplotypes (-964/2905/4603) | HIV 2n = 420 (%) | Control 2n = 480 (%) | OR (95% CI) | *P* value |
|---|---|---|---|---|
| ATG | 261 (62.0) | 294 (61.2) | 1.03 (0.79 – 1.35) | 0.805 |
| GTG | 93 (22.1) | 82 (17.0) | 1.39 (1.00 – 1.93) | 0.053 |
| GTA | 44 (10.5) | 43 (8.9) | 1.20 (0.77 – 1.87) | 0.417 |
| GGG | 17 (4.1) | 58 (12.1) | 0.31 (0.18 – 0.55) | 0.001 |
| GGA | 2 (0.4) | 1 (0.1) | 3.16 (0.30 – 33.63) | 0.503 |
| AGG | 1 (0.3) | 2 (0.3) | 0.92 (0.09 – 10.05) | 0.947 |
| ATA | 1 (0.3) | 2 (0.3) | 0.94 (0.09 – 9.58) | 0.955 |
| AGA | 1 (0.2) | 0 (0.0) | NA | NA |

NA: not available as of null cell.

# Chapter 6

## Summary and Perspectives

### 6.1 Summary

In this study, we described the seroprevalence of HBsAg positivity, HCV infection among HIV positive population in Shenzhen for the first time, and demonstrated plasma IL-27 titer was significantly elevated in HIV mono-infected persons and HIV, HBV, HCV co-infected individuals; we also found a significant positive correlation between $CD4^+$ T cell counts and plasma IL-27 titer within HIV mono-infected people ($r = 0.177$, $P = 0.034$), a significant positive correlation between HIV viral load and IL-27 titer in HIV/HCV co-infected individuals ($r = 0.362$, $P = 0.049$), and IL-27 p28 2905T/G was associated with HIV susceptibility in Chinese MSM population.

A cohort study was first conducted in Shenzhen regarding the seroprevalence of HBV, HCV infection among HIV-infeted population. Totally 914 HIV positive individuals were recruited in the study and tested for HBsAg and anti-HCV antibodies positivity, results showed a 10.9% (100/914) HIV/HBV co-infection rate, 14.6% (133/914) HIV/HCV co-infection prevalence, and 3.7% (34/914) HIV/HBV/HCV triple-infection prevalence. Multivariate logistic regression revealed that HIV transmission risk behavior was significantly associated with HIV, HBV, HCV co-infections. Most HIV/HBV co-infection people were mediated through sexual contacts include heterosexual and homosexual behaviors (95/100); while most HIV/HCV co-infected individuals were IDUs (89/133); in HIV/HBV/HCV triple-infection group, IDUs accounted for a huge part (28/34). Among IDUs population, most of them were male (108/122) and nearly half aged among 27 to 32 years old (56/122). Near half of people who got HIV through blood and blood products were HIV/HCV co-infected (10/23). Gender has a significant relationship with HIV risk behavior, with most IDUs were male.

In these clinical samples, ELISA was applied. Our results showed that plasma IL-27 was significantly elevated in HIV mono-infected, HIV/HBV co-infected and HIV/HCV co-infected people when compared to healthy controls. Later on, we further revealed that plasma IL-27 titer was not varied among HIV, HBV and HCV co-infections except HIV/HCV co-infections and HIV/HBV/HCV triple-infections. We also observed a significant positive correlation between $CD4^+$ T cell counts and plasma IL-27 titer within HIV mono-infected people (r = 0.177, $P$ = 0.034).

We further analyzed the impact of HIV, HCV viral loads on plasma IL-27 titer and found there was no significant correlation between HIV quantity and IL-27 titer among HIV mono-infected individuals (r = - 0.063, $P$ = 0.679) while a significant positive correlation between HIV viral load and IL-27 titer in HIV/HCV co-infected individuals (r = 0.362, $P$ = 0.049). For HIV/HCV co-infection, there was no significant linear correlation between HIV and HCV viral loads (r = - 0.072, $P$ = 0.704) but exist an obvious subdivision of samples with significant IL-27 titer variance ($P$ = 0.014), and lack of correlation for HCV viral load and IL-27 titer (r = - 0.119, $P$ = 0.530).

IL-27 p28 polymorphisms were genotyped by TaqMan® Allelic Discrimination Assay in Chinese men who have sex with men (MSM) population in Shenzhen, results revealed that proportions of IL-27 p28 -964A/G and 4603G/A genotypes were not significantly different from healthy controls, IL-27 p28 -964A/G and 4603G/A allele frequencies were similar between HIV positive MSM group and healthy control MSM group. Results also showed that for IL-27 p28 2905T/G, TG genotype had a 2.77-folds decreased risk of HIV susceptibility and subjects with G allele had a 2.72-folds decreased risk of HIV susceptibility. LD coefficients were observed between IL-27 p28 -964A/G and and 2905T/G (|D'| = 0.942), -964A/G and 4603G/A (|D'| = 0.930). Haplotype analyses revealed that haplotype GGG has a protective role against HIV infection in MSM population (OR = 0.31; $P$ < 0.001).

## 6.2 Perspectives

In this study, seroprevalence of HBV, HCV infection among HIV positive population in Shenzhen was surveyed and risk factors associated with co-infections were analyzed. Plasma IL-27 titer was measured and was significantly elevated in HIV mono-infected people, HIV/HBV co-infected and HIV/HCV co-infected individuals. IL-27 has a significant positive correlation with $CD4^+$ T cell counts within HIV mono-infected people, a significant positive correlation between HIV viral load and IL-27 titer in HIV/HCV co-infected individuals. IL-27 p28 2905T/G was associated with individual susceptibility to HIV infection and haplotype GGG has a protective role against HIV infection in MSM population. Some topics towards following perspectives may worthy further illustration.

**(1) More information, samples to be collected:**

In the cohort study, we collected age, gender and HIV transmission risk behaviors from participants. More demographic information should be collected in the future studies, such as family HBV infection history, anti-HcAb and HBeAg positivity, HBV DNA and HCV RNA levels, liver diseases markers, to clearly define the status of HBV, HCV infection, so as to reveal the scenario exist in Shenzhen, which may provide more in-depth information for proper policy formation and implementation. As moderate correlations were observed between $CD4^+$ T cell counts and IL-27 titer in HIV mono-infected and HIV/HCV co-infected people, more samples may needed to clarify the potential correlation.

**(2) The relationship between IL-27 and HIV, HBV and HCV viral loads**

In the study, we revealed that plasma IL-27 titer and $CD4^+$ T cell counts were significantly differed between HIV/HCV co-infection and HIV/HBV/HCV triple-infection people, but the underline mechanism was not revealed. As HBV infection in China mainly occurs during perinatal and early childhood, the above mentioned difference may be resulted from chronic HBV infection. How chronic HBV infection related liver diseases and HBV DNA quantity affect HIV/HBV/HCV progression is

worthy further elucidation as it provides important reference for treatment.

The snapshot impact of HIV, HCV viral loads on plasma IL-27 titer was studied in the thesis, further follow-up and continuous monitor of HIV, HCV viral loads, together with IL-27, $CD4^+$ T cell counts and liver diseases related markers should be collected to reveal the long-term impact of HIV, HCV viral loads on disease progression.

**(3) The relationship between cytokines and HIV, HBV, HCV co-infections**

HIV, HBV and HCV infections induce a cascade of cytokine changes. As IL-27 is responded to HIV, HBV and HCV infections, and it modulate the immune response, other cytokines may be regulated by IL-27 and have impact on HIV, HBV and HCV infection. So information about changes of these cytokines may help to understand the picture of HIV, HBV and HCV infection in a more comprehensive way.

**(4) The impact of IL-27 with liver diseases progression.**

As HIV, HBV, HCV co-infected people has elevated liver-related mortality and IL-27 plays crucial pathogenic role in T cell-mediated hepatitis, it will worth to correlate IL-27 titer in plasma with inflammation and hepatic injuries. A longitudinal monitor of IL-27 titer changes, liver cirrhosis and hepatocelluar carcinoma develpoment together may help to reveal the function of IL-27 on liver diseases progression and liver-related mortality. Treatment based studies in hospitals would help to solve these questions in future studies.

**(5) IL-27 titer in HIV-exposed uninfected individuals.**

HIV-exposed uninfected individuals form a specific population of great importance in understanding immune responses and genetic susceptibility to HIV infection. As IL-27 was secreted by APCs several hours after stimulation and mediated innate and adaptive immunity; and IL-27 inhibited HIV replication *in vitro*; then it is of great importance to understand whether IL-27 was significantly elevated in HIV-exposed

uninfected population, and whether IL-27 was partly responsible for the early elimination of invaded HIV and control of HIV infection.

**(6) SNPs with HIV infection.**

In the study, a Chinese MSM population was recruited and revealed that IL-27 p28 2905T/G was associated with decreased risk of HIV susceptibility. Other SNPs of IL-4, IL-4 receptor, IL-10, IRF-1, IL-32; gene variants of CD4, CCR5, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5 and SDF-1, which have been proved to be associated with HIV susceptibility, should be tested for a potential interaction with IL-27 SNPs and their overall contribution to HIV susceptibility.

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