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CICLO XXV

TESI DI DOTTARATO DI RICERCA:

HOST GENETIC INFLUENCE ON HIV AND HCV INFECTIONS

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INTRODUCTION

CHAPTER 1

THE HUMAN IMMUNODEFICIENCY VIRUS

The human immunodeficiency virus (HIV) is a Retrovirus belonging to the family of Lentivirus. HIV is the etiologic agent of the acquired immunodeficiency syndrome (AIDS) that results in extremely variable clinical outcomes, such as a severe immunodeficiency accompanied by the establishment of opportunistic infections and tumors, organ decay and central nervous system degeneration [1]. Two different HIV viruses exist: HIV-1, the pandemic type, and HIV-2, more represented in West Africa [2].

1.1 STRUCTURE AND GENOME

HIV has a spherical form with an external lipoproteic envelope and an internal nucleocapside called core. The envelope is formed by a double phospholipid layer of host-cell origin in which the viral glycoproteins gp120 and gp41 are embedded. Gp120 and gp41 originate from the proteolitic cleavage of the gp160 precursor encoded by the env gene, they are exposed on the envelope in a trimeric form and control viral tropism by mediating the fusion of the virus membrane with the target cell. The core is made by p24 proteins and it encloses HIV genome, and virus-specific enzymes like reverse transcriptase, integrase and protease. The integrity of virion particle is ensured by the viral protein p17, which forms the matrix and surrounds the internal core (fig.1).

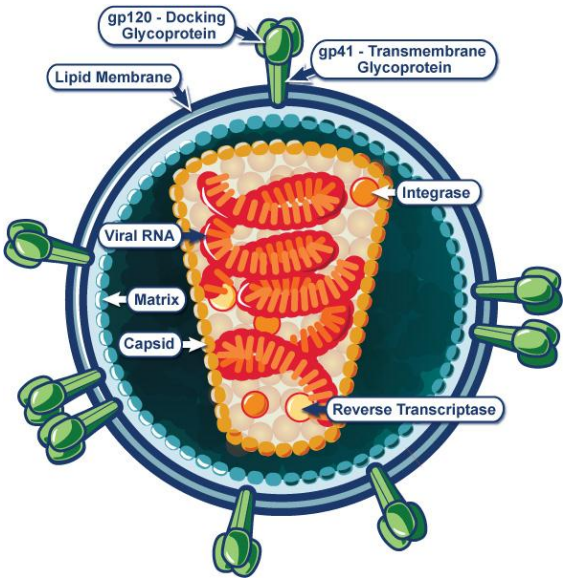


Figure 1. *Human Immunodeficiency Virus* type-1 (HIV-1): section of the structure of the virion.

HIV genome (fig.2) is composed by two identical copies of positive-sense single-stranded RNA (ssRNA+), each 9.2kb long, associated with two nucleocapside proteins (p7 and p9). HIV genome shows the typical genomic organization of virus belonging to *Retroviridae* family. It encodes for structural genes (gag, pol and env), regulator genes (tat, rev, nef, vpr, vpu and vif) and regulator sequences. Gag encodes for nucleocapsid and matrix proteins, pol for reverse transcriptase, integrase and protease while env for gp160 glycoprotein, this is a precursor from which gp120 and gp41 proteins derive [3]. A peculiar characteristic of HIV consists in its capability to create overlapping transcripts, to operate alternative splicing and to produce polyproteins which are subsequently split into different functional proteins.

At each end of the ssRNA molecule there is a sequence called Long Terminal Repeat (LTR) that mediates the integration of HIV genome into the genome of a host cell and regulates viral replication. The LTRs contain promoters and enhancers of gene transcription specific for host transcription factors such as Nuclear Factor kappa B (NF-kB), activator protein 1 (AP1), nuclear factor of activated T cells (NFAT), as well as virus-encoded factors [4]

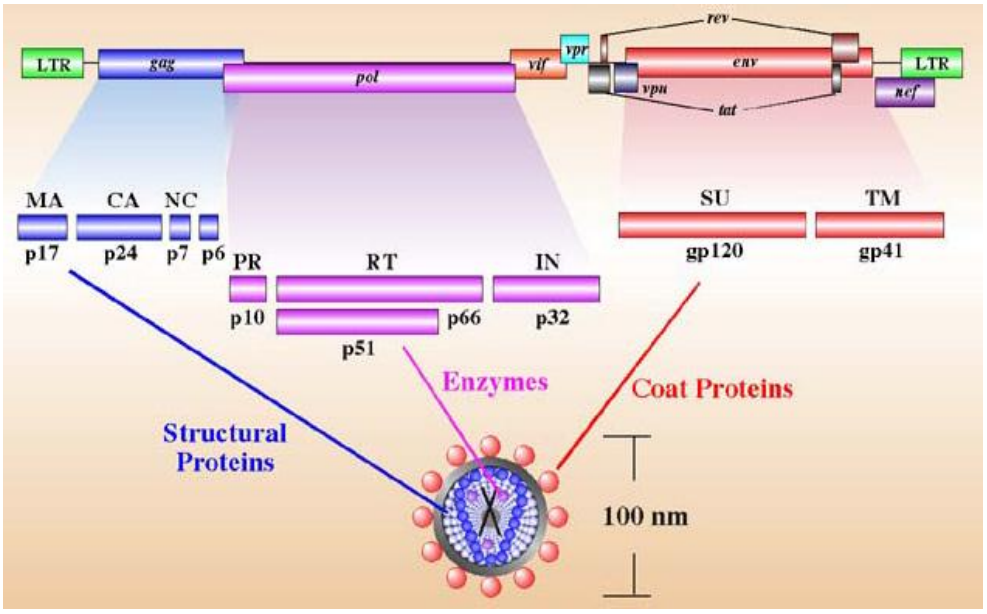


Figure 2. Organization of the HIV-1 genome.
(From Haseltine W.A., "Molecular biology of human immunodeficiency virus type 1", The FASEB Journal, Vol. 5, no. 10, 1991, pp. 2349-2360).

1.1.1 The viral envelope

The HIV envelope is a phospholipid bilayer acquired from the host cell during virion budding and release. The envelope contains host-derived proteins, including Human leukocyte antigens (HLAs) and adhesion molecules, and a series of spikes of 9-10 nm in length, called Env. Each spike is a trimeric complex of a glycoprotein of 120 kDa, called gp120 that from a cap non-covalently bound to a stem of a trimeric complex of transmembrane glycoprotein of 41 kDa, called gp41. The *env* gene encodes a protein called gp160 that, after glycosylation in the Golgi apparatus, is cleaved into gp120 and gp41. Respectively, these proteins enable HIV to attach to and fuse with the target cell membrane [5, 6].

1.1.2 The viral core

The core of HIV is a nucleocapsid made of an elongated conical shell with icosahedral symmetry composed of proteins of 24 kDa in weight, called p24, which contains the HIV genome. The nucleocapsid is surrounded by a spherical shell, known as matrix, made of proteins of 17 kDa, called p17, anchored to the viral envelope through a myristic acid residue. The two ssRNA+ molecules in the capsid are associated with two proteins of 7 and 9 kDa, called p7 and p9 respectively that function as packaging proteins. The proteins p7, p9, p17 and p24 are encoded by the gene *gag*, which transcript is translated in a poly-protein of 55 kDa, called p55 that is then cleaved by viral proteases in the above mentioned components during virion maturation [7]. In addition to the ribonucleoproteic complex, the core contains several virus-encoded enzymes: the reverse transcriptase (RT), a heterodimer constituted of two proteins of 66 and 51 kDa, the integrase, called p32 that facilitates the insertion of HIV genome into the host genome, and the protease, a homodimer composed of a protein of 10 kDa. These enzymes are encoded by the gene *pol*, which transcript is translated into a poly-protein of 180 kDa, called Gag-Pol, that is then cleaved by the viral potease itself [8]. The core also contains molecules that are important for the early stages of the biologic cycle of HIV and accessory proteins. Some of these proteins are virus-encoded such as Trans-activator of transcription (Tat or p14), Regulator of virion expression (Rev or p19), Negative regulatory factor (Nef or p27), Viral infectivity factor (Vif or p23), Viral protein U (vpu or p16) and Viral protein R (Vpr or p15) (or Viral protein X in case of HIV-2), and some derive from the host cell, such as cyclophilin A and a $tRNA^{lys}$ that serves as primer for the RT to initiate HIV genome reverse transcription [9].

1.2 TROPISM

The term viral tropism refers to which cell types HIV infects. HIV can infect a variety of immune cells such as $CD4^+$ T cells, macrophages, and microglial cells. HIV-1 entry to macrophages and $CD4^+$ T cells is mediated through interaction of the virion envelope glycoproteins (gp120) with the CD4 molecule on the target cells and also with chemokine coreceptors [10].

Macrophage (M-tropic) strains of HIV-1, or non-syncitia-inducing strains (NSI) use the *β*-chemokine receptor CCR5 for entry and are, thus, able to replicate in macrophages and $CD4^+$ T cells [11]. This CCR5 coreceptor is used by almost all primary HIV-1 isolates regardless of viral genetic subtype. Indeed, macrophages play a key role in several critical aspects of HIV infection. They appear to be the first cells infected by HIV and perhaps the source of HIV production when $CD4^+$ cells become depleted in the patient. Macrophages and microglial cells are the cells infected by HIV in the central nervous system. In tonsils and adenoids of HIV-infected patients, macrophages fuse into multinucleated giant cells that produce huge amounts of virus.

T-tropic isolates, or syncitia-inducing (SI) strains replicate in primary $CD4^+$ T cells as well as in macrophages and use the *α*-chemokine receptor, CXCR4, for entry [11, 12, 13]. Dual-tropic HIV-1 strains are thought to be transitional strains of HIV-1 and thus are able to use both CCR5 and CXCR4 as co-receptors for viral entry.

The *α*-chemokine SDF-1, a ligand for CXCR4, suppresses replication of T-tropic HIV-1 isolates. It does this by down-regulating the expression of CXCR4 on the surface of these cells. HIV that use only the CCR5 receptor are termed R5; those that use only CXCR4 are termed X4, and those that use both, X4R5. However, the use of coreceptor alone does not explain viral tropism, as not all R5 viruses are able to use CCR5 on macrophages for a productive infection [11] and HIV can also infect a subtype of myeloid dendritic cells, [14] which probably constitute a reservoir that maintains infection when $CD4^+$ T cell numbers have declined to extremely low levels.

Some people are resistant to certain strains of HIV [15]. For example, people with the CCR5-Δ32 mutation are resistant to infection with R5 virus, as the mutation stops HIV from binding to this coreceptor, reducing its ability to infect target cells.

Sexual intercourse is the major mode of HIV transmission. Both X4 and R5 HIV are present in the seminal fluid, which is passed from a male to his sexual partner. The virions can then infect numerous cellular targets and disseminate into the whole organism. However, a selection process leads to a predominant transmission of the R5 virus through this pathway [16, 17 and 18]. How this selective process works is still under investigation, but one model is that spermatozoa may selectively carry R5 HIV as they possess both CCR3 and CCR5 but not CXCR4 on their surface [19] and that genital epithelial cells preferentially sequester X4 virus [20]. In patients infected with subtype B HIV-1, there is often a co-receptor switch in late-stage disease and T-tropic variants appear that can infect a variety of T cells through CXCR4 [21]. These variants then replicate more aggressively with heightened virulence that causes rapid T cell depletion, immune system collapse, and opportunistic infections that mark the advent of AIDS [22]. Thus, during the course of infection, viral adaptation to the use of CXCR4 instead of CCR5 may be a key step in the progression to AIDS. A number of studies with subtype B-infected individuals have determined that between 40 and 50 percent of AIDS patients can harbor viruses of the SI and, it is presumed, the X4 phenotypes [23, 24].

1.3 REPLICATIVE CYCLE OF HIV-1

HIV begins its life cycle when the viral glycoproteins gp120/gp41 (ENV complex) bind to a CD4 receptor and one of two co-receptors on the surface of a target cell (fig.3). Physiologically HIV-1 co-receptors belong to chemokine receptor family the most important of which are CCR5 and CXCR4. CCR5 is typically expressed on macrophages therefore viral strains that exploit it are named M-tropic strains, conversely CXCR4 is mainly expressed on T cells and virus strains that use it are known as T-tropic strain.

The Env complex mediates a multistep fusion process. Gp120 binds to CD4 receptor and subsequently to the chemokine coreceptor, allowing gp41 to change its conformation and to expose a hydrophobic region called fusion peptide; this peptide inserts into the cell membrane and allows the fusion between the target cell membrane and the viral membrane.

After the fusion, the nucleoprotein core is disrupted; reverse transcriptase transcribes genomic RNA in double strand DNA which enters into the nucleus together with the integrase enzyme. Integrase promotes the integration of viral DNA in the cellular genome.

The integrated viral DNA is called provirus and it can be latently inactive for long periods of time without being transcribed.

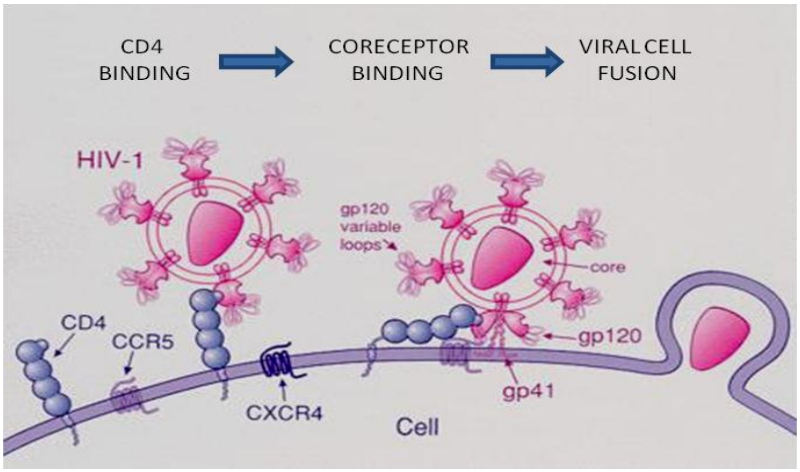


Figure 3. Schematic representation of HIV entry into CD4+ cell via CCR5 co-receptor.

Transcription of the provirus genes is regulated by the LTR sequences. LTR are regulator sequences at the ends of HIV genome which form during retrotranscription of RNA in proviral DNA; they are important because they contain polyadenylation signal sequences, the TATA box promoter sequence and binding sites for two host cell transcription factors, NF-κB and AP1 which regulate the provirus transcription following the interaction with viral and host factors. Initiation of HIV gene transcription in T-cells can be initiated only after T-cell activation induced by antigen presentation or cytokine action. HIV genes expression can be divided in an early and late phase. Regulator genes (tat, rev, nef) are transcribed in the early phase of HIV life cycle, in a full-length transcript which is spliced and translocated from nucleus to cytoplasm to be translated in proteins. Rev promotes nuclear export of incompletely spliced and unspliced viral mRNAs. Tat is required for elongation of viral transcripts by direct binding to mRNA and by increasing the processivity of host RNA-polymerase. Moreover Tat can be secreted by infected cells, as a matter of fact, its concentration is consistent in sera of HIV-infected and extracellular Tat can act as a viral toxin triggering T cell apoptosis and the secretion of many cytokines by monocytes contributing to immune dysfunction and progression of HIV disease.

Nef plays a pivotal role in accelerating endocytosis and degradation of CD4 and MHCI host cell molecules other than enhancing the release of virus from infected cells. The role played by Vif, Vpr and Vpu: in viral replication is not completely clarified. Vif enhances infectivity of viral particles, Vpr promotes nuclear import of viral DNA and Vpu plays a similar role to that played by nef and also promotes maturation and trafficking of gp160

protein.The switch from early gene to late gene is regulated by Rev, which allows the translocation of unspliced late genes to the cytoplasm.

As previously mentioned late genes (pol, gag and env), are synthesized as precursor protein and are subsequently cleaved. Pol gene encodes for reverse transcriptase, protease, ribonuclease and integrase protein. Gag encodes for a precursor protein that it is subsequently cleaved in p24, p17 and p15 by pol protease, these are core proteins, necessary for assembly of viral particles. Env gene encodes for gp160, precursor of gp120 and gp41.

Assembly of viral particles begins by packaging full-length RNA transcripts of the proviral genome with proteins encoded by gag genes and pol enzymes. Viral particles are released from the target cell through a process of budding and in this passage they acquire the phospholipid layer and expose gp120 and gp41 proteins on viral particles surface (fig.4). [25].

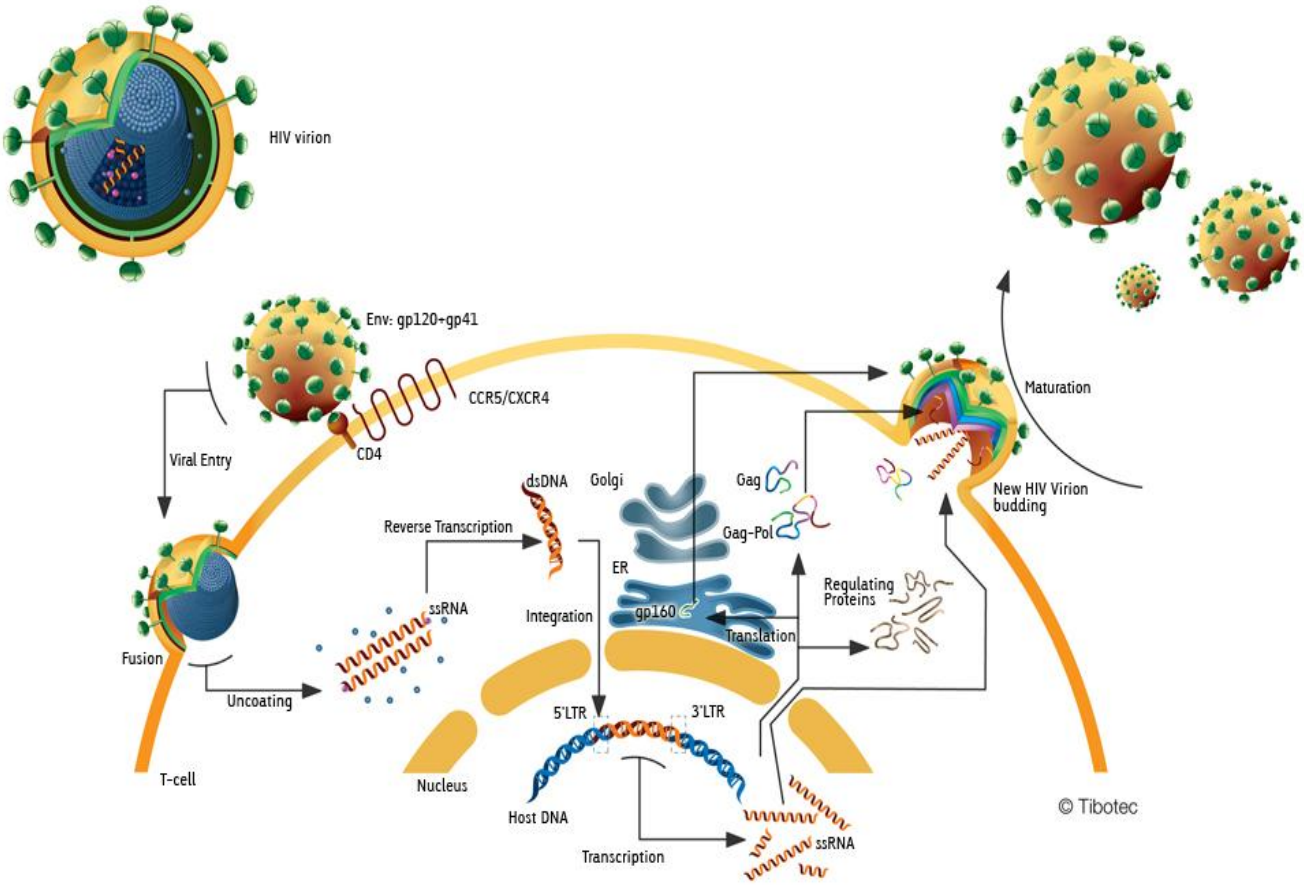


***Figure 4**. Mechanism of action of HIV-1 virus*

CHAPTER 2:

NATURAL HISTORY OF HIV INFECTION

2.1 PATHOGENESIS of HIV-1 INFECTION

The pathogenesis of HIV-1 infection begins as an acute infection that after a long period of latency, which can last 3 to 20 years, leads to a severe status of immunodeficiency, called acquired immunodeficiency syndrome (AIDS) [26]. The major determinant of the pathogenesis and disease caused by HIV-1 is the virus tropism for $CD4^+$ T cells, which results in a reduction in the number of these cells and consequent abolition of the helper function of adaptive immunity. HIV-1 induces several cytopathology effects that may kill the infected T cell. In the acute phase of infection, HIV-induced cell lysis and killing of infected cells by CTLs account for $CD4^+$ T cell depletion, although apoptosis may also be an important factor [27]. During the chronic phase, the consequences of generalized immune activation, coupled with the gradual loss of the ability of the immune system to generate new T cells, account for the slow decline in $CD4^+$ T cell number [28,29].

The course of HIV disease parallels the reduction in $CD4^+$ T cell numbers and the amount of virus in the blood (Fig. 5). During the acute phase, there is a large burst of virus production and infection spread systemically, in particular to secondary lymphoid organs. T cell proliferation and responses to the infected lymphoid and myeloid cells promotes a mononucleosis-like syndrome. The $CD8^+$ T cell response is thought to be important in controlling virus levels, which peak and then decline, as the $CD4^+$ T cell counts recover. Virus levels in the blood decrease during a clinically latent period, but viral replication continues in the secondary lymphoid organs [31]. At the same time, virus can remain latent in macrophages, dendritic cells and resting T cells, establishing reservoirs in many organs. Continuous HIV-1 replication results in a state of generalized immune activation persisting throughout the chronic phase [28]. Immune activation, which is reflected by the increased activation state of immune cells and release of pro-inflammatory cytokines, results from the activity of several HIV-1 gene products and the immune response to ongoing HIV-1 and co-infecting pathogen replication. Immune activation has also been linked to the breakdown of the defenses of the gastrointestinal mucosal barrier caused by the depletion of mucosal $CD4^+$ T cells during the acute phase of disease [32, 33].

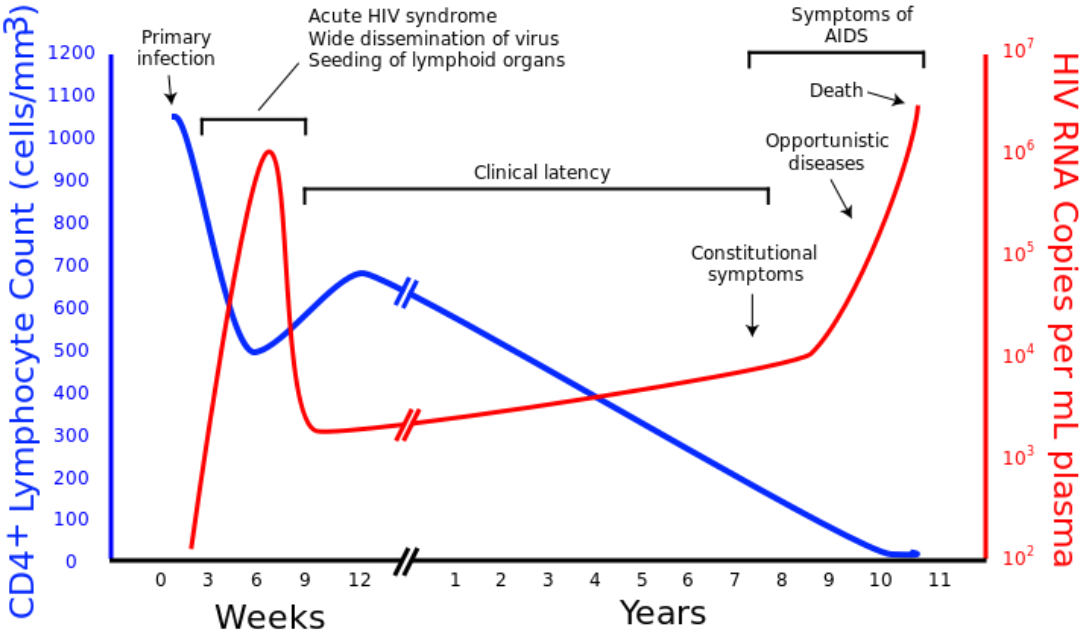


Figure 5. Time course and stages of HIV disease.

Late in the disease, virus levels in the blood increase, $CD4^+$ levels are significantly decreased, $CD8^+$ levels also decrease, X4-tropic virus rises (in about 50% of the infected individuals), the structure of the lymph nodes is destroyed [34], and the patient becomes immunosuppressed (Fig 5). Stage IV or AIDS, as defined by the World Health Organization, is characterized by severe symptoms which includes toxoplasmosis of the brain, candidiasis of the esophagus, trachea, bronchi or lungs and Kaposi's sarcoma, and a $CD4^+$ T cell count of less than 200/μL [35].

The central role of the $CD4^+$ helper T cells in the initiation of an adaptive immune response is indicated by the extent of the loss of function caused by HIV-1 infection. When $CD4^+$ T cells are unavailable or not functional, antigen-specific immune responses (especially cellular immune responses) are incapacitated, and humoral responses are uncontrolled. The loss of the $CD4^+$ T cells responsible for activating macrophages allows the outgrowth of many of the opportunistic intracellular infections characteristic of AIDS (e.g., fungi and intracellular bacteria). Of importance, the decrease in number and the inability to activate $CD8^+$ T cells increases the potential for recurrence of latent viruses, including JC polyomavirus progressive multifocal leukoencephalopathy (PML), herpes simplex virus, and cytomegalovirus infections, and even Epstein-Barr-associated lymphomas and human herpesvirus-8-associated Kaposi sarcoma [30].

2.2 CLINICAL COURSE OF DISEASE PROGRESSION:

A typical characteristic of HIV-1 infection is the huge variability of the progression of the disease in the infected patients. Based on the duration of the HIV-1 infection and on the kinetic of the virologic and immunologic events observed during the disease, the clinical course of disease progression can be divided into three phases: 60-70% of HIV-1 infected people are "*Typical Progressors*" and the median time from infection to development of AIDS is approximately 10 years; 10-20% of HIV-1 infected people are "*Rapid Progressors"* and they progress rapidly to AIDS within 5 years; 5-10% of people infected showed no clinical progression for several years (10-15) and they are called "S*low Progressors*" (SP) and they maintain a stable CD4+ count ≥ 500 cell/ul with plasma viremia ≤ 10.000 copie/ml. To this category belongs also a subgroup of 2-3% of infected people called "long term non-progressors" LTNP, (36), fig 6.

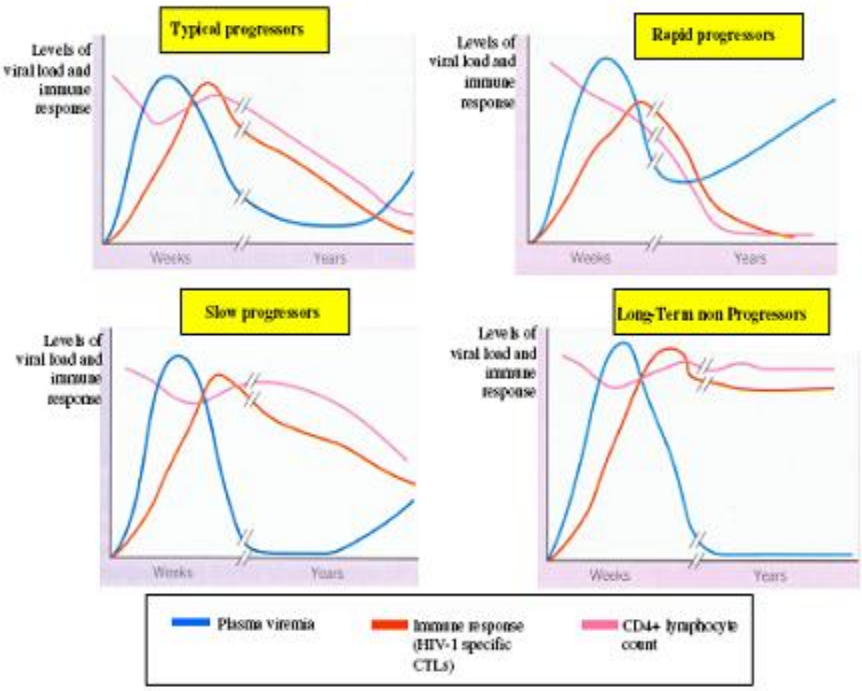


Figura 6. Plasma viremia variation, lymphocytes T-CD4+ count and immune response in 4 stages of the disease.

More recently, a subset of infected individuals, called Elite Controllers, with spontaneous and sustained control of HIV infection, with viral loads <50 RNA copies/ml in the absence of therapy have been described [37, 38],(tab.1). This spontaneous control occurs in approximately 1 in 300 HIV-infected persons, and the HIV Controller Consortium was recently established to study host genetics together with functional immunology studies in elite controllers.

	EC	LTNP
CD4+ t-cell level (cell/ ul)	≥500*	≥500
viral load (copies/ml)	≤50	≤10000
antiretroviral therapy	No	No
Years without AIDS	Months-years	≥7-20*

*values can vary in studies

Table 1. Definitions of major controller populations

Interest in non-progressive HIV-1 infection has increased over time in the hope of understanding HIV pathogenesis and the mechanisms of protection involved, and providing insights relevant to viral attenuation, novel therapies and vaccine design [38]. SP or LTNP cohorts provide a unique opportunity to study the complexity of the virus-host interaction with the aim of understanding the biological and molecular correlates of protection against HIV-1 infection and disease progression. The duration of clinical latency following HIV-1 infection is regulated by the interplay of at least three virus-host factors: (i) genetic susceptibility of the host, (ii) the hosts' ability to mount an effective immune response, and (iii) the genotypic properties of the infecting viral quasispecies [39, 40].

2.3 LONG TERM NON PROGRESSORS (LTNP)

2.3.1 Definition

Infectious diseases can be extremely variable in their clinical manifestations. In the case of HIV, the time from infection to the development of AIDS varies widely. Typically, the

median time from infection to AIDS is estimated to be 10 years [41, 42] with peripheral CD4+ T lymphocytes typically declining at a rate of 50-90 cells/mm3/year [43,44,45]. Although rapid progression is rare, there have been reports describing progression to AIDS within 2-4 months after primary infection [46,47] and within 2 years in infants infected by blood transfusions [48]. At the other extreme, investigators have also identified untreated HIV-1 infected individuals who remain healthy and AIDS free for more than 13 years [49].

In the HIV-infected population, the proportion of individuals classified as long term non progressors (LTNP) varies widely due to differences in study design, the length of available follow-up and inconsistency in the criteria's used to define LTNP. In the Tricontinental and Italian Seroconverted Study, five definitions were used to categorize LTNP. They found that the proportions of LTNP varied from 0.5 – 25 % depending on the defining criteria such as time to AIDS, CD4 count thresholds and use of ARVs [50, 51, 52]. There is evidence today of yet another category of potentially slow progressing HIV infected individuals. A criterion incorporating viral load (VL) measures found that approximately 1 patient in 300, naive to ARV therapy, has undetectable viremia below the detection threshold of 50 RNA copies/ml (Elite HIV controllers or EC). EC must be infected for at least one year and unlike typical LTNP definitions, CD4 counts are not always considered [53-56]. In contrast, a typical LTNP definition requires that CD4 counts be above 500 cells/mm after at least 7 years of untreated infection [57]. This definition is supported by the observation that in untreated HIV infection, the median time for CD4+ T cell counts to fall below 500 cells/mm3 is 1.7 yrs (95% confidence intervals 1.29 to 1.89 yrs) [58]. Additionally, while untreated HIV disease progressors have a declining peripheral CD4 count of approximately 50-90 cells/mm3/year, LTNPs loose on average 6 cells/mm /year, a rate not significantly different from that seen in HIV seronegative controls [45,59]. It is commonly accepted that LTNPs represent the tail end of a continuous distribution of progression rates and that individuals who never progress to AIDS are extremely rare [57-60]. The findings that LTNPs are not a discrete homogenous group could be seen as a study population devoid of relevant information on mechanisms of disease progression.
However, it has become clear over the years that multiple virologic, genetic and immunologic factors influence the rate of disease progression. Studying factors that impact on the natural course of HIV is crucial to the rational design of therapeutic vaccines.

Translating this knowledge into novel therapies will hopefully prolong the life of infected individuals and reduce the rate of HIV transmission.

2.3.2 Demographic cofactors of progression

Psychological, behavioral and environmental factors can potentially alter the natural history of HIV infection. LTNP have been identified in all HIV-infected risk groups: heterosexuals, MSM, IDU, transfusion recipients and perinatally-acquired infections [61]. Studies of LTNP have not found a significant association between age and rates of disease progression. However, older age at seroconversion is one factor that is associated with more rapid progression to AIDS and death [62]. The extrapolated median time to AIDS for a person infected at age 20 is 10.2 years and for a person infected at age 40 is 7.4 years [63-65]. The overall evidence for epidemiologically linked cofactors such as geographical location (ethnicity), route of transmission, social status or lifestyle cofactors has not shown strong correlations with delayed AIDS progression [66-69].

2.3.3 Virologic cofactors of progression

A number of viral factors have been recognized to influence disease progression. Unlike typically progressing individuals, LTNP are reported to have lower VL, proviral DNA and multiply spliced RNA. These observations are consistent with a large number of published reports which suggest that the rate of disease progression is driven by viremia and immune activation. Individual case reports or small patient cohort studies have described infections with attenuated forms of HIV-1 leading to decelerated disease progression. Viral polymorphisms associated with LTNP status have been identified in the non-coding LTR region and in structural, regulatory and accessory genes. Kestler et al. first demonstrated that Nef-defective SIV causes attenuated disease in primates. Nef is required for maintaining viral replication and pathology during the course of persistent infection in rhesus monkeys. Subsequent studies confirmed the relationship between Nef and disease progression in a murine model of HIV. In humans, the best known example of Nef-attenuated virions is provided by the Sydney Blood Bank Cohort (SBBC). The cohort consisted of 8 individuals inadvertently transfused with an attenuated HIV strain lacking a functional Nef gene. All except one recipient and the donor remained clinically free of symptoms, with stable and normal CD4+ T cell counts 10 to 14 years after infection.

Genetic analysis of sequences from virus isolates and peripheral blood mononuclear cells (PBMC) revealed a 29 base-pair deletion in the Nef gene and in the overlapping region of Nef and LTR. Nef has multiple functions responsible for pathogenesis and immune evasion. These include the downregulation of CD4, MHC class I and CD28. Nef is also described to mediate cellular signalling, activation and enhancement of virus infectivity. Investigators show that LTNPs harboring Nef/LTR deletion mutants have low levels of immune activation and strong HIV-specific proliferative responses. However, follow up information documented immunological damage and progression in 3 individuals suggesting that although they experienced a delay in disease progression, infection with attenuated viral quasispecies is not completely protective.

Accounting for a small proportion of cases of non-progression, mutations within Gag pl, LTR and accessory genes (Vif, Vpr, Vpu, Tat and Rev) have been associated with slow HIV disease progression. Changes in Env that affect viral tropism are also important modulators of HIV-1 disease progression. A switch in the specificity of HIV-1 Env from R5 to X4 or R5X4 is considered an indicator of poor prognosis, partly because it increases the number of CD4 cells that are susceptible to cytolytic infection by HIV-1. Although some studies have indicated that LTNP and EC harbor slow replicating defective viruses, these findings do not account for slow disease progression in most individuals experiencing a benign course of disease. A recent study described the transmission of HIV-1 from a LTNP to two individuals that subsequently developed progressive disease. This indicated that host characteristics are important determinants of non-progression.

2.3.4 Host genetic cofactors of progression.

Host genetic factors associated with disease progression have focused on HLA genes, as discussed in a previous section, but also on chemokine coreceptors, their ligands and cytokines. While a 32-base-pair deletion in the CCR5 chemokine receptor gene is associated with resistance to HIV infection60, heterozygous individuals for the CCR5A32 deletion exhibit slower disease progression taking 2 to 4 years longer to develop AIDS. This is caused by a lower expression of surface CCR5 coreceptors which translates into slower HIV-1 replication and reduced pathogenesis. The association between CCR5 genotype and rate of disease progression however is modest and the effect of CCR5 genotype on disease can vary in different risk groups or at different stages of infection. In addition, the protective effect exerted by CCR5 heterozygosity accounts for no more than

one third of LTNPs. Single nucleotide polymorphisms (SNP) in the CCR5 promoter region appear to influence progression. The CCR5 PI haplotype comprising 13 distinct SNP upstream of the promoter confer rapid disease progression by influencing CCR5 abundance on lymphoid cells. A Valine to Isoleucine mutation at position 64 (V64I) of CCR2 mediates delay in AIDS progression indirectly as it causes no allele-specific quantitative difference in the amount of CCR2 produced. The variant CCR2 protein preferentially heterodimerizes with CXCR4 polypeptides reducing expression and delaying disease progression.

RANTES is the principal chemokine ligand for CCR5. Elevated circulating levels of RANTES have been detected in individuals who have a delayed onset of AIDS. Conversely, SNP in an intronic regulatory sequence downregulates RANTES production by a factor of 4 leading to rapid progression. Polymorphisms at the 5' promoter region of IL-10 and IFN-y have been implicated in modulating disease progression. The IL-10 SNP variant reduces IL-10 transcription by a factor of 2-4. Heterozygous and homozygous individuals experience an accelerated course of disease presumably caused by a heightened state of immune activation446. Individuals heterozygous for a functional polymorphism identified in the IFN-y promoter progress to AIDS more rapidly. This SNP (G-179T) represents a Guanidine to Thymidine transition and confers TNF-a inducibility. It is hypothesized that increased IFN-y production induced by TNF-a causes CD4 cell depletion by apoptosis.

Using a genome-wide association strategy, researchers identified polymorphisms that account for 15% of the variability in the viral load set point during the asymptomatic period. Among them, HLA complex P5 (HCP5) is found within an endogenous retroviral element and is in high linkage disequilibrium with HLA-B*5701 while the second SNP is located in the 5' region of the HLA-C gene. A third polymorphism encoding an RNA polymerase subunit contributed to 5.8% of the variation in the rate of disease progression.

Given the importance of the gut in the pathogenesis of HIV, investigators evaluated gene expression profiles in intestinal biopsies sampled from LTNPs. A significant increase in mucosal gene expression regulating immune activation and inflammation was detected in high VL patients compared to LTNPs. However both groups showed deregulation in genes associated with cell cycle regulation, lipid metabolism and enterocyte function.

2.3.5 Immunologic cofactors of progression

NAbs directed against HIV-1 have the potential to play an important role in preventing viral infections both in vitro and in animal models [70-72]. With the help of cellular immune responses, evidence suggests that Nab potentially prevent or delay the progression to AIDS. Studies have shown that Nabs induced by infection or passively transferred contribute to controlling viral replication [73,74] and are more avid and more abundant in LTNP than in other HIV-infected individuals [75-76]. However other reports failed to detect effective NAb responses in LTNPs suggesting that their contribution in the prevention of disease progression remains unclear Immune pressure mediated by NAb induces rapid escape variants and studies have shown their protective effects only when present at high doses [74].

Both innate and adaptive immune responses potentially contribute to the initial containment of infection and hence may be crucial in modulating the subsequent course of disease. DCs are the most potent antigen-presenting cells involved in the generation of both innate and acquired immune responses464'465. Data suggests they are impaired in number and function in HIV-infected individuals. Several studies have reported a reduction in the number of blood myeloid dendritic cells DC (mDC), plasmacytoid dendritic cells (pDC) and tissue Langerhans cells (LC) in HIV-infected progressors. LTNPs maintain their pDC numbers suggesting that control of viremia is mediated by the secretion of large amounts of IFN-a. In addition, mDC and pDC isolated from progressing patients showed an impaired ability to stimulate allogeneic T cell proliferation [75].

Natural Killer cells (NK) exert their antiviral effect both by direct killing of infected cells and by secretion of cytokines and chemokines which help direct the adaptive arm of the immune response. Supernatants from NK cell cultures were found to suppress endogenous HIV replication in autologous CD4+ T cells by secreting CC chemokines such as RANTES, MlP-1 alpha and MIP-1 beta. NK cells may also lyse infected targets through the secretion of perforin, granzyme and by ADCC. A previous study found a genetic association between an activating NK cell receptor KIR3DS1 and delayed progression to AIDS when coexpressed with HLA-B molecules expressing an Isoleucine at position 80. Functional and phenotypical differences have also been observed between NK cells isolated from LTNP and progressors. Both natural killer cytotoxicity receptors and cytotoxic activity were found to be decreased in viremic compared to LTNP individuals [76].

Regulatory T cells (Tregs) are a subset of CD4+ lymphocytes that limit the expansion and activation of autoreactive T cells effectively preventing autoimmune disease. The role Tregs play in the pathogenesis of HIV is still controversial. On the one hand, a decrease in circulating Tregs has been reported in the periphery of chronically infected individuals potentially through direct infection and killing. These findings support the view that lack of immunosuppresion favors immune activation and disease progression. On the other hand, the expression of Treg markers was found to be increased in the tonsils of HIV-infected individuals suggesting that altered trafficking or accumulation of Tregs in lymphoid tissues is responsible for their decreased frequency in the periphery.

Although an accumulation of Tregs at sites of viral replication can limit the damage caused by hyperactive immune responses, it has been hypothesized that it can also prevent the development of a protective adaptive immune response. In support of this view, LTNPs have low numbers of Tregs at multiple lymphoid sites suggesting that Tregs prevent the mounting of efficient HIV-specific immune responses in progressing individuals. A critical balance between positive and negative signals is crucial for effective immunity. Suppression may limit protective HIV-specific immune responses but it can also contain viral replication by inhibiting CD4+ T cell activation and infection. Several additional immunological differences exist between LTNPs and progressors.

LTNPs appear to exhibit a balanced Thl/Th2 response with increased expression of IL-2, IFN-y, IL-4 and IL-10 production. LTNP maintain higher proportions of end stage CD8+ (CD45RA+CCR7"CD62L") effector cells and mount potent HIV-specific CD4+ and CD8 proliferative responses with the capacity to produce larger quantities of perforin [77].

2.3.6 *Co-infections.*

Human co-infection of HIV-1 with other viruses such as Human T-cell lymphotropic Virus, Herpes simplex virus type 2 [78], Varicella zoster virus (Hung et al., 2005) and cytomegalovirus (Saillour et al., 1998) are associated with an increased rate of disease progression to AIDS. Interestingly, infections with helminths shift the immune response from Th1 response to a less protective Th0/2 type response (reviewed in [79]). It appears that the aforementioned infections result in a more rapid progression to AIDS. Similarly, malaria has been associated with increases in HIV-1 viral load that may impact HIV progression in coinfected individuals (reviewed in [80]).

By contrast, coinfection with GBV-C (hepatitis G virus) has been shown to result in a lower mean viral load and a higher mean CD4 cell count and subsequent retardation of disease progression. The latter findings have however, been disputed although these effects may be only noted in advanced HIV infection.

CHAPTER 3: TRL9 AND IMMUNITY TO INFECTIOUS DISEASE

3.1 TLR IN THE IMMUNE SYSTEM

TLRs play an important role in innate immune recognition; they are the first line of innate defense against microorganisms. TLRs belong to the family of pattern recognition receptors (PRR) due to their ability to recognize pathogen conserved structures, known as pathogen-associated molecular patterns (PAMPs). PRRs are expressed mainly by macrophages, dendritic cells, endothelial cells mucosal epithelial cells and lymphocytes; they can be localized at intracellular level or on the surface of these cellular populations. Other receptors are involved in innate recognition but TLRs have been studied most extensively, to date 13 receptors (TLR1-TLR13) have been described. They are distinct on the base of their cellular localization and their specific ability in PAMPs recognition.

TLR-1, -2, -4, -5, -6 are expressed on cell surface and they are mainly involved in detecting microbial products while TLR-3, -7, -8, -9 are localized on late endosome-lysosome vesicles and do recognize nucleic acid of both viral and bacterial origin. So far, TLR-10, -11, -12, -13 are orphan receptors and less is known about them.

All the TLRs show the same structural organization, they are type I integral membrane glycoproteins characterized, at the extracellular side by leucine-rich regions responsible for the recognition of pathogens and at the transmembrane and cytoplasmic level by Toll/interleukin-1 receptor (TIR) domain. Signal transduction is activated only after TLRs dimerization because this allows the binding of adaptor proteins like MyD88 or TIR-domain containing adapter inducing IFNβ (TRIF). Recruitment of MyD88 leads to the activation of MyD88 dependent pathway while the binding of TIR domain with TRIF induces the activation of the MyD88 independent pathway. All of the TLRs, except for TLR3 and in some cases also TLR4, bind MyD88 adaptor protein.

3.2 MyD88 dependent and independent pathways

After recognition of a pathogen-specific molecular pattern, TLRs are capable of differentially activating distinct downstream signaling events via different cofactors and adaptor proteins mediating diverse immune responses (fig.7).

MyD88-dependent pathway culminates in the activation of NF-κB and JNK transcription factors. This pathway is activated via the conserved, cytoplasmic TIR domain, which provides a scaffold for recruitment of the adaptor molecule MyD88 and serine/threonine

kinases of the IL-1R-associated kinase (IRAK) family. Four IRAK have been identified: IRAK-1, IRAK-2, IRAK-4 and IRAK-M. IRAK-1 and IRAK-4 possess intrinsic serine/threonine protein kinase activities, IRAK-2 and IRAK-M lack this activity, suggesting that they negatively regulate TLR-mediated signaling. IRAK1 and IRAK4 are sequentially phosporilated and dissociated from MyD88, which results in activation of tumor necrosis factor receptor-associated factor 6 (TRAF6). The TRAF6 adaptor protein activates transforming growth factor-β-activated protein kinase 1 (TAK1) which in turn triggers the IKK complex that leads to the transcription factor NF-κB activation.

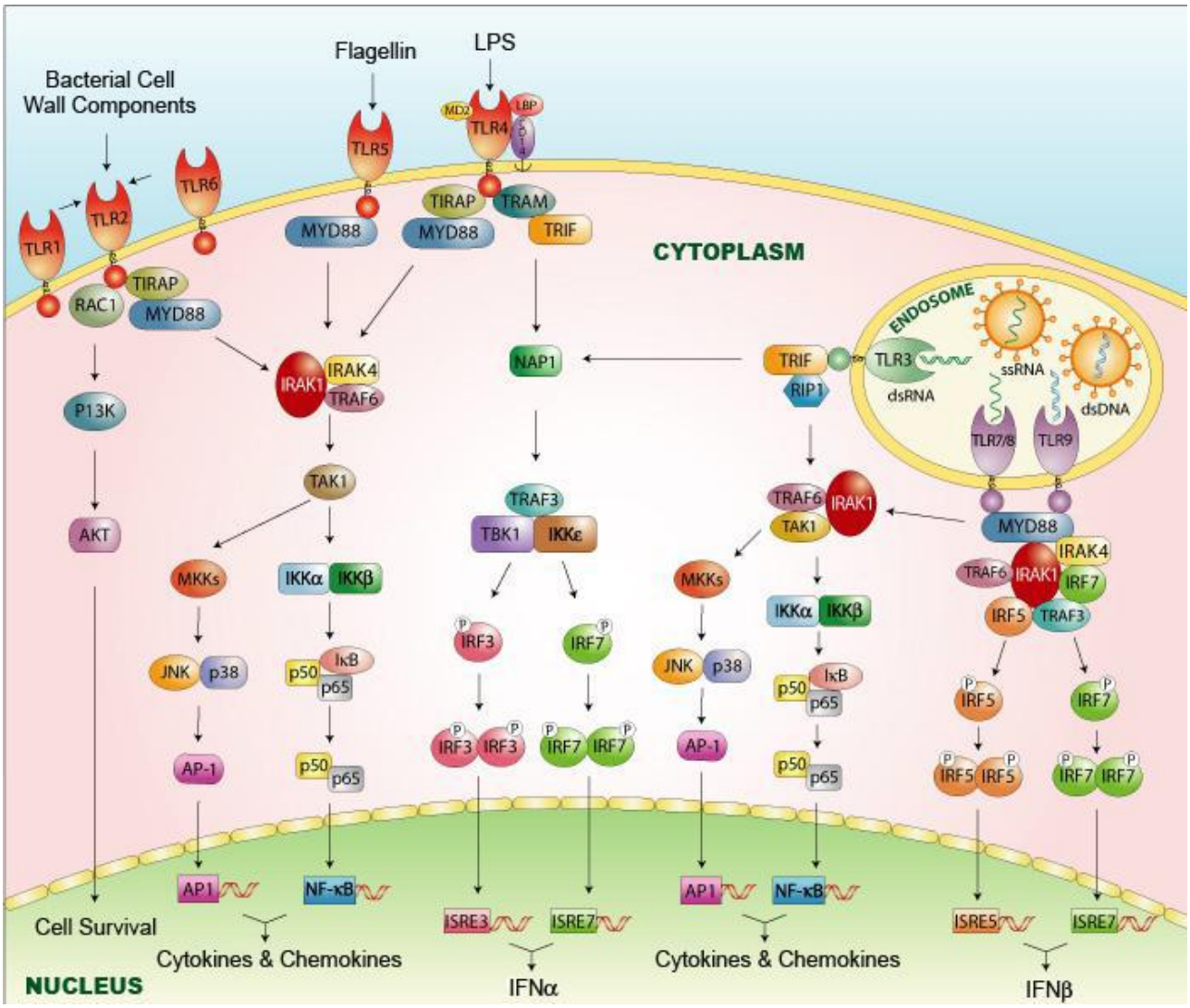


Figure 7. TLR signaling pathway. MyD88-dependent and independent pathways are illustrated.

TAK1 simultaneously phosporilates two members of the MAP kinase kinase family (such as JNK) which in turn activate AP-1. NF-κB and AP-1 promote the transcription of genes codifying macrophage inflammatory mediators, inducing B cell proliferation and endotoxin

shock. Experiments conducted in mice elucidated that this pathway is essential for TLR-mediated inflammatory responses.

MyD88-independent pathway activation is based on IFN-regulatory factor 3 (IRF-3) activity a transcription factor that promotes IFNβ synthesis. Recently, it has been discovered that IRF-3 is associated with IκB kinases (IKKs). IKKs are composed of IKKα and IKKβ, both of which phosphorylate IκBα thereby inducing NF-κB activation. In addition, there are two noncanonical IKKs, TANK-binding kinase 1 (TBK1) and IKKε/IKKi, which have distinct kinase activities compared with the canonical IKKα and IKKβ and both of them are able to promote the phosporilation of IRF-3.

This signaling pathway could also be activated via the TIR-domain-containing adaptor protein (TIRAP; also designated Mal for “MyD88-adapter-like‟) and results in activation of the dsRNA-binding protein kinase PKR. This protein has been proposed to be a central downstream component of both the TIRAP- and MyD88-dependent signaling pathways and could mediate potential crosstalk between them. The MyD88-independent pathway appears to utilize both IRF3 and NF-κB, and results in the expression of IFN-inducible genes including IP-10.

It has been demonstrated that NF-κB can be induced in a later phase thanks to the activation of IRF-3. Moreover TLR4 activation might lead to the induction of both MyD88-dependent and -independent pathway, according to the adaptor protein that is recruited to the TIR domain of the receptor. Adaptor protein TIRAP promotes the activation of the MyD88-dependent pathway while TRIF is the inducer of the independent one [81, 82, 83].

Following HIV infection, TLR2, TLR4, TLR7 and TLR8 are triggered. TLR2 binds to peptidoglycan and bacterial lipoprotein, TLR4 recognizes LPS, TLR7 and TLR8 bind to ssRNA.

Moreover experimental evidences show that TLRs expression is not restricted to APC but they characterize other cellular population like T regulatory cells (Tregs).

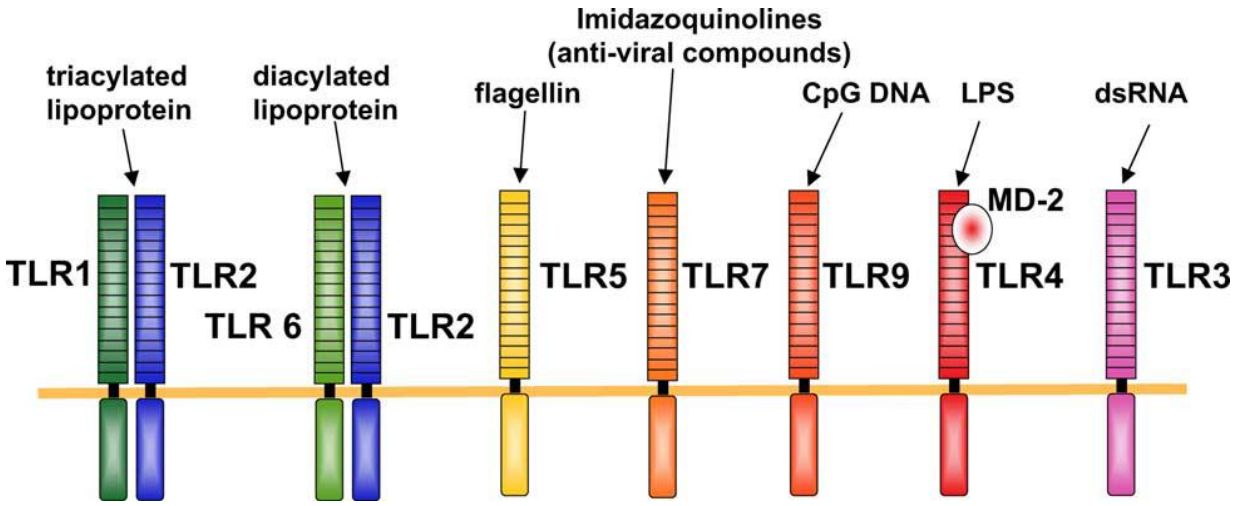


Figure 8. TLRs and their ligands. TLR1–TLR7 and TLR9 have been characterized to recognize microbial components. TLR2 is essential for the recognition of microbial lipopeptides. TLR1 and TLR6 associate with TLR2, and discriminate subtle differences between triacyl- and diacyl lipopeptides, respectively.TLR4 recognizes LPS. TLR9 is the CpG DNA receptor, whereas TLR3 is implicated in the recognition of viral dsRNA. TLR5 is a receptor for flagellin. Thus, the TLR family discriminates between specific patterns of microbial components

TLR9

TLR9 is localized in the intracellular endolysosomal compartment, it's activated by unmethylated CpG motifs within single strand DNA that are present in bacteria and viruses. TLR9 is expressed on pDC, B cells and monocytes/macrophages. CpG DNA is recognized in the endosome following nonspecific uptake into the cells and the activation of TLR9 leads to the induction of MyD88 dependent signaling pathway. Stimulation of TLR9 triggers B-cell proliferation and secretion of antibodies, secretion of Type 1 cytokines and chemokines. [84]

Through the intracellular domains, TLRs interact with several adaptor proteins to activate transcription factors, leading to the production of inflammatory cytokines and the activation of the adaptive immunity. Common polymorphisms in TLR genes have been associated with increased susceptibility to or protection against several infectious diseases, such as tuberculosis (TLR2), bacterial sepsis (TLR4), meningococcemia (TLR4), Legionnaire's disease (TLR4, TLR5) and infection with respiratory syncytial virus (TLR4) [85-92]. Rare inherited immunodeficiencies are associated with mutations affecting molecules involved in TLR signaling pathways (IRAK4, IKKg and IkBa) [93]. These data clearly demonstrate that mutations in TLRs and their associated signal transduction molecules can have a dramatic influence on individual susceptibility to human diseases [94-95].

TLR9 and HIV

While most HIV-positive patients develop AIDS within 10 years, some progress to profound immunodeficiency in 1–5 years (rapid progressors) and others remain immunocompetent for up to 20 years. It has been clearly demonstrated that genetic variations in the host can influence disease progression. In the context of HIV infection, the role of SNPs in TLRs may provide relevant information on HIV pathogenesis because the in vitro activation and signaling trough TLR9 (and TLR4, TLR2) and might enhance HIV replication. Furthermore, HIV may impair the in vitro immune response after TLR4 and TLR2 stimulation. Genetic variants of TLR9 have been associated with the rate of disease progression in adults [96-98]. Specific variants of TLR9 were found to be associated with risk of mother-to-child transmission (MTCT) of HIV-1, and data regarding their role in pediatric HIV-1 disease progression are available in Fregujas' study. Functional study demonstrated a critical role of the G allele of the rs352140 SNP on TLR9 expression.
In this way, TLR9 may also contribute to effects of HIV on the liver. TLR9 binds CpG-DNA of bacterial origin but more recently, it has been shown that TLR9 can also be activated by hepatocyte-derived apoptotic DNA fragments. HIV can induce hepatocyte apoptosis in vitro via binding of gp120 to CXCR4 even in the absence of productive infection and intrahepatic apoptosis is increased in the setting of HIV-HBV coinfection. Signalling through TLR9 has also been shown to suppress HIV replication in ex vivo lymphoid tissue blocks which correlated with production of chemokines such as CXCL-10 and 12 and CCL 3, 4, and 5. Therefore, indirect effects of HIV on TLR9 may also contribute to liver disease in the setting of HIV infection.

TLR9 and HBV

HBV has also been shown to impair TLR9 signaling in plasmacytoid DCs (pDCs) leading to reduced IFN-α production [100] which may be another mechanism for evasion of the immune system. TLR9 expression is increased in patients with chronic HBV compared to uninfected controls, and TLR9 expression significantly correlates with HBV DNA levels in plasma, suggesting a link between TLR9 expression and viral replication. Given the direct and indirect effects of HIV on TLR9, it is possible that, in the setting of HIV-HBV coinfection, TLR9 signaling in the liver by both HIV and HBV could result in an increase in chemokine production and a proinflammatory response.

TLR9 and HCV

HCV has adapted multiple methods of evading the host innate immune response by down regulating signaling through TLRs 2, 4, 7, and 9 by binding to and interfering with MyD88 and TRIF in multiple cell types including hepatocytes, macrophages, and plasmacytoid (p)DCs [99-100]. HCV has also been shown to cleave mitochondrial antiviral signaling protein (MAVS) which forms part of the viral sensing TLR signaling cascade, inhibiting IFN-α/β production, and transcription of antiviral ISGs [101]. Recent studies have also shown a potential role for TLR7 and 9 agonists in promoting clearance of HCV [102–104]. This would suggest that HCV would contribute to a reduction in the antiviral response in patients with HIV-HCV coinfection.

CHAPTER 4

HEPATITIS C VIRUS

Hepatitis C virus is a major causative agent of liver disease worldwide. An estimated 3% of the world's populations are chronic carriers of the virus, including approximately 1,6 million of Italians. Each year, 3 to 4 million people are newly infected with HCV, primarily through contact with infected human blood by sharing needles in intravenous drug use or by contaminated blood transfusions. Acute infection is most commonly asymptomatic but can be accompanied by fatigue and jaundice with 80% of infected individuals progressing to a chronically infected state. Chronic infection is often characterized by the development of hepatitis, steatosis, fibrosis and liver cirrhosis approximately 20 to 30 years after infection. Some patients will eventually die of HCV due to extensive liver damage, primarily due to the host immune response, or the development of hepatocellular carcinoma (HCC). Currently there is no vaccine for HCV and only 54% of HCV infected individuals respond positively to combined therapy of pegylated interferon alpha-2b plus ribavirin, depending on HCV genotypic variation and other underlying health problems. Continued HCV research is clearly necessary to develop an effective vaccine or antiviral therapy for this devastating disease.

4.1 HCV structure

4.1.1 Virus particles

HCV is an enveloped virus with a positive sense RNA genome in the *Hepacivirus* genus of the *Flaviviridae* family. Each virus particle is approximately 55-65 nm in size [105,106] Electron microscopy (EM) of the virus has been hampered by the lack of a cell culture system that produces a sufficient amount of 2 virus for visualization. However, recent work using the JFH-1 cell culture system has allowed for further characterization of the virus. By examining other viruses of the *Flaviviridae* family, it is believed that HCV has an icosahedral arrangement in which the structural glycoproteins E1 and E2 are embedded into a bi-layer lipid envelope derived from the host cells. The core protein forms the nucleocapsid of the virus that encloses the RNA genome [107]. There are three forms of the virus present in the serum of infected individuals including free virions, immunoglobulin

associated virions and virions associated with very-low-density and low-density lipoproteins [108,109].

4.1.2 Positive sense RNA genome

Hepatitis C virus contains a positive sense, single stranded RNA genome of approximately 9.5 kb. The genome consists of a single open reading frame flanked by 5' and 3' untranslated (UTR) regions which are important for replication of the genome. The highly conserved 5' UTR is 341 nucleotides long and contains the internal ribosome entry site consisting of four major RNA domains with extensive secondary structure. The entire 5' UTR is believed to be important for IRES activity and HCV translation. The 3' UTR is potentially important in initiating viral replication. It consists of a poly (U)/polypyrimidine tract, a variable 40 nucleotide sequence, and a highly conserved 98 nucleotide sequence with stable secondary structure[110,111].

4.1.3 HCV genotypes and quasispecies

The discovery of HCV was unique in that the existence of the virus was confirmed using molecular cloning techniques rather than direct biological methods. The viral sequence was first obtained by extracting all nucleic acid from the serum of a non-A non-B hepatitis infected chimpanzee, creating cDNA clones and identifying the clone corresponding to the HCV genome [112]. Using these methods, different strains of HCV were indentified and categorized into at least six major genotypes based on nucleic acid sequence alone. Genotypes are approximately 65% identical across the whole HCV genome. Within each genotype, subtypes are also evident with approximately 80% nucleic acid similarity [113]. The different genotypes have different geographical distributions and prevalence, with genotype 1 being the most prevalent genotype in North America and Europe while genotype 4 is most common in Egypt and North Africa and genotypes 5 and 6 are most common in South Africa and Hong Kong. Genotypes 2 and 3 are common in North America, Europe and Japan, but to a lesser extent than genotype 1. A high number of subtypes are evident in Africa and Southeast Asia, suggesting that this region may be the original source of HCV as fewer subtypes are evident in Europe and North America [103]. Currently, genotyping of the virus in an infected individual is accomplished by DNA hybridization, restriction length fragment polymorphism, direct nucleotide sequencing using

polymerase chain reaction, and serologic genotyping. Many of the current methods, excluding direct nucleotide sequencing, cannot discriminate between viral subtypes.

It has become very clear that the HCV genotype is clinically relevant. In acute infection, the rate of progression to chronic infection has been related to genotype, with an increased number of genotype 1 infected individuals progressing to chronicity in comparison with other genotypes [114]. Perhaps more importantly, 4 treatment response rates to combination therapy are directly correlated with HCV genotype. Genotype 1 or 4 infected individuals will only respond to treatment approximately 40-50% of the time, and only with a longer course of therapy than genotype 2 and 3 infected individuals who are able to clear the virus in approximately 70 to 80% of cases with a shorter treatment duration. The reason for this dramatic difference between genotypes is largely unknown, but may be related to an interferon sensitivity determining region in the NS5a protein. Interestingly, HCV genotype has also been correlated with the development of steatosis in HCV infected individuals. Several studies have shown that a substantially increased number of HCV genotype 3a infected patients have steatosis, even in the absence of contributing factors such as obesity, diabetes and alcoholism. Furthermore, steatosis is abolished upon successful treatment and clearance of the virus, suggesting that HCV genotype 3a may play a direct role in altering hepatic lipid metabolism that is not as evident with other genotypes.

Within each HCV infected individual, many different sequences of the virus exist which are 90-99% identical at the nucleotide level and are members of the same infecting genotype. These populations of virus, called quasispecies, are generated by the error prone nature of the HCV RNA-dependent RNA polymerase during genomic replication. These quasispecies populations exist in high numbers during acute infection; as infection progresses, the number of dominant quasispecies population decreases with the major quasispecies strain changing intermittently. The role of these quasispecies populations is twofold. Firstly, high mutation rates can result in the development of new viral strains that have increased viral fitness over the parent strain. Secondly, viral quasispecies likely serve as a mechanism for immune evasion and viral persistence. As the immune system mounts an antibody response against a particular viral epitope, the virus quickly mutates and the dominant viral population changes to one that the immune system no longer recognizes. In a similar fashion, the virus can also quickly generate mutants that are resistant to antiviral drugs. The high mutability of the HCV virus poses several challenges to the development of an effective vaccine or antiviral drug.

4.2 HCV proteins

The HCV genome is translated into a polyprotein that is subsequently cleaved into approximately 10 proteins in the sequence Core-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b. The Core, E1, E2 and p7 proteins are considered structural proteins of the virus while NS2, NS3, NS4a, NS4b, NS5a and NS5b are non-structural proteins.

4.2.1 Structural proteins

The core protein is cleaved from the polyprotein via host signal peptidase to form a 191 amino acid immature form of the protein. The core protein is then cleaved between amino acids 173 to 179 by host Signal Peptide Peptidase (SPP) to form the mature protein [115]. The core protein is a dimeric alpha-helical protein that can bind RNA and consists of two major domains. The D1 domain is primarily hydrophilic and is located in the N-terminus while the D2 domain is primarily hydrophobic and located in the C-terminus. D2 assists in the folding of the protein as well as membrane and lipid droplet association. The core protein is localized on the endoplasmic reticulum and on the surface of lipid droplets in infected cells. Some studies have also shown a small amount of core protein in the nucleus and on mitochondria, but this localization has not been observed in infected cells. Localization of core with lipid droplets is also mediated by proteolitic cleavage by SPP [116]. Mature core proteins form the nucleocapsid of the virus. However, many studies have shown alternative roles for the core protein in the pathogenesis of HCV including apoptosis, insulin resistance, cell cycle, and lipid metabolism. Interestingly, the core protein-encoding region also contains a +1 alternative reading frame that results in translation of the ARF (alternative reading frame) protein. The F protein does not contain an AUG start codon, so translation of this protein is a random event. No known function of the F protein is currently known. Although antibodies against it are present in HCV infected individuals, it is not required for viral replication [117].

The E1 and E2 proteins of HCV are envelope glycoproteins that are essential for virus entry and form heterodimers on the viral envelope. The E2 protein contains a hypervariable region that mutates frequently to allow for the development of immune escape variants [117,118].

The p7 protein has recently been found to have ion-channel activity and it is essential for virus infectivity. As such, new research has been directed toward developing antiviral compounds to block the function of this protein [119].

4.2.2 Non-structural proteins

The NS2 protein is a hydrophobic protease that cleaves the HCV polyproteins between NS2 and NS3 and it may function as a novel cysteine protease. Efficient cleavage of NS2/3 is required for HCV replication, but the presence of NS2 is not required for genome replication. Following cleavage, NS2 is localized in the ER membrane. Besides its protease activity, NS2 appears to be involved in apoptosis, cell proliferation, innate immunity and lipid metabolism. The NS3 protein is a protease and RNA helicase. Protease activity of NS3 is mediated by the NS4a protein. Both proteins are localized on the ER. The NS3/4a protease complex cleaves the HCV polyprotein downstream of NS3 and this action is essential for formation of the viral replication complex. NS3/4a is able to inhibit the host innate immune response via disrupting the RNA helicase
retinoic acid-inducible gene-I (RIG-I) pathway by cleaving Cardif, an adaptor protein of interferon regulatory factor-3. The function of the NS3/4a helicase activity is still unknown.
The NS4b protein is present on the ER membrane and induces intracellular membrane changes that may be involved in the formation of the replication complex membranous webs. It also contains a nucleotide binding motif that hydrolyzes GTP. NS4b is thought to play a major role in viral replication, assembly and release as well as lipid metabolism. NS5a is also an ER-associated protein that is particularly multi-functional. It can bind to HCV RNA and it is essential for viral replication [120]. It can be hyperphosphorylated in order to inhibit viral replication. NS5a is also able to decrease the immune response via inhibition of interferon-induced double stranded RNA activated protein kinase PKR. NS5a may also play a role in mediating lipid metabolism as it interacts with core proteins on the surface of lipid droplets and it also interacts with apolipoproteins. NS5b is an RNA dependent RNA polymerase that synthesizes RNA using an RNA template. It is also localized on the ER membrane and it is essential for viral replication. As such, it is a prime target for the development of antiviral compounds [121].

4.3 HCV LIFE CYCLE

4.3.1 Receptors and entry

The entry of HCV into a naïve host cell appears to be a complicated, multi-step process involving several different host receptors. The initial entry steps have only been recently characterized thanks to the newly developed infectious cell culture system, and many details are still to be discovered. Heparin, a glycosaminoglycan, has been long suspected to play a role in viral entry with several studies showing inhibition of viral attachment in the presence of heparinase, an enzyme that disrupts heparin. Low-density lipoprotein receptor (LDLR) is very likely a critical component of viral attachment. HCV in the serum of infected patients is bound to low density and very low-density lipoproteins. Adsorption of HCV from the serum of infected patients can be inhibited through the use of antibodies against LDLR suggesting that the association of HCV with lipoproteins is an important step in attachment of the virus. CD81, a cell surface protein in the tetraspanin family, is considered one of the major players in HCV attachment. Antibodies against CD81 effectively inhibit HCV infectivity in different models. In addition, HepG2 and HH29 hepatoma cells, which are not normally permissive to HCV infection, can be infected following ectopic expression of CD81. However, expression of CD81 in other non-permissive cell lines does not allow HCV infection to occur, suggesting that other host cell factors may be involved in the attachment and entry process. Scavenger receptor class B type I (SR-BI) is another cell surface protein that may be involved in viral attachment and entry. SR-BI is a receptor for lipoproteins and it can also change the lipid composition of membranes. Blocking of SR-BI with antibodies can prevent virus entry. It is still unclear whether SR-BI is interacting directly with the virus proteins or if it is interacting with virus associated lipoproteins to mediate virus entry as both mechanisms have been observed [122-126]. Recently, two tight junction cell proteins Claudin-1 and Occludin have been shown to be involved in HCV entry. Claudin-1 can be ectopically expressed in non-permissive, non-hepatic 293T cells and allow HCV infection to occur. Knock-down of Claudin-1 in permissive Huh7 cells can reduce HCV infectivity. It has also been found that Claudin-1 and Occludin are involved in late entry of the virus into the cell, which likely occurs through interaction with the tight junctions. The use of the tight junctions by HCV may also allow for cell to cell spread of the virus. It has been shown that HCV entry is mediated by endocytosis in a pH-dependant manner. In addition, knock-down of clathrin, a critical component of the endocytotic

vesicles, prevents HCV infection. Fusion of the virus envelope with the endosome membrane is not well characterized and both E1 and E2 are likely involved in the process [127].

4.3.2 Replication, assembly and release

HCV replication occurs in a membrane associated replication complex that contains several viral and host proteins and replicating RNA. The positive strand of the virus RNA genome is made into a negative strand that is then used to make more positive strand copies for packaging. The NS5b protein is the major player in the synthesis of both the positive and negative strands. The HCV replication complex appears to contain a membrane alteration called a membranous web that can be observed by electron microscopy in infected cells *in vitro* and *in vivo*, as well as in cells expressing the NS4b protein alone. The source of the membranous web is likely the ER as most of the HCV proteins are associated with the ER upon translation. It is also possible that viral replication is occurring on lipid rafts that contain large amounts of cholesterol and sphingolipids. Inhibitors of sphingolipid synthesis can prevent HCV replication and both non-structural proteins and replicating RNA are found on lipid. Recently, it has been determined that lipid droplets play an important role in virus assembly. Core protein localizes around lipid droplets where it recruits non-structural proteins and replication complexes. Inhibiting lipid droplet localization of core protein decreased the production of infectious virus particles. There are also many different host cell proteins with putative roles in HCV replication. For example, vesicle-associated membrane protein-associated proteins A and B (VAP-A and VAP-B) localize in the ER and bind both NS5a and NS5b and are essential for viral replication. Cyclophilin B, a peptidyl-prolyl cis-trans isomerase, binds to NS5b and enhances its RNA binding activity. Similarly, p68, and RNA helicase, binds NS5b and moves into the cytoplasm from the nucleus to assist in viral replication. Very little is known about HCV assembly and release. There are several different forms of the virus found in infected serum, including free mature virions, virions bound to low-density and very low-density lipoproteins, virions bound to immunoglobulins and non-enveloped nucleocapsids. It is believed that viral RNA can interact with the core protein, which then oligomerizes to form the nucleocapsid around the viral RNA. The envelope of the virus is then obtained through core interaction with E1 glycoprotein on the ER membrane, from which budding occurs. From this point it is thought that the virus is

released from the cell after transit through the Golgi apparatus and the secretory pathway [128].

4.3.3 Virus-like particles

In the serum of HCV infected individuals, several different forms of HCV can be found that differ in both size and density. Currently very little is known about HCV assembly and how these different types of HCV particles are formed and secreted. HCV RNA can be found in infected serum at a density between 1.03 and 1.25 g/ml. Interestingly, the lower density particles appear to be more infectious than the higher density particles when tested in chimpanzees. The low-density particles contain high amounts of triglycerides, as well as HCV RNA, core protein and apolipoproteins B and E (ApoB and ApoE). Triglycerides along with ApoB and ApoE are the major components of very-low density lipoproteins (VLDL) and low-density lipoproteins (LDL) which are particles packaged in the ER of hepatocytes to be secreted into the bloodstream and transport their triglyceride load to adipocytes for storage. During HCV infection, assembled virus particles are hypothesized to use this pathway to acquire ApoB, ApoE and triglycerides prior to secretion from the cells. Virus particles that do not obtain the necessary triglycerides and apolipoproteins are not secreted from the cell and undergo degradation. Exploitation of the VLDL assembly pathway by HCV ultimately decreases lipid export from the cells and could be an important factor in the development of steatosis in infected patients. The addition of apolipoproteins and triglyceride to the virus particle could also be advantageous to the virus as it could be providing a mechanism of immune escape by hiding viral epitopes and also by increasing the number of potential receptors (ie. CD81, LDLR and SR-BI) for attachment [129-133]. Another fraction of the HCV population in infected serum appears to be nonenveloped. These particles have a higher density and vary in size. The core protein is displayed on the surface of these particles and antibodies against core protein are a hallmark of HCV infection. Currently it is unknown whether these nonenveloped particles are infectious or what role they play in the course of HCV infection and pathogenesis [134].

4.4 HCV MOLECULAR PATHOGENESIS

4.4.1 Cell survival and apoptosis

Apoptosis is a very important mechanism for the removal of virus infected cells and many viruses inhibit apoptotic pathways in order to ensure their continued survival. However, in chronic HCV infection an increased amount of apoptosis is observed in the liver although it is unknown whether HCV infected or uninfected hepatocytes are undergoing apoptosis [135,136]. Three major death ligands, CD95 Ligand, Tumor Necrosis Factor α (TNFα) and Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL), are considered to be the major players in inducing hepatocyte apoptosis. Infection with HCV up-regulates all three of these death ligands. In addition, CD95 Ligand induced apoptosis was observed in uninfected bystander cells of HCV infected livers [135-138]. Interestingly, TRAIL can also induce the development of steatosis [39]. When specific HCV proteins were examined for their influence on apoptosis, results showed that all HCV proteins have both pro-apoptotic and anti-apoptotic effects depending on the model being used and it is currently unclear as to what occurs *in vivo.* Core proteins have been shown to inhibit TNFα and CD95 Ligand induced apoptosis in cell culture, while other studies have shown that the presence of core protein, including those expressed in transgenic mice, cannot inhibit CD95 Ligand induced apoptosis [140-142]. Core proteins can directly interact with several pro-apoptoic proteins including CD95, TNF-R1 and lymphotoxin-β. However, it can also bind to FADD, C-FLIP and Smad3 to prevent apoptosis. Core can also increase phosphorylation of Akt, an important protein involved in cell survival [143]. Core proteins also induce oxidative stress that can lead to increased susceptibility to apoptosis (Okuda et al., 2002; Machida et al., 2006). The E1 protein inhibits TRAIL-induced apoptosis, while E2 can induce caspase dependent apoptosis. NS2 can inhibit CIDE-B induced apoptosis, while NS3 can cleave Cardif and prevent RIG-I associated apoptosis. NS4a can induce caspase-8 independent apoptosis from the mitochondria. NS5a has shown both anti-apoptotic and pro-apoptotic effects. It can inhibit Baxmediated apoptosis by acting as a bcl-2 homologue. It can also activate the PI3K/Akt pathway and prevent p53- mediated apoptosis. However, direct apoptosis has been observed in cells expressing NS5a. All of these results suggest an important but complex role of HCV and its proteins in mediating cell survival and apoptosis that is yet to be fully explained. The anti-apoptotic potential of this virus may influence the development of hepatocellular carcinoma, a common complication of HCV infection [144].

4.4.2 Immunopathogenesis

It is generally accepted that liver damage during HCV infection is primarily caused by the reaction of the immune system to virus infection, although some cytopathic effects of the virus such as increased apoptosis and the development of steatosis have been observed. HCV itself has also been shown to alter both the innate and adaptive immune responses. The NS3/4a protease is able to induce cleavage of mitochondrial antiviral signaling protein (MAVS), which results in the impairment of RIG-I mediated interferon production in the presence of dsRNA. During viral infection, cytotoxic lymphocytes are critical to the efficient removal of infected cells. In acute HCV infection, the appearance of HCV specific cytotoxic CD8+ T cells (CTLs) is associated with decreased viremia. Activation of HCV specific CD38+ CD69+ T cells coincides with inflammation and hepatitis during acute infection, suggesting a role for these non-interferon γ producing CTLs in causing immune mediated liver damage. Regulatory T cells have been shown to have an important role in suppressing HCV-specific CTL responses, as increased numbers of regulatory T cells are present in chronically infected patients versus those who have spontaneously recovered from their infection. Therefore, it is likely that CTLs play a role in both controlling viremia and causing liver damage during HCV infection.

Natural Killer (NK) cells are also important lymphocytes involved in clearing viral infection. NK cells can directly or indirectly induce apoptosis in virally infected hepatocytes that may contribute to both viral clearance and liver damage. HCV infected cells have decreased MHC class I protein expression, which is a primary inducer of NK mediated cell lysis. Conversely, NK cells may actually protect the liver from progression to fibrosis by removing activated hepatic stellate cells, the main fibrogenic cell type in the damaged liver. Indeed, increased NK cytolytic activity is inversely correlated with the fibrosis stage in the liver [145].

γδ T cells are also important lymphocytes in the liver during viral infection. Increased levels of γδ T cells are present in the livers of chronic HCV patients [146]. However, γδ T cells have also been shown to have a protective effect on the liver during chronic viral infection, suggesting that they too may also decrease liver injury during infection [147].

4.4.3 Lipid metabolism

One of the major functions of the liver is to metabolize lipids. As such, infection of hepatocytes with HCV is likely to affect cellular pathways involved in lipid metabolism. Indeed, patients infected with HCV show clinical disease associated with lipid metabolism defects such as steatosis and hypolipoproteinemia [148]. *In vitro* studies have also demonstrated an important role for the virus in exploiting lipid metabolism pathways in order to produce virus particles and evade the immune system that contribute to HCV pathogenesis [149].

4.5 HCV CLINICAL CHARACTERISTICS

4.5.1 Acute viral infection

Upon infection with HCV, most patients enter an acute phase that is largely asymptomatic. Most of the infected individuals (~80%) will progress to chronic HCV infection while the other 20% will clear the virus within the first 3 months after infection. The asymptomatic nature of the acute stage of infection results in difficulty in diagnosing and reporting HCV infection, as well as difficulty in studying the aspects of the immune response that allow for viral clearance during this time. The greatest risk of infection with HCV is through intravenous drug use, unprotected sex, exposure during medical procedures, vertical transmission from mother to child, or needle injury in health care professionals. Blood transfusion prior to 1992 is also an important risk factor (Centers for Disease Control and Prevention. Surveillance for acute viral hepatitis United States, 2005. 56th ed. Atlanta, GA: Centers for Disease Control and Prevention; 2007. P. 1-24). HCV RNA can be detected in the serum approximately 1-3 weeks after exposure. Symptoms include fatigue, jaundice, dyspepsia, and abdominal pain but are generally not specific or severe enough to warrant consultation of a doctor. Elevated alanine aminotransferase (ALT) levels can be detected 4-12 weeks after exposure and are the first indication of liver injury. Serum HCV RNA and anti-HCV antibody seroconversion is generally used to detect HCV infection. Host and viral factors appear to contribute to viral clearance in acute infection including genotype of the virus, HIV co-infection, gender, race, age and HLA. A strong cellular immune response is also thought to increase viral clearance, and symptomatic infection appears to correlate

with viral clearance. Patients who spontaneously clear the virus need to be monitored as disease relapse may occur up to 6 months after clearance [150].

4.5.2 Chronic viral infection

Infection with HCV is one of the leading causes of liver disease worldwide. As previously discussed, a small proportion of people are able to clear the virus following an acute infection but the majority progress to a chronically infected state. Chronic infection can persist for many years without causing the death of the patient. However, most cases eventually lead to the development of hepatitis, fibrosis, steatosis and cirrhosis with the eventual need for a liver transplant if left untreated. A small number of HCV infected individuals will also develop hepatocellular carcinoma. Host factors such as age, obesity, alcohol use, gender, race and co-infection with HIV or HBV can increase the rate of progression of liver. Genotype does not appear to play a role in disease progression. However, the presence of non-alcoholic steatohepatitis can increase progression of disease, and infection with genotype 3a is associated with a higher prevalence of steatosis. In general, the rate of progression to cirrhosis is unpredictable in chronic HCV infection [151].

4.6 HCV GENOTYPING METHODS

4.6.1 Molecular genotyping

Because differences in geographical distribution, disease outcome, and response to therapy among HCV genotypes have been suggested, reliable methods for determining the HCV genotype may become an important clinical test. The HCV genotype can be determined by nucleotide sequencing of a specific PCR-amplified portion of the HCV genome obtained from the patient, followed by composition of a phylogenetic tree, which is presently the 'gold standard' for the detection and identification of the various HCV genotypes and subtypes. Investigators of HCV genotyping have used sequence analysis of HCV NS5, core, E1, and 5'-UTRs. Other methods focus on the amplification of defined regions of the HCV genome by reverse transcription (RT)-PCR followed by digestion with restriction enzymes and restriction length polymorphism analysis (RFLP) (31), amplification with genotype-specific primers, hybridization of genotype-specific probes with

the amplified products heteroduplex mobility assay, melting curve analysis with fluorescence resonance energy transfer probe and DNA enzyme assays. HCV typing can also be done by analyzing the sequences of several regions of the virus genome. A commercial kit (InnoLipa) for HCV genotyping has been introduced in Europe by Innogenetics (Zwijndre, Belgium), which is based on hybridization of 5'-UTR amplification products with genotype specific probes . It has been shown that genotyping methods using 5'- UTR, including InnoLipa, may not discriminate subtype 1a from 1b in 5 to 10% of cases and also may not distinguish between subtypes 2a and 2c. Others have used restriction enzymes to determine a restriction fragment length polymorphism. In this method, a PCR amplified DNA fragment is digested into fragments with different lengths by enzymes (restriction endonucleases) that recognize cleavage sites specific for each genotype. Investigators have used different regions of the HCV genome for restriction fragment length polymorphism, including NS5 and the 5'-UTR. Although the 5'-UTR region is sufficiently variable to allow HCV genotypes in most clinical situations, it cannot always characterize HCV subtypes and may fail to trace the origin of a de novo infection. The NS5b region contains a subtype-specific motif which makes it suitable for epidemiological applications. It is difficult to assess the actual prevalence of mixed-genotype infections by currently available assays, including direct DNA sequencing, since they are designed to identify only the HCV genotype dominant in the population.

4.6.2 Serologic genotyping

More recently, investigators identified genotype specific antibodies that could be used as indirect markers for the HCV genotype. Serological typing uses enzyme immunoassays to detect the antigenic properties of several specific epitopes encoded by the NS-4 or the core regions of the HCV genome. Serologic genotyping has several advantages that make it suitable for large epidemiologic studies. These advantages include the low risk of contamination and the simplicity of the assay. However, serologic typing seems to lack specificity and sensitivity, which limits its usefulness.

4.7 Clinical relevance of HCV genotype

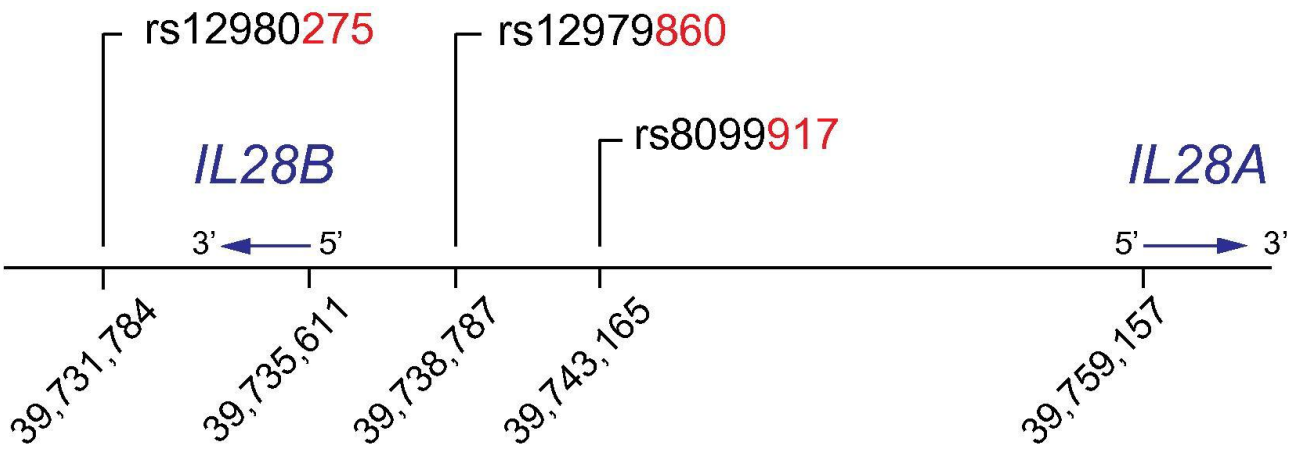
Genotype, viral load, and liver histology are important parameters used in selecting an antiviral therapy with the greatest chance of success. Genotyping and subtyping of HCV is relevant to the epidemiology of HCV, vaccine development, clinical management, and

assessment of the risk-benefit ratio of therapeutic measures against chronic HCV infection. It has been postulated that differences in nucleotide sequence could result in differential activity of HCV proteins that could alter the rate of HCV replication, sensitivity to the antiviral activity of interferon, or pathogenicity of the virus. In recent years, substantial evidence has emerged indicating that typing and subtyping for HCV is important clinically; genotype 1 in particular cannot be treated efficiently with IFN-alpha, while genotypes 2 and 3 respond favorably. The causes of variation in treatment response are not well understood. Studies of Japanese patients infected with subtype 1b indicated that the outcome of interferon therapy was correlated with genetic variability in a portion of the NS5A gene (the interferon sensitivity determining region, ISDR), although subsequent studies of European patients did not confirm this result. Moreover, genotype 1 infection may proceed more rapidly to severe forms of chronic hepatitis, cirrhosis and hepatocellular carcinoma, when compared with genotype 2 and 3.

CHAPTER 5

INTERFERON LAMBDA 3 GENE POLYMORPHISM (IL28B)

Host genetic factors may predict the outcome and treatment response in hepatitis C virus (HCV) infection. Recently, three landmark genome-wide association studies identified single nucleotide polymorphisms near the interleukin 28B (IL28B) region, which were more frequent in responders to treatment. IL28B encodes interferon (IFN) λ3, a type III IFN involved in host antiviral immunity. Favorable variants of the two most widely studied IL28B polymorphisms, rs12979860 and rs8099917, are strong pretreatment predictors of early viral clearance and sustained viral response in patients with genotype 1 HCV infection. IL28B has been also implicated in the development of chronic HCV infection versus spontaneous resolution of acute infection and suggest that IL28B may be a key factor involved in host immunity against HCV.



***L28B* gene locus on human chromosome 19.** The 3 most strongly associated SNPs are in the genome regions flanking the *IL28B* gene. Nucleotide numbers on chromosome 19 are indicated for three SNPs and for the start of the coding region of the *IL28A* and *IL28B* gene.

5.7.1 IL28B polymorphism *Rs12979860*

Rs12979860 is a SNP on chromosome 19q13 and is strongly associated with sustained virologic response (SVR) in genotype 1 HCV-infected subjects [152]. Results in three possible genotypes: the C/C genotype was associated with 2.5 or greater rate (depending on ethnicity) of SVR compared to the T/T genotype, and the C allele may favor

spontaneous clearance of HCV [152]. The rs12979860 polymorphism also may explain much of the difference in response between different population groups: in fact, the genotype leading to better response presents with greater frequency in European than in African populations [152]. Moreover, HCV-infected patients with C/C genotype showed association with lower steatosis severity grade in HCV genotypes 2, 3, and 4, and also in HCV/HIV-infected patients even though prior non-responders [153-158]. The C allele also appears to affect positively early viral kinetics in patients with chronic hepatitis C receiving interferon-free treatment [159,160]. The C allele may differentially regulate the course of genotype 2 and 3 infection [161,162]. IL28B plays a determinant role in natural clearance of HCV and spontaneous resolution of HCV infection [163]. However, rs12979860 homozygosity is not associated with resistance to HCV infection in exposed uninfected patients [164]. Studying the impact of donor and recipient genotypes of IL28B rs12979860C>T SNP on hepatitis C virus (HCV) liver graft reinfection revealed a dominant but not exclusive impact of the donor rather than the recipient genetic background on the natural course and treatment outcome [165]. Interestingly, the risk of developing post-transplant diabetes mellitus is significantly increased in recipients carrying the IL28B rs12979860C>T SNP [166].

5.7.2 IL28B polymorphism rs8099917

The minor allele (T/G or T/T) was associated with progression to chronic HCV infection and also with failure to respond to therapy, with the strongest effects in patients with HCV genotype 1, 2, or 4. However, T/T genotype may contribute to the increased viral clearance rate and better virological responses in Taiwanese patients with a lower daily viral production rate than Western patients. Even though there are no studies considering multiethnic cohorts, a recent meta-analysis evidenced that rs8099917 T/T had slight predictive value in Asian patients. A further study reported that combination analyses of SNP of rs8099917 in recipient and donor tissues and mutations in HCV RNA allowed prediction of SVR to PEG-IFN/RBV therapy in patients with recurrent HCV infection after orthotropic liver transplantation. Another study revealed the high prevalence of the rs8099917 G allele in HCV/HIV-1-coinfected patients as well as its strong association with treatment failure in HCV genotype 1-infected patients. The rs8099917 T/T genotype is associated with higher levels of apoB-100 and LDL cholesterol in genotype 1-HCV-infected patients.

5.7.3 Combined IFN3λ Polymorphisms

Both favorable genotypes for rs12979860 (C/C) and rs8099917 (T/T) were associated with spontaneous HCV clearance, possibly interacting in synergy with female sex. On the other side, IL28B variants associated with poor response to interferon therapy may predict slower fibrosis progression, especially in patients infected with non-1 HCV genotypes. Concerning HCV genotype 3-infected subjects, IL28B polymorphisms are associated with RVR but not SVR to PEG-IFN therapy [167]; other studies showed that IL28B polymorphisms are strongly associated with the first phase viral decline during PEG-IFN/RBV therapy of chronic HCV infection, irrespective of HCV genotype, and in genotype 1– 4 HIV/HCV-coinfected patients [166-169]. These reports suggest that IL28B polymorphisms could play a role in blocking the production or release of virions in the first phase viral decline [170]. Treatment outcome in HCV-infected patients may be also influenced by viral polymorphisms within the viral core and NS5A proteins, even though it has been clearly demonstrated that IL28B polymorphisms and HCV core amino acid 70 substitutions contribute independently to an SVR to PEG-INF/RBV therapy [171]. A further study demonstrated that SNPs rs8099917 and rs12979860 used alone may be useful for predicting the outcome of HCV treatment, and in a rational and cost-effective approach, determination of only one of these two SNPs is sufficient for predicting SVR. Because of the highest predictive SVR associated with rs12979860 C/C compared with the rs8099917 T/T, rs12979860 determination alone is sufficient for predicting interferon response [176, 177], even in HIV/HCV-coinfected patients [178]. However, there is evidence that a significant proportion of heterozygous carriers of the rs12979860 T nonresponder allele can profit with respect to SVR prediction by further determination of the rs8099917 SNPs [179].

AIM

HIV

Toll-like receptors (TLR) play a crucial role in the host's innate immunity and may influence HIV-1 disease progression. The transcription factor IRF-5 is an important player in the TLR-MyD88 signaling cascade. We investigated the impact of two SNPs in TLR9 gene, rs352139 and rs352140, and two SNPs in IRF5 gene, rs10954213 and rs11770589, on CD4 count, HIV viral load, and clinical progression in a cohort of HIV-infected patients.

HCV

In patients with chronic hepatitis C, the hepatitis C virus (HCV) RNA level is an important predictor of treatment response. To explore the relationship of HCV RNA with viral and demographic factors, as well as *IL28B* genotype, we examined viral levels in an ethnically diverse group of injection drug users (IDUs).

MATERIAL AND METHODS

METHODS

1. HIV STUDY*:*

1.1 Patients:

All individuals of this study were enrolled by the Departments of Infectious diseases of the Luigi Sacco Hospital in Milan and written informed consent was obtained before enrolment. For this project a total of 262 HIV-infected patients were enrolled of which 99 were LTNP and 163 progressors.

- Progressor patient under ARV therapy;
- LTNP with HIV plasma viremia has been under the threshold for at least 8 years, CD4+ ≥ 500 cell/ul years, never under teraphy, in absence of HIV sympthoms and opportunisitc infections

  .

1.2 Patients collection and samples processing

Whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (ethylenediaminetetraacetic acid) (Becton Dickinson, Rutherford, NJ, USA). Samples were centrifugated 1400rpm for 10 minutes, plasma obtained was collected and stored at -20°C for subsequent analyses. The remaining sample, composed by erythrocytes, PBMC, granulocytes and platelets, was diluited in PBS (phosphate buffered saline) (PBI International, Milano, Italy) and separated thanks to a density-gradient centrifugation on lymphocyte separation medium (Ficoll-Hypaque) (Cedarlane Laboratories Limited, Hornby, Ontario Canada) for 25 minutes at 2300rpm. PBMC are localized between the phase of fycoll-hypaque and the phase including granulocytes and erythrocytes, they are collected and washed twice in PBS for 10 minutes at 1900 rpm. Cellular pellet was resuspended in PBS and washed twice in phosphate-buffered saline (PBS) (PBI International, Milano, Italy). Cellular count was conducted using an automatic cell counter.

1.3 Cell count

The number of viable leukocytes was determined by the use of an automatic cell counter ADAM-MC (Digital-Bio, NanoEnTek Inc., Corea). ADAM-MC automatic cell counter measures total cell numbers and cell viabilities by cutting edge detection technologies. The

instrument is able to count the cells thanks to the use of propidium iodide, a fluorescent dye that intercalates in the DNA of cell characterized by damage cell membranes. It‟s based on two different solution, AccuStain Solution T is composed by propidium iodide and lysis solution while AccuStain Solution N is composed by propidium iodide and PBS. AccuStain Solution T allows to count all cells present in the sample while AccuStain Solution N just non-viable cells.

To evaluate cell concentration, a small quantity of sample (30µl) is combined with an equal volume of each solution. Samples prepared in this way are directly transferred in a disposable slide and this is read by the instrument. A green laser (532nm) is focalized on the slide and the cells colored are detected by a CCD camera (B/W CCD). On the screen it‟s shown total cell count, the count of non-viable cells and the vitality of each sample is calculated by ADAM-MC software. The number of cell/ml is obtain with the substraction of total number of non-viable cells from the number of total cells.

1.4 Genotyping of the SNPs in TLR9 and IRF5 gene

Two single nucleotide polymorphisms (SNPs) rs352139 and rs352140 of TLR9 were genotyped by sequencing 163 progressors and 99 LTNP HIV-1-positive patients. PCR was carried out in a reaction mixture containing 40 ng of DNA, 10 µl of HotStar Taq Master Mix (Qiagen, Germantown, MD) and 100 pmol of each of the following primers: for rs352139 TLR9, forward 5'-GCCAGGGATTGGTTAAGTGA-3' and reverse5'-GTAGGAAGGCAGGCAAGGTA-3'; for rs352140 TLR9, 5'-CAGTCAATGGCTCCCAGTTC-3' and reverse 5'- TTGGCTGTGGATGTTGTTGT-3'; for rs10954213/rs11770589, forward 5-CCCTGATTTCCCTGGTTTG-3 and reverse 5'-AGCCAGCCAGGTGAGTGTT-3. The reaction mixture was denatured at 95°C for 15 minutes and cycled 35 times at 94°C for 45 seconds, Ta = 55°C for 45 seconds, 72°C for 60 seconds, with final extension at 72°C for 10 minutes. PCR product was treated with Exosap-IT (USB Corporation, Cleveland, OH) to removed excess primers. 3.5 µl out of 20 of purified DNA product was combined with 2.0 µl of Big Dye terminator (ABI Prism Big Dye Terminator cycle sequencing ready reaction kit v3.1, Applied Biosystems, Foster City, CA) and 100 pmol of forward primer.

The sequencing reaction was carried out for 30 cycles of denaturation (96°C/1 m), annealing (50°C/30 s) and extension (60°C/ 4 m). Excess dye terminators were removed

using DyeEx 96 Kit columns as per Manufacturer's instructions (Qiagen Inc. Germantown, MD). Electrophoresis was performed on ABI Prism 3730 XL instrument (Applied Biosystems, Foster City, CA).

1.5 Statistical analysis

Association analyses were conducted by chi-square test using 2 × 2 and 2 × 3 contingency tables. When 1 or more variables in the contingency table were ≤ 5, Fisher's exact test was used. Two side probability values (p2-value) < 0.05 were considered to be statistically significant.

Calculation of linkage disequilibrium (LD) parameters (r2 and D') based on genotype data from 262 patients was performed using LDPlotter tool from https://pharmgat.org/Tools

2. HCV STUDY METHODS*:*

2.1 Subjects and Data Collection

As previously reported, UHS investigators recruited IDUs from six San Francisco Bay area neighborhoods (10). All individuals 18 years of age or older who had injected illicit drugs within the past 30 days or who had previously participated in UHS were eligible for enrollment. Study participants received modest monetary compensation. Although some participants had received hepatitis B vaccine (9), few, if any, were treated for HBV or HCV infection. Participants were not asked about treatment for HCV infection during 1998-2000, but in 2002 only 3% of UHS participants reported interferon-based treatment for HCV infection, (11) thus the vast majority of subjects in this study had never received treatment for chronic hepatitis C. Among the 237 subjects in this analysis who tested positive for human immunodeficiency virus type 1 (HIV-1), 47 (19.8%) reported taking at least one anti-retroviral drug at the time of enrollment.

Trained staff obtained informed consent from the participants, including explicit written consent for host genetic testing. Participants were interviewed using a standardized instrument, counseled on reducing infection risks, and referred to appropriate medical and social services. Participants were asked about socio-demographic characteristics and their injection drug history, including age at first injection. Blood samples were collected by a trained phlebotomist. Further details about UHS are provided elsewhere.(10) The study

was approved by the Committee on Human Subjects Research at University of California, San Francisco and an Institutional Review Board of the National Cancer Institute.

We assessed possible repeat enrollment by comparing demographic information, including gender, birth date, race and site of enrollment. Enrollees who appeared very similar demographically were evaluated by DNA testing (as described below). Among 2296 UHS participants, 2092 were positive for HCV antibody of whom 2073 had sufficient specimen to be tested for HCV RNA. Among these 2073 participants, 1701 had detectable HCV RNA in the plasma and were included in the current study.

2.2 Viral Serology

As previously described,(9) to define HCV infection status, we first tested for HCV antibody by HCV version 3.0 ELISA Test System (Ortho-Clinical Diagnostics, Raritan, NJ). Participants who were positive by HCV EIA were considered to have been infected with HCV and those with sufficient archived plasma (n=2073) were tested for HCV viremia by a branched-chain DNA assay [VERSANT® HCV RNA 3.0 Assay (bDNA), Bayer-Diagnostics, Tarrytown, NY; analytic sensitivity, $2.5x10^3$ copies/ml]. Those positive for HCV RNA were considered to have chronic HCV infection and those with a negative result were considered to have resolved HCV infection. Methods of testing for HIV-1 and HBV infection status in these subjects have been described.(9)

2.3 Determination of Viral Genotypes

Total nucleic acid was isolated from 500 µl of serum (Roche MagNa Pure LC Total Nucleic Acid Isolation Kit-Large Volume, Roche Diagnostics Corporation, Indianapolis, IN) and reverse transcription was performed. PCR was carried out in a reaction mixture containing 3 µl of cDNA, 10 µl of HotStar Taq Master Mix (Qiagen) and 1 µl of each of the following primers: forward 5'- TGGGGTTCTCGTATGATACCC-3' and reverse 5'-CCTGGTCATAGCCTCCGTGAA-3' to amplify the 5'-NS5b region. PCR product was purified with Exosap-IT (USB Corporation) and combined with 2.0 µl of Big Dye terminator (ABI Prism Big Dye Terminator cycle sequencing ready reaction kit v3.1) and 100pmol of primer forward (5'-NC or 5'-NS5B). The sequencing reaction was carried out for 30 cycles and electrophoresis was performed on an ABI Prism 3730 XL instrument (Genewiz, South Plainfield, NJ, USA).

Raw sequence data were analyzed by Sequencher 4.8 Gene codes to trim ambiguous sequences. To query HCV genotype, sequences were compared to an HCV database operated by the Los Alamos National Laboratory (http://hcv.lanl.gov/content/sequence/BASIC_BLAST/basic_blast.html) using BLAST. Viral genotype call was based on the highest score and lowest e-value, using the NS5B sequence unless those results were negative or missing, in which case genotype was based on the 5NC region.

2.4 IL28B Genotyping

DNA was extracted from cryopreserved lymphocytes using a modified salt precipitation-extraction method (Gentra Systems, Minneapolis, MN) or from granulocytes using silica membrane binding method using Qiagen DNA purification columns (Qiagen Inc, Valencia, CA). The NCI Core Genotyping Facility performed genotyping for *IL28B* rs12979860 using an optimized TaqMan™ assay (available at http://variantgps.nci.nih.gov/cgfseq/pages/snp500.do)

2.5 Statistical Analyses

All analyses were cross-sectional and based on a single study visit. We determined median HCV RNA levels ($\log_{10}$ copies/ml) overall and among subgroups. As neither $\log_{10}$ HCV RNA nor alternative transformations of these data were normally distributed, non-parametric statistical methods were the basis of the analysis. We used the Wilcoxon (Kruskal–Wallis) to compare the distribution of HCV RNA levels for variables in SAS PROC NPAR1WAY. To perform multivariate analysis we divided HCV RNA into quintiles and examined determinants of higher HCV RNA in unconditional ordinal logistic regression models that included age [or duration of injection drug use], gender, race/ethnicity, HBV infection, HIV-1 infection and HCV genotype (SAS PROC LOGISTIC). All analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC).

RESULTS

1. HIV RESULTS :

1.1 POPULATION STUDY

A total of 163 HIV-1 infected Progressors and 99 LTNP patients were included in this cohort. The characteristic of both are summarized in table 2 and 3 respectively.

Table 2. Characteristics of the HIV-Infected **LTNP** Patients

N*		58	
Age (yrs)		49	(35-72)
Sex (% women)		20	(33.9)
Risk (%)			
	Heteroexual	11	(19)
	Homo BS TRSF**	21	(36)
	Transfusion	21	(36)
	homosex	3	(4.5)
	other	3	(4.5)
CD4 ount		780	(273-1837)
HIV RNA		12524	(49-200,000)
HCV+ (%)		34	(60)
HBsAg+ (%)		12	(20)

* clinical data available only on 58 patients (out of 99)
** Homo BS TRSF= Homosexual, Bisexual, Transfusion patients

Table 3. Characteristics of the HIV-Infected **Progressor** Patients at Their Entry in the Cohort

N*	148	
Age (yrs)	50.7	(33 - 75)
Sex (% women)	41	(27.7)
Risk (%)		
Heteroexual	50	(34,7)
Homosexual	23	(15,9)
PTD	50	(34,7)
other	21	(14,5)
HCV+ (%)	63	(42)
HBsAg+ (%)	3	(2)

* clinical data available only on 148 patients (out of 163)

Clinical data were available for 58 patients out of 99 of the Progressors patients and 148 out of 163 of the LTNP patients. Progressors patient was an extremely

heterogeneous group, some patients had already started the antiretroviral therapy (not homogeneous treatment, i.e. TDF/FTC + ATV; ABC/3TC + ATV; fAMP) at the time of the beginning of the study whereas others were naïve to the therapy; the leading factor for this cohort was that the progression of the disease showed a CD4 cell decay <500 cells/ml within 3 years from the date of HIV-1 positive diagnosis. Unfortunately, HIV viral load was only available in LNTP patients.

1.2 GENETIC ASSOCIATION

1.2.1 TLR9 SNPs association results

Rs352139 TLR9 SNP amplification was possible in 162 samples in the progressors and 98 in the LTNP, whereas all the samples were genotyped for rs352140. Genotype and allele frequencies for the two SNPs were in Hardy-Weinberg equilibrium in both groups and are shown in table 4 and 5. Rs352140 G allele was more frequent among LTNP (51% G allele vs. 49% A allele) and conversely A allele was more frequent in Progressors (57% A allele vs. 43% G allele).

Table 4. TRL9 genotype frequencies

		LTNP	**PR**	Totale
rs352139	**AA**	24	29	61
		24.50%	17.90%	19.90%
	AG	48	74	146
		49.00%	45.70%	47.70%
	GG	26	59	99
		26.50%	36.40%	32.40%

		LTNP	**PR**	Totale
rs352140	**AA**	23	58	94
		23.20%	35.60%	30.40%
	AG	51	70	146
		51.50%	42.90%	47.20%
	GG	25	35	69
		25.30%	21.50%	22.30%

Table 5. TRL9 SNPs allelic frequencies and HWE p values.

rs352140 LTNP	AA	AG	GG
Observed	23	51	25
Expected	23.76	49.48	25.8

Exact P=0.84 P(A)= 0.49 P(G)= 0.51

rs352140 Progressors	AA	AG	GG
Observed	58	70	35
Expected	53.1	79.9	30.1

Exact P=0.11 P(A)= 0.57 P(G)= 0.43

rs352139 LTNP	AA	AG	GG
Observed	24	48	26
Expected	23.51	48.98	25.5

Exact P=0.841 P(A)= 0.49 P(G)= 0.51

rs352139 Progressors	AA	AG	GG
Observed	29	74	59
Expected	26.9	78.2	56.9

Exact P=0.52 P(A)= 0.41 P(G)= 0.59

The presence of the AA genotype in rs352140 was predominant in Progressors (rs352140: AG+GG: 76% LTNP vs. 64.4% progressors, AA: 23.2 % LTNP vs.35.6% Progressors, P = 0.025), (Table 6).

Table 6. TLR9 rs352140 SNP association to HIV progression
P=0.025

rs352140	PR	LTNP
AA	58 (35.6 %)	23 (23.2 %)
AG+GG	105 (64.4%)	76 (76.8 %)
	163 (62.2%)	99 (37.8 %)

No statistical association was found between the progressors group and the LTNP according to the frequencies of rs352139 TLR9 SNPs.

No additional associations were found between TLR9 SNPs and viral load or CD4 cell count progression. This association analysis were only performed among the LTNP group because and it was not possible perform it among progressors due to lack of availability of clinical data.

1.2.2 IRF5 SNPs association results

IRF5 SNPs amplification was possible in 163 samples in the progressors and 99 in the LTNP. Genotype and allele frequencies for the two SNPs were in Hardy-Weinberg equilibrium (data showed below). Rs10954213/ rs11770589 GG genotype was less frequent among LTNP (Rs10954213: 11% LNTP vs. 26% PR; rs11770589: 20,2% LTNP vs 22.8% PR) but no differences among those frequencies were statistically significant.

IRF5 genotype frequencies

rs10954213		LTNP	PR	Totale
	AA	33	63	107
		33,3%	38,9%	35,0%
	AG	55	73	154
		55,6%	45,1%	50,3%
	GG	11	26	45
		11,1%	16,0%	14,7%

rs11770589		LTNP	PR	Totale
	AA	24	48	78
		24,2%	29,6%	25,5%
	AG	55	77	158
		55,6%	47,5%	51,6%
	GG	20	37	70
		20,2%	22,8%	22,9%

IRF5 SNPs allelic frequencies and HWE p values.

rs10954213 LTNP	AA	AG	GG
Observed	33	55	11
Expected	36.97	47.06	14.97

Exact P=0.137 P(A)= 0.611 P(G)= 0.389

rs10954213 Progressors	AA	AG	GG
Observed	63	73	26
Expected	61.11	76.77	24.11

Exact P=0.51 P(A)= 0.614 P(G)= 0.386

rs11770589 LTNP	AA	AG	GG
Observed	24	55	20
Expected	26.79	49.42	22.79

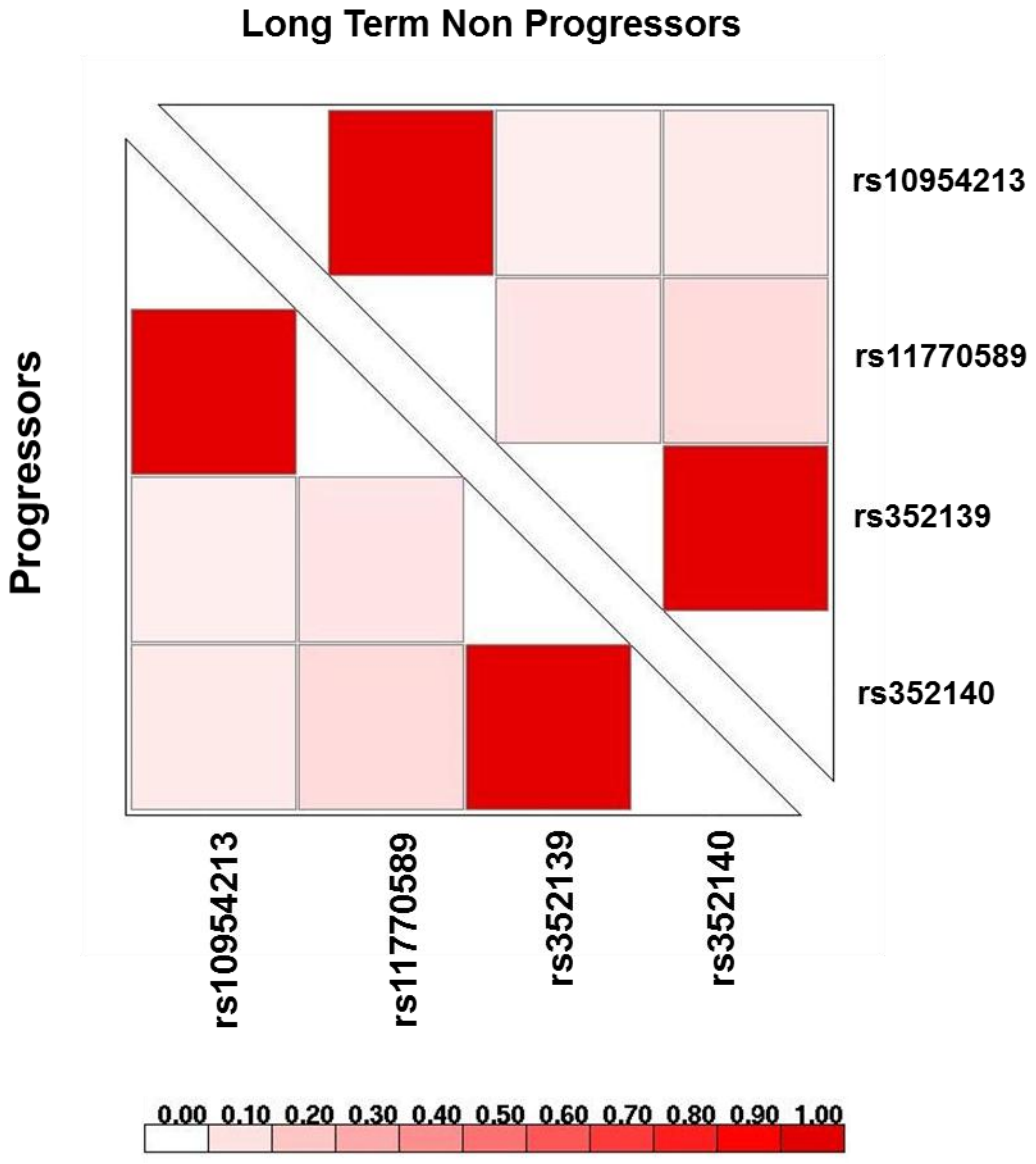
Exact P=0.317 P(A)= 0.52 P(G)= 0.48

rs11770589 Progressors	AA	AG	GG
Observed	48	77	37
Expected	46.19	80.63	35.19

Exact P=0.635 P(A)= 0.534 P(G)= 0.466

1.2.3 Linkage Disequilibrium and Haplotype analyses

Linkage disequilibrium analysis confirmed the results of previous studies that a strong disequilibrium exists between the two SNPs of TLR9 and between the two SNPs of IRF5 with D'= 1, in both Progressors and LTNP.



To investigate their combined effect, given their close proximity, haplotype frequencies were estimated in both groups. However, no association was found in this analysis for none of the SNPs considered in this study (data not showed).

HCV results

1.1 STUDY POPULATION:

A total of 2092 UHS subjects had antibody to HCV. Among these, 2073 participants had sufficient plasma to be tested for HCV RNA of whom 1701 (82.1%) had detectable HCV RNA. Demographic and clinical features for the 1701 participants with HCV viremia were generally similar to those among all UHS subjects with HCV antibodies (Table A). Among those with detectable HCV RNA, the median age at enrollment was 46 years, the median age at which a drug was first injected was 18 years and the median time from first use of injection drugs to enrollment was 26 years. Most participants (72.4%) were men. Over half (56.0%) of the participants considered themselves African American, 34.0% white (non-Hispanic), 6.8% Latino (non- African American), 1.1% were Asian or Pacific Islanders and 2.2% were American Indian or Alaska natives. Infection with HIV-1 was present in 237 (13.9%) participants. As previously reported in this and other cohorts (7, 8, 12), chronic hepatitis B was less frequent and HIV-1 infection was more frequent among participants with chronic hepatitis C.

1.2 PREDICTOR OF HCV RNA LEVELS

1.2.1 All Subjects

Among participants with detectable virus, the median HCV RNA level was 6.45 $\log_{10}$ copies/ml [inter-quartile range, 5.97-6.89]. Median viral levels were progressively higher in each older age category, ranging from 6.15 $\log_{10}$ copies/ml among participants 18-29 years at enrollment to 6.59 $\log_{10}$ copies/ml among those >50 years of age ($p<0.0001$; Table B). Duration of injection drug use is highly correlated with age at enrollment in UHS participants ($r^2=0.74$) and there was also a strong trend toward higher HCV RNA levels with longer duration of drug use (<0.0001). HCV RNA levels were higher in men (6.52 $\log_{10}$ copies/ml) than women (6.29 $\log_{10}$ copies/ml; $p<0.0001$). With regard to race and ethnicity, the highest levels were found in African American participants (6.49 $\log_{10}$ copies/ml), intermediate viral levels were found in white participants of non-Hispanic origin (6.35 $\log_{10}$ copies/ml) and

Latinos (6.39 $\log_{10}$ copies/ml), and the lowest levels were found in those who reported their ancestry as Asian, Pacific Islander, American Indian or an Alaska native (6.24 $\log_{10}$ copies/ml; Table B) with similar median HCV RNA levels among Asian/Pacific Islanders (6.24 $\log_{10}$ copies/ml; n=19) and American Indians/Alaska natives (6.18 $\log_{10}$ copies/ml; n=37). Regarding infection with other viruses, for subjects overall, those without antibody to HBV had lower HCV RNA levels than those with resolved or chronic HBV infection (Table 2). HCV RNA levels were higher in HIV-infected participants (6.73 $\log_{10}$ copies/ml) than in HIV-uninfected IDUs (6.40 $\log_{10}$ copies/ml; $p<0.0001$). We also performed analyses stratified by HIV-1 infection status (Table B). Among the HIV-1-uninfected participants, the patterns of association were very similar to those seen among all viremic subjects except the HCV RNA level was consistently lower for each characteristic examined. Among the 237 HIV-1-infected participants, differences by age, gender, and duration of drug use were blunted or absent, but differences between African American and white participants were preserved (0.01).

Among the participants with detectable virus, 1669 had a specimen available for viral genotyping and 1524 (91.3%) of those subjects were successfully genotyped (Table C). Most participants were infected with an HCV-genotype 1 strain (1a, 69.0%; 1b, 10.0%), but 9.3% were infected with a genotype 2 strain, 10.6% with a genotype 3 strain and 1.1% with genotype 4a. The median HCV RNA level did not differ significantly between participants infected with 1a (6.50 $\log_{10}$ copies/ml) and those infected with 1b (6.63 $\log_{10}$ copies/ml; p=0.11). In comparison to participants who were infected with genotype 1 (median HCV RNA, 6.50 $\log_{10}$ copies/ml), HCV RNA was lower in those infected with genotype 3 (6.34 $\log_{10}$ copies/ml; p=0.0003) or genotype 4 (6.12 $\log_{10}$ copies/ml; p=0.03). We observed the lowest median HCV RNA level (5.64 $\log_{10}$ copies/ml) among participants who had detectable HCV RNA, but could not be genotyped.

1.2.2 *IL28B* rs12979860 Genotype

IL28B genotyping was performed for a subset of the participants with chronic hepatitis C (table D). Among 347 African American participants, we saw no differences in viral levels by *IL28B* genotype. Among 391 European American IDUs, those with the *IL28*-CC genotype had a higher median HCV RNA level (6.67 $\log_{10}$

copies/ml) than those with *IL28*–TT (6.12; p=0.01); the median HCV RNA level among European American participants with the *IL28*–CT genotype was 6.26 $\log_{10}$ copies/ml. Among 88 participants of Hispanic ethnicity, median HCV RNA levels for those with the *IL28*-CC (6.63 $\log_{10}$ copies/ml) and *IL28*–TT (6.19 $\log_{10}$ copies/ml) genotypes were similar to those seen in European American participants, but this difference was not statistically significant among participants of Hispanic ethnicity, which is a much smaller group.

1.3 MULTIVARIATE ANALYSES

Results from the multivariable ordinal logistic regression analysis (Table E) confirmed the unadjusted findings. HCV RNA levels were higher for older participants, men and those infected with HIV-1. Compared to African Americans, HCV RNA levels were lower in all other ancestry groups, although this difference did not approach statistical significance for the comparison with Latinos (p =0.44). Regarding viral genotype, compared to those infected with genotype 1, participants infected with genotype 2 had higher HCV RNA (p=0.01). The *IL28B*-CC genotype was associated with higher HCV RNA (p = 0.001). A model that substituted duration of drug use for age produced similar results. In view of the findings in Table D, we also conducted a multivariate analysis that including a term for an interaction between *IL28B* rs12979860 genotype and race/ethnicity, but this interaction was not statistically significant (p>0.10). but levels in those with the heterozygous genotype were not significantly higher than those with the TT genotype (p = 0.17). A model that substituted duration of drug use for age produced similar results.

DISCUSSION

Discussion (HIV)

There is increasing evidence for a role for TLRs in HIV-1 pathogenesis. In the present study, we found that the minor allele A of rs352140 TLR9 SNPs was more frequent among rapid progressors than among LTNP patients. While our observations support the hypothesis of a role for TLRs in HIV-1 infection, the precise mechanisms of the interaction between HIV-1 and innate immune cells remain poorly understood. During reverse transcription, HIV-1 produces double-stranded DNA containing CpG motifs; these are transferred from the cytoplasm to the nucleus and integrated in the host cell genome. Since TLR9 is exclusively located in the endosomal compartment, debris phagocytosed from HIV-1-infected cells containing sufficient amounts of proviral DNA may activate macrophages through TLR9.

In addition, TLR9 as well as other TLRs, may be activated by pathogens that are frequently associated with HIV-1 infection (e.g., mycobacteria, cytomegalovirus, herpes simplex virus and hepatitis C virus), thereby inducing the production of inflammatory cytokines and modulating viral replication. TLRs activation results in the production of inflammatory cytokines that can enhance viral replication. HIV-1 replication is directly dependent on NF-kB, a critical TLR-induced transcription factor that promotes activation of the HIV-1 long terminal repeat. This might be of importance in the mechanism underlying the increased HIV-1 replication and disease progression associated with concomitant and opportunistic infections. This study suggests an important role for TLR9 and serves to generate several hypotheses for the pathogenesis of HIV-1 infection.

Recent studies have analyzed the role of SNPs in the clinical context of HIV infection. Ferweda et al. reported a potential association between the TLR4 Asp299Gly SNP and a higher susceptibility to develop active tuberculosis in HIV-infected patients in Tanzania. Bochud's study reported association between CD4 cell depletion and the rs352140 A/G SNP in the TLR9 gene among rapid progressors, failing to find an association with HIV viral load. Ricci's group focused their research on the role of rs352139 and rs352140 TLR9 polymorphisms in mother to child transmission of HIV-1 and they showed no association of the two polymorphisms with rapid progression; however, the rs352140 GG and AA haplotype were associated with higher of rapid progression compare to the prevalent AG haplotype. Freguja's group recently reported that TLR9 rs352139 AA associated with

better prognosis and was significantly associated with a slow disease progression of the disease and conversely, that TLR9 rs352140 AG genotype associated with Rapid Progression in HIV-infected children.

This study show lack of an association of TLR9 polymorphisms with the rapid CD4 cell decline or HIV viral load as it has been found in the studies mentioned above. The role of rs352139 and rs352140 TLR9 SNPs differences between LTNP and Progressors patient has been analyzed. However, this study was limited by several factors. The cohort of Progressors was extremely heterogeneous, some patients had already started the antiretroviral therapy at the time of the beginning of the study whereas others were naïve to the therapy; the leading factor for this cohort was that the progression of the disease showed a CD4 cell decay <500 cells/ml within 3 years from the date of HIV-1 positive diagnosis. Furthermore, in our study, HIV viral load was only available in LNTP patients, and therefore our analyses were restricted in this variable. Finally, genetic associations can be biased by population stratification. Our cohort of LNTP was not limited to the analyses to one single homogeneous population and this factor could have played a major role on the meaning and lack of an association with CD4 count and HIV viral load.

In conclusion, specific genotypes and haplotypes in the TLR9 gene may affect the functional ability of their encoded proteins to modulate innate immunity and immune activation, thus contributing to the variability of clinical outcome in HIV-1 infected patients.

DISCUSSION (HCV)

In this large multi-racial cohort of IDUs with chronic hepatitis C infection, HCV RNA levels were independently associated with six factors: age, gender, racial ancestry, HIV-1 infection, HCV genotype and host *IL28B* genotype.

HCV RNA levels tended to be higher with older age and longer duration of drug injection, variables which are highly correlated in this study. The average time since initiation of drug use in these IDUs is 19 years and, at least until recently, most IDUs

who enrolled in UHS became infected with HCV relatively soon after initiating drug injection. We believe, therefore, that reported years of injection drug practices is a reasonable proxy for the time since initial infection with HCV. Our data suggest that HCV RNA levels may increase ~2.4% (0.15 “logs”) per year or ~25% per decade. Consistent findings were previously reported in another cross-sectional study of IDUs, but results from longitudinal studies of HCV RNA are mixed. The study with the longest follow-up period (median, 9.2 years) found that HCV RNA levels increased over time, but studies based on shorter follow-up periods (average 1-5 years) did not. These shorter studies may have lacked the statistical power to exclude a 2.4% annual increase (13% increase in 5 years), therefore, those results are not necessarily inconsistent with our own. Our findings suggest that HCV may propagate more efficiently over time. The explanation for this observation is beyond the scope of this investigation, but we speculate it could reflect selection of HCV variants with high replicative efficiency or host loss of immunological control of HCV over time, perhaps due to immune senescence. Higher HCV RNA levels are associated with lower rates of sustained virological response after treatment with pegylated interferon and ribavirin, therefore, evidence that HCV RNA levels increase over time may weigh for earlier treatment of chronic HCV infection.

In the absence of HIV-1 infection, HCV-RNA levels tended to be lower for women in this study compared to men and this difference remained even after potential confounding variables were considered. Among the 237 HIV-infected UHS participants, however, median HCV RNA levels were similar in women and men. In the ALIVE study of IDUs, Thomas et al saw somewhat lower HCV RNA levels in women compared to men among HIV-uninfected subjects, although that association was not statically significant in a multivariate analysis. As in our study, HCV RNA levels did not differ by gender among the HIV-infected ALIVE participants. Among HCV-infected Alaska Natives, women had much lower levels of HCV RNA than men.

Comparing HCV RNA by racial ancestry, UHS participants who were African American tended to have higher levels than participants of European or Asian ancestry, even after we considered other factors, including *IL28B* genotype. Few previous studies have been able to make such comparisons. In ALIVE, no difference in HCV RNA levels was seen between African American subjects and those of other races, however, only 40 non-African American subjects were included in that

analysis. Among patients enrolled in treatment trials for chronic hepatitis C, pre-treatment HCV RNA levels did not different between African American and European American subjects in either the VirahepC or IDEAL studies.

HCV-RNA levels were considerably higher among UHS participants who were infected with HIV-1 (6.73 $\log^{10}$ copies/ml) compared to those who were not (6.40 $\log^{10}$ copies/ml), which is consistent with the results from a number of previous studies. In our study, we were able to control for a fuller range of potential confounding, but this association remained strong even when these factors were considered.

Among the subjects for whom we could determine viral genotype, almost 80% were infected with HCV genotype 1A or 1B; the median HCV RNA level in this group was 6.51 $\log^{10}$ copies/ml. UHS participants who were infected with HCV genotype 2 had higher HCV RNA levels (median, 6.69 $\log^{10}$) than those infected with genotype 1, although this difference reached statistical significance only when other factors were considered in the analysis. We observed lower viral levels in participants who were infected with genotype 3 (median, 6.35 $\log^{10}$; n=162) or genotype 4 (median, 6.12 $\log^{10}$; n=17), but those findings were not statistically significant in the multivariate analysis, perhaps due to insufficient statistical power. Consistent with our findings, an earlier report of Swiss blood transfusion recipients co-infected with HIV-1 showed the highest HCV RNA levels in patients with genotype 2, and the lowest levels in patients with genotype 4. In a multi-national study (predominantly IDUs), HCV RNA levels were lowest among subjects infected with genotypes 3 or 4, and similar among those with genotypes 1 and 2, although relatively few subjects with genotype 2 were included in this analysis. Among Alaska Natives, the lowest HCV RNA levels were found in persons infected with HCV genotypes 3a and the highest in those infected with genotype 2b. There were no patients with genotype 4 in the analysis.

Several variables that we found to be associated with higher HCV RNA among UHS participants (older age, male gender, African ancestry and HIV infection) were previously associated with failure to spontaneously clear HCV infection in this cohort, as well as in other studies. An exception to this pattern is the *IL28B-CC* genotype, which is associated with increased spontaneous HCV clearance in UHS (Shebl, submitted) (and other studies), but also higher HCV RNA (among the European

American participants and UHS subjects overall). Paradoxically, the rs12979860-CC genotype, which is associated with better response to pegylated interferon /ribavirin therapy and a higher frequency of spontaneous HCV clearance, has been associated with higher HCV RNA levels in those with chronic hepatitis C. Some prior reports found higher baseline HCV RNA to be associated with the otherwise favorable *IL28B* genotype, but other studies did not. Comparing participants by racial ancestry, African American UHS participants had the highest HCV RNA levels despite having the lowest frequency of the *IL28B* –CC genotype. Furthermore, we did not see the association between higher HCV RNA and *IL28B* –CC among the African American participants. It is possible, therefore, that there are additional genetic factors that lead to poorer viral control among persons of African ancestry.

Our study has a number of strengths. Because UHS is a multi-racial cohort of street-recruited IDUs we could compare HCV RNA across ancestral groups without the potential biases caused by markedly differing sources of HCV infection or socioeconomic status. Few, if any, of the UHS participants had been treated for HCV infection, therefore, the HCV RNA values among these subjects were not subject to selection by previous HCV treatment. The relatively large size of the cohort provided good statistical power for many comparisons, although our power was low for certain variable categories, including Hispanic or Asian ancestry and viral genotypes 3 or 4. The limitations of our study should be considered as well. The cross-sectional design did not allow us to determine the timing of HCV, HBV and HIV infections among the participants and we also could not differentiate the effect of duration of infection (as estimated by number of years of drug injection) from the effect of age because these factors are highly correlated. As mentioned above, we could not determine whether the relationship between duration of infection might represent super-infection, immune senescence or some other factor that varies with time or age.

We performed viral genotyping by direct sequencing, the ‘gold standard’ technique for discriminating HCV types and subtypes. This genotyping was based on the NS5B region, which tends to produce more accurate results than the 5’NC region. An important concern in this analysis is whether methodological differences may account for the discrepancies in HCV RNA levels between different genotypes. We used a third generation bDNA assay with an analytic sensitivity of 2.5×10^3 copies/ml to measure viral levels. This method amplifies signal rather than target, which is the

basis for classical reverse transcription polymerase chain reaction (PCR) and transcription-mediated amplification assays. First-generation bDNA assays underestimated levels of HCV genotype 2 and 3, but third-generation bDNA tests are accurate, reproducible and well calibrated to the World Health Organization HCV RNA standard. In support of our findings, a previous report of an association between HCV genotype 4 infection and lower HCV RNA levels was based on measurement by PCR and determined that the results were not influenced by viral genotype-specific amplification bias.

In conclusion, the level of HCV viremia, an important predictor of response to HCV treatment, is itself influenced by a wide range of demographic, viral and host genetic factors. A better understanding of the determinants of HCV viremia could lead to improved treatment of patients with chronic hepatitis C.

Table A. Characteristics of HCV-infected injection drug users, San Francisco Bay area, 1998–2000.

	HCV antibody (n=2092)		HCV viremia (n=1701)		Viral genotype (n=1524)		*IL28B* genotype (n=882)	
Characteristic	**Median**	**IQR** [a]	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Age at enrollment	45	40-50	46	40-50	46	40-50	44	39-49
Years of injection drug use	25	17-31	26	18-32	26	18-32	25	18-31
Age at first injection drug use	18	16-24	18	16-24	18	16-24	18	16-22
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Gender [b]								
Male	1461	70.9	1222	72.4	1104	73.1	615	69.7
Female	600	29.1	465	27.6	407	26.9	267	30.3
Race								
White	759	36.3	577	33.9	522	34.3	391	44.3

African American	1078	51.5	951	55.9	861	56.5	347	39.3
Latino	167	8.0	115	6.8	92	6.0	88	10.0
Asian or Pacific Islander	28	1.3	19	1.1	15	1.0	18	2.0
American Indian	58	2.8	37	2.2	32	2.1	36	4.1
Other	2	0.1	2	0.1	2	0.1	2	0.2
Hepatitis B virus infection								
Acute	18	0.9	7	0.4	7	0.5	5	0.6
Chronic	53	2.5	29	1.7	26	1.7	28	3.2
Resolved	1641	78.4	1348	79.2	1197	78.5	680	77.1
Vaccinated	78	3.7	70	4.1	65	4.3	38	4.3
Uninfected and unvaccinated	302	14.4	247	14.5	229	15.0	131	14.9
HIV-1 infection	262	12.5	237	13.9	211	13.8	107	12.1

a. IQR: inter-quartile range (25th percentile-75th percentile); b. Missing data: gender, 31

Table B. Median HCV RNA levels (log^{10} copies/ml) among injection drug users with detectable HCV RNA[a], by selected demographic and viral variables - San Francisco Bay area, 1998-2000.

	All Subjects			HIV-1 Uninfected Subjects			HIV-1 Infected Subjects		
Characteristic	**No.**	**HCV RNA**	**p-value**	**No.**	**HCV RNA**	**p-value**	**No.**	**HCV RNA**	**p-value**
Overall	1701	6.45		1461	6.40		237	6.73	
Age									
18-29 years	84	6.15		76	6.11		8	6.32	
30-39 years	303	6.29		243	6.25		60	6.74	
40-49 years	845	6.47		724	6.43		119	6.73	
≥50 years	469	6.59		418	6.56		50	6.79	
			<.0001			<.0001			0.15
Years injection drug use									
0-9 years	176	6.08		158	6.04		18	6.42	
10-19 years	316	6.29		265	6.21		51	6.75	
20-29 years	602	6.47		504	6.44		96	6.62	
≥ 30 years	573	6.61		508	6.59		64	6.84	
			<.0001			<.0001			0.08
Gender									
Male	1222	6.52		1053	6.49		167	6.71	
Female	465	6.29		397	6.21		67	6.79	
			<.0001			<.0001			0.47
Race									
African American	951	6.49		806	6.45		144	6.78	
White	577	6.35		500	6.34		76	6.56	

Latino	115	6.39		106	6.37		9	6.58	
Asian or American Indian	56	6.24		47	6.23		8	6.49	
			<.0001			0.002			0.04
HBV infection									
Resolved	1348	6.48		1143	6.44		203	6.73	
Chronic	29	6.64		22	6.22		6	6.96	
			0.57			0.67			0.40
Uninfected	247	6.30		230	6.25		17	6.77	
			0.0001			0.0005			0.45

a. Subjects with undetectable HCV RNA were excluded

Table C. Median HCV RNA levels (log^{10} copies/ml) among injection drug users with detectable HCV RNA[a], by HCV genotype - San Francisco Bay area, 1998-2000.

HCV Genotype*	Frequency	Percent	Percent (excludes NR)	Median	p-value	Median	p-value
1A	1057	63.07	69.08	6.50			
1B	152	9.07	9.93	6.63	0.11	6.51	
2A	15	0.89	0.98	6.71	0.92		
2B	127	7.58	8.30	6.69	0.19	6.69	0.31
3A	160	9.55	10.46	6.35	0.001		
3B	2	0.12	0.13	6.08	0.37	6.35	0.0004
4A	17	1.01	1.11	6.12	0.04	6.12	0.03
NR	146	8.71		5.64	<0001	5.64	<0.0001
Total	1676						

*Viral genotype is based on NS5B sequence

Table D: HCV RNA levels load among injection drug users with chronic HCV, by *IL28B* rs12979860genotype- San Francisco Bay area, 1998-2000.

	African American (n=347)			European American (n=391)			Hispanic (n=88)			Asian/American Indian (n=54)		
Genotype	Number	RNA Median	p-value	Number	RNA Median	p-value	Number	RNA Median	p-value	Number	RNA Median	p-value
TT	123	6.44		41	6.12		10	6.19		7	6.28	
TC	177	6.51	0.33	185	6.26	0.34	48	6.53	0.21	20	6.27	0.46
CC	47	6.48	0.68	165	6.67	0.01	30	6.63	0.15	27	5.89	1.00

Table E. Multivariate ordinal logistic regression analysis of HCV RNA levels (log^{10} copies/ml) among 874 injection drug users with chronic hepatitis C, San Francisco Bay area, 1998-2000. The analysis is limited to subjects with results for *IL28B* rs12979860 genotype.

	Subjects with *IL28B* Genotype (n=874)		
Characteristic	**OR**	**95% CI**	**p-value**
Age	1.28	1.10 - 1.50	0.001
Female Gender	0.73	0.56 - 0.95	0.02
Race			
White	0.70	0.52 - 0.94	0.02
Latino	0.84	0.54 - 1.31	0.44
Asian or American Indian	0.39	0.23 - 0.69	0.001
Chronic HBV	1.06	0.53 - 2.14	0.86
HIV-1	1.95	1.34 - 2.83	0.0004
HCV Genotype*			
1A/1B			
2A/2B	1.78	1.14 - 2.77	0.01
3A/3B	0.80	0.55 - 1.17	0.25
4A	0.37	0.12 - 1.15	0.09
NR	0.17	0.11 - 0.28	<.0001

IL28B genotype			
TT			
CT	1.10	0.80-1.51	.17
CC	1.69	1.17-2.44	0.001

Supplemental table 1. Multivariate ordinal logistic regression analysis of HCV RNA levels (log^{10} copies/ml) among injection drug users who were chronically infected with HCV, San Francisco Bay area, 1998-2000 - all subjects and the subset of subjects with *IL28B* rs12979860 genotype results.

	All Subjects (n=1,650)			**Subjects with *IL28B* Genotype** (n=874)		
Characteristic	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Age	1.33	1.18 - 1.49	<.0001	1.28	1.10 - 1.50	0.001
Female Gender	0.64	0.53 - 0.78	<.0001	0.73	0.56 - 0.95	0.02
Race						
White	0.86	0.70 - 1.06	0.16	0.70	0.52 - 0.94	0.02
Latino	0.90	0.63 - 1.28	0.55	0.84	0.54 - 1.31	0.44
Asian or American Indian	0.48	0.29 - 0.80	0.01	0.39	0.23 - 0.69	0.001
Chronic HBV	1.25	0.64 - 2.46	0.51	1.06	0.53 - 2.14	0.86
HIV-1	2.13	1.65 - 2.75	<.0001	1.95	1.34 - 2.83	0.0004
HCV Genotype*						
1A/1B						
2A/2B	1.30	0.94 - 1.79	0.11	1.78	1.14 - 2.77	0.01
3A/3B	0.68	0.50 - 0.93	0.02	0.80	0.55 - 1.17	0.25
4A	0.50	0.20 - 1.20	0.12	0.37	0.12 - 1.15	0.09
NR	0.20	0.15 - 0.28	<.0001	0.17	0.11 - 0.28	<.0001

IL28B genotype						
TT						
CT				1.10	0.80-1.51	.17
CC				1.69	1.17-2.44	0.001

Supplemental table 2. Unadjusted and multivariate ordinal logistic regression analysis of HCV RNA levels (log^{10} copies/ml) among 874 injection drug users with *IL28B* rs12979860 genotype results who were chronically infected with HCV[a], San Francisco Bay area, 1998-2000

	Unadjusted			**Adjusted (Without *IL28B* Genotype)**			**Adjusted (With *IL28B* Genotype)**		
Characteristic	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Age	1.28	1.11 - 1.47	0.001	1.27	1.09 - 1.48	0.002	1.28	1.10 - 1.50	0.001
Female Gender	0.66	0.51 - 0.85	0.002	0.73	0.56 - 0.94	0.02	0.73	0.56 - 0.95	0.02
Race									
White	0.76	0.59 - 0.98	0.04	0.78	0.58 - 1.04	0.09	0.70	0.52 - 0.94	0.02
Latino	0.83	0.55 - 1.26	0.39	0.91	0.59 - 1.41	0.68	0.84	0.54 - 1.31	0.44
Asian or American Indian	0.47	0.28 - 0.80	0.006	0.46	0.27 - 0.80	0.01	0.39	0.23 - 0.69	0.001
Chronic HBV	1.04	0.53 - 2.05	0.91	1.17	0.59 - 2.35	0.65	1.06	0.53 - 2.14	0.86

HIV-1	1.73	1.20 - 2.49	0.003	1.95	1.34 - 2.82	0.0004	1.95	1.34 - 2.83	0.0004
HCV Genotype*									
1A/1B									
2A/2B	1.64	1.07 - 2.51	0.02	1.90	1.22 - 2.95	0.004	1.78	1.14 - 2.77	0.01
3A/3B	0.77	0.54 - 1.10	0.15	0.87	0.60 - 1.27	0.48	0.80	0.55 - 1.17	0.25
4A	0.48	0.16 - 1.48	0.20	0.43	0.14 - 1.33	0.14	0.37	0.12 - 1.15	0.09
NR	0.17	0.11 - 0.28	<.0001	0.18	0.11 - 0.30	<.0001	0.17	0.11 - 0.28	<.0001
IL28B genotype									
TT									
CT	1.05	0.77 - 1.43	0.45				1.10	0.80-1.51	0.17
CC	1.33	0.95 - 1.85	0.05				1.69	1.17-2.44	0.001

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