EVELI KALLAS

The influence of immunological markers
to susceptibility to HIV, HBV, and HCV
infections among persons who inject drugs

TARTU ÜLIKOOL

UNIVERSITAS TARTUENSIS

1632

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

252

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Dissertation has been accepted for the commencement of the degree of Doctor of Philosophy in Medicine on February 15, 2017 by the Council of the Faculty of Medicine, University of Tartu, Estonia

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Commencement: April 18th, 2017

Publication of this dissertation is granted by University of Tartu.

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LIST OF ORIGINAL PUBLICATIONS

1. Kallas, E.; Huik, K.; Pauskar, M.; Jõgeda, E.-L.; Karki, T.; Des Jarlais, D.; Uusküla, A.; Avi, R.; Lutsar, I. (2015). Influence of interleukin 10 polymorphisms −592 and −1082 to the HIV, HBV and HCV serostatus among intravenous drug users. Infection, genetics and evolution, Mar; 30, 175–180.

2. Kallas, E.; Huik, K.; Türk, S.; Pauskar, M.; Jõgeda, E.-L.; Šunina, M.; Karki, T.; Des Jarlais, D.; Uusküla, A.; Avi, R.; Lutsar, I. (2016). Differences in T cell distribution and CCR5 expression in HIV-positive and HIV-exposed seronegative persons who inject drugs. Medical Microbiology and Immunology, Jun; 205(3), 231–239

3. Kallas, E.; Huik, K.; Türk, S.; Pauskar, M.; Jõgeda, E.-L.; Šunina, M.; Karki, T.; Des Jarlais, D.; Uusküla, A.; Avi, R.; Lutsar, I. (2016). T Cell Distribution in Relation to HIV/HBV/HCV Co-infections and Intravenous Drug Use. Viral Immunology, Oct; 29(8):464–470.

Author's personal contribution:

In article 1: participated in the study design, conducted data analyses, and wrote the article.

In article 2: participated in the study design, conducted flow cytometry laboratory experiments, data analyses, and wrote the article.

In article 3: participated in the study design, conducted flow cytometry laboratory experiments, data analyses, and wrote the article.

ABBREVIATIONS

af	–	Allelic frequency
AIDS	–	Acquired immunodeficiency syndrome
ART	–	Antiretroviral treatment
BIM	–	Bcl2-interacting mediator
CCL5	–	CC chemokine ligand 5
CCR5	–	CC chemokine receptor 5
CCR5 Δ32	–	32 base pair deletion in CCR5 gene
CCR7	–	CC chemokine receptor 7
CD	–	Cluster of differentiation antigen
CI	–	Confidence interval
CMV	–	Cytomegalovirus
CSW	–	Commercial sex workers
CTL	–	Cytotoxic T lymphocyte
CXCR4	–	CX chemokine receptor 4
DC	–	Dendritic cell
DNA	–	Desoxyribonucleic acid
DPBS	–	Dulbecco's phosphate-buffered saline
ESN	–	HIV exposed seronegative individuals
EU	–	European Union
GBVC	–	GB virus type C
HAART	–	Highly active antiretroviral therapy
HBV	–	Hepatitis B virus
HCV	–	Hepatitis C virus
HESN	–	Heavily exposed HIV seronegative individuals
HIV	–	Human immunodeficiency virus
HLA	–	Human leukocyte antigen
HTLV	–	Human T-lymphotropic virus
HWD	–	Hardy-Weinberg Disequilibrium
IVDU	–	Intravenous drug use
IFN	–	Interferon
IL	–	Interleukin
MSM	–	Men having sex with men
MTC	–	Mother-to-child transmission
NK	–	Natural killer
OR	–	Odds ratio
PBMC	–	Peripheral blood mononuclear cells
PCR	–	Polymerase chain reaction
PWID	–	Persons who inject drugs
SNP	–	Single nucleotide polymorphism
TNF	–	Tumor necrosis factor
UNODC	–	United Nations Office of Drugs and Crime

1. INTRODUCTION

The human immunodeficiency virus 1 (HIV) causes lifelong infection that can lead to acquired immunodeficiency syndrome (AIDS) and death. In 2015, approximately 36.7 million people globally were living with the HIV/AIDS infection and although the infection is manageable using antiretroviral drugs, there are still major medical and socio-economic issues related to the infection: accessibility to treatment; side-effects; and risk behavior (www.unaids.org; www.who.int). Despite positive advances in treatment, research is ongoing to clarify the molecular mechanisms of HIV infection and find more effective treatment options.

In addition to HIV, the hepatitis B virus (HBV) and hepatitis C virus (HCV) cause major health care issues around the world. All three infections can be transmitted via blood and therefore HIV/hepatitis co-infections are common among risk groups, such as persons who inject drugs (PWID).

Despite repeated exposure, some individuals do not get infected with HIV, HBV, or HCV. Based on the frequency of their exposure, these individuals are defined as heavily exposed seronegative subjects or exposed seronegative subjects. Such individuals are interesting research subjects, because the reasons they are resistant are not fully understood. Only one gene variant, the 32-base pair deletion in the CC chemokine receptor 5 (CCR5) gene, is known to provide complete resistance against HIV R5-tropic viruses (Samson et al., 1996). As homozygosity of this mutation is quite rare, the reasons of resistance to HIV, HBV, and HCV viruses are still of great interest. The focus of HIV exposure studies has mainly been on sexually exposed individuals and data regarding persons who inject drugs (PWID) is limited.

Factors protecting people from HIV and hepatitis infections can be divided into genetic, immunological, and viral factors. Genetic factors, such as single nucleotide polymorphisms and copy number variation, do not change during an individual's life. However, immunologic factors are influenced by risk behavior and co-infections. Little is known about immunological aspects of HIV, HBV, and HCV co-infected/exposed PWID populations or how intravenous drug use itself influences immunological factors among PWID.

The HIV epidemics is an especially important problem in Estonia, because Eastern-Europe is one of the few regions in the world where numbers of new HIV infections continues to increase (DeHovitz et al., 2014). The number of HIV infected and HIV/hepatitis co-infected individuals in Estonia is high, especially among PWID (Uusküla et al., 2007). The HIV virus transmitted in Estonia is very homogeneous, providing an opportunity to investigate the effects of genetic and immunological factors in terms of HIV and its co-infections, without considering the viral divergence. Therefore, the aim of this study was to investigate genetic and immunological factors associated with HIV, HBV, and HCV infections and intravenous drug use among PWID.

2. REVIEW OF THE LITERATURE

2.1. HIV, HBV, and HCV epidemics around the world

The HIV epidemic is a major global health care problem, having already caused 35 million deaths. Approximately 37 million individuals have HIV and about two million people became infected with HIV in 2015 (www.who.int). Although the majority of people infected with HIV live in Sub-Saharan Africa, Eastern Europe and Central Asia are one of the few regions where HIV incidence continues to increase (DeHovitz et al., 2014; www.who.int). HIV is not curable; however, it is controllable using antiretroviral treatment (ART), which has lowered the number of people reaching the acquired immuno-deficiency syndrome (AIDS) stage and AIDS related deaths. In 2015 over 15 million people received ART, however, 1.1 million people still died from AIDS related illnesses (UNAIDS, 2015; www.who.int). The main risk groups of HIV infections are commercial sex workers (CSW), men having sex with men (MSM), and persons who inject drugs (PWID, formerly known as intravenous drug users) (Beyrer, 2007). Although the risk of acquiring the HIV infection via intravenous drug use is higher than via heterosexual intercourse, the latter still accounts for 70% of new HIV cases worldwide (Mathers et al., 2008; Shaw and Hunter, 2012).

The hepatitis C virus (HCV) is also a blood borne infection that has reached an epidemic proportion globally. HCV leads to acute and chronic infections that cause liver fibrosis, cirrhosis, or hepatocellular carcinoma in 15–30% of chronically infected individuals within 20 years (www.who.int). It is not exactly known how many patients recover from the HCV infection without treatment, but it could be approximately 15% (Di Bisceglie et al., 1991; Villano et al., 1999). Currently, about 130–150 million people globally are chronically infected with HCV (www.who.int). Although the infection is curable, access to treatment is low and approximately 500,000 people die annually from HCV related liver diseases (Lozano et al., 2012; Vos et al., 2015). The groups at risk of HIV and HCV infection are similar, including MSM, PWID, and hemophiliacs.

The third infection common among groups at high risk of contracting HCV and HIV, is hepatitis B (HBV). Similar to HCV, HBV causes acute and chronic infections, and leads to hepatocellular carcinoma in ≈5–10% of infected indi-viduals (www.who.int). Approximately 248 million people are chronically infected with HBV (Schweitzer et al., 2015). Although virus-specific treatment is not available, an effective vaccine is used in 99% of countries globally as part of a routine immunization plan (www.who.int). In the absence of vaccination, the HBV infection is self-limiting in over 90% of cases, and causes chronic infection mainly among infants and children (Liaw and Chu, 2009). The other groups of individuals at risk of chronic HBV infection are individuals with a compromised immune system, such as HIV positive patients, and PWID.

HIV, HCV, and HBV have similar routes of transmission and co-infections are common, especially among risk groups such as PWID. It is estimated that

over two million people are HCV/HIV co-infected, of whom more than half are PWID (Platt et al., 2016). Co-infection status is important, because co-infection with HIV/HCV increases the pathogenic effects of both viruses. Among people with HIV, co-infection quickens disease progression, and co-infections involving HCV increase liver fibrosis (Eyster et al., 2016; Graham et al., 2001; Kovacs et al., 2008). In the case of HIV/HBV co-infections, the effects upon HIV progression are small; however, the possibility of a self-limiting HBV infection decreases and the risk of chronic hepatitis increases (Thio et al., 2002).

2.2. HIV, HBV, and HCV epidemics in Estonia

The first HIV-positive patient in Estonia was diagnosed in 1988. During the 1990s, the prevalence of HIV remained low, spreading mainly through hetero- and homo-sexual contact (Ustina et al., 2001). An epidemic occurred in 2000, when the virus spread rapidly among the PWID population (Figure 1) (Laisaar et al., 2011). After the first outbreak – since 2001 – the number of newly infected individuals has decreased; however, Estonia remains one of the countries with the highest HIV incidence in Europe (DeHovitz et al., 2014; Laisaar et al., 2011). The main group affected by the epidemic were young male PWID, but over more recent years the situation has changed, with the number of new diagnoses increasing among individuals infected during heterosexual contact (Soodla et al., 2015). Currently, the age of newly infected individuals and number of women infected is increasing. Although sexual contact is one of the main causes of HIV transmission, the number of reported HIV cases is low among MSM (Laisaar et al., 2011; Soodla et al., 2015). Since the outbreak, the epidemic has been mainly localized in the northern part of the country (mainly the capital Tallinn and Eastern-Viru County).
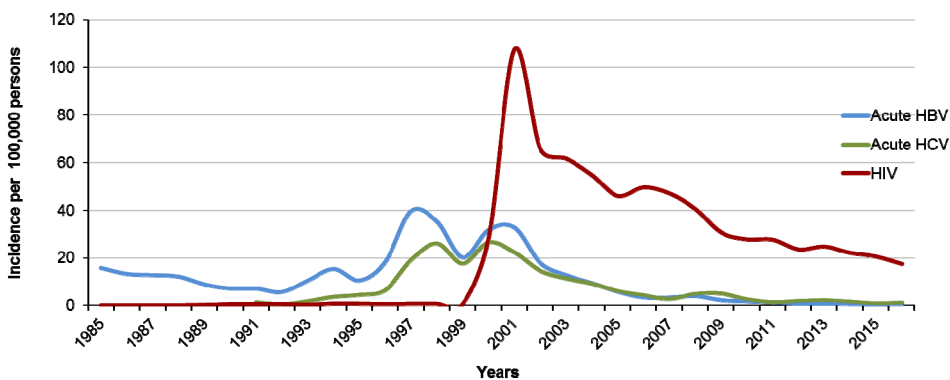
Figure 1. The incidence of HIV, HBV, and HCV infections in Estonia. The incidence per 100,000 persons of HIV is marked in red, acute HCV in green, and acute HBV in blue. The data are reported by the Estonian Health Board (www.terviseamet.ee/en/information.html).

The HIV epidemic in Estonia mainly involves HIV-1 CRF06_cpx viruses and to a lesser extent subtype A1 or recombinants between CRF06_cpx and A1 viruses (Adojaan et al., 2005; Avi et al., 2009, 2010). Similar to other Eastern European countries, the HIV epidemic is monophyletic and the rates of drug resistant mutations are low (Avi et al., 2014).

A peak in chronic HCV infections in Estonia occurred during the 1990s – preceding the HIV epidemic – remained high at the beginning of 2000s (Figure 1 and Figure 2), and then decreased steadily mirroring the HIV infection rate (Tefanova et al., 2006). The main population affected by HCV has also been PWID. According to genotype data, 1b and 3a are the most prevalent HCV genotypes among chronic HCV patients and HIV/HCV positive individuals (Kase et al., 2015; Zusinaite et al., 2005).
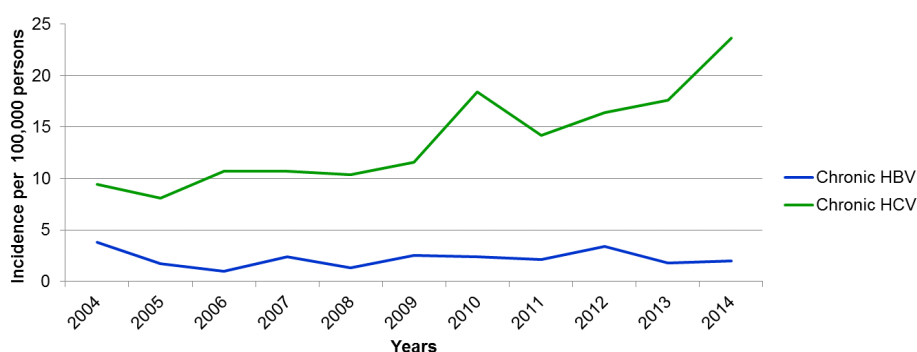
Figure 2. The incidence of chronic HCV and HBV infections in Estonia. The incidence per 100 000 persons of chronic HCV is marked in green and chronic HBV in blue. The data are reported by the Estonian Health Board (www.terviseamet.ee/en/information.html).

The prevalence of HBV infections has followed similar trends as HCV infections, peaking during the mid-1990s and thereafter gradually decreasing (Figure 1 and Figure 2). The main population affected has also been PWID (Tefanova et al., 2006). The nationwide HBV vaccination strategy was implemented in 1999 among teenagers and in 2003 expanded to include newborns; however, vaccination coverage among the general population is low (Paat et al., 2009). Considering HBV genotypes, A and D have been recorded in the Estonian population, with genotype D accounting for 86% of total HBV cases (Tallo et al., 2004).

Among HIV positive individuals, rates of HCV and HBV co-infections are high, especially among PWID. HCV is the most frequent co-infection among HIV positive individuals; depending on the studied group, 54–96% of Estonian HIV positive PWID are seropositive for HCV (Soodla et al., 2015; Uusküla et al., 2007). Data regarding HBV is more complex, showing that more than 20% of needle exchange visiting PWID are HBsAg positive, and 6% of HIV positive individuals in the Estonian E-HIV database are HBsAg positive (Soodla et al.,

2015; Uuskula et al., 2006). Numbers of triple-infected individuals are not precisely known, however, previous cross-sectional studies have indicated that 12–40% of PWID may be triple-infected (Huik et al., 2013; Huik et al., 2010).

2.3. Factors influencing susceptibility to HIV

Some individuals remain HIV negative despite repeated exposure (Horton et al., 2010). The main exposure routes are through unprotected sexual intercourse, and intravenous drug use (previously also blood transfusion, especially among hemophiliacs). Based on the frequency of exposure, individuals can be classified as heavily exposed seronegative subjects (HESN) or exposed seronegative subjects (ESN). However, this classification is not very strict and varies across studies. Usually study subjects are classified per the frequency of risk behavior(s), mostly the number of times they had unprotected sex with a HIV positive partner or shared a syringe. The percentages of individuals remaining HIV-seronegative vary from 6% among hemophiliacs, 5–10% among commercial sex workers, and up to 85% of individuals in serodiscordant relationships (Fowke et al., 1996; Kroner et al., 1994; Peterman et al., 1988; Salkowitz et al., 2001). Resistance against HIV has been associated with HIV co-receptor CCR5 genetic poly-morphisms, its cell surface density on CD4+ T cells (Samson et al., 1996), and with multiple other genetic factors, for example CCR5 ligand polymorphisms (Liu et al., 1996; Zhao et al., 2004). However, the genetic associations found have not explained whole resistance to HIV.

2.3.1. HIV pathogenesis and route of infection

HIV infection can be acquired via parenteral, sexual, or vertical routes. Infection risk varies by transmission route, being highest via blood transfer, because this route avoids the mucosal barrier (Boily et al., 2009; Msellati et al., 1990). The risk of HIV transmission from contaminated blood products is 90–100% (Donegan et al., 1990; Msellati et al., 1990). The risk of infection during sexual exposure is 1 in 200–2000, and depends on multiple co-factors, such as type of intercourse, HIV stage, and male circumcision (Boily et al., 2009; Powers et al., 2008).

Of all the routes of infection, no significant differences in the overall course of HIV infection have been found. The virus is transmitted via mucosal or parenteral routes, and establishes a lymphatic reservoir (Haase, 1999). Depending on the route of infection, the first viral targets are CD4+ and dendritic cells in blood or mucosa (Ayehunie et al., 1997; Blauvelt et al., 1997; Geijtenbeek et al., 2000; Schacker et al., 2001). The virus enters the target cells using a CD4 receptor and co-receptor, mainly CCR5 or to a lesser extent the CX chemokine receptor 4 (CXCR4) (Berger et al., 1999; Hladik et al., 2007; Jakobsen et al., 2010). Viruses using CCR5 as a co-receptor usually prevail in the early phases

of infection, and in some cases are replaced by CXCR4 viruses during the later stages of infection (Gorry and Ancuta, 2011). The reasons for co-receptor switching are not completely understood, but it has been proposed to be linked with a declined fitness of CCR5-using viruses or the presence of CXCR4 on more cell types than CCR5 (Coetzer et al., 2008).

The primary clinical infection results in peak HIV viremia and an initial decrease in CD4+ T cells, which rise and plateau after the creation of a virus specific immune response (Koup et al., 1994). After the initial acute infection, a steady state of clinical latency is reached, and for multiple years little change in blood immune factors occur. This latency is dependent on the host's genetics and immune reactions. During this stage there is a slight decrease in CD4+ T cells, an increase in HIV viral load, and increased immune activation (Deeks et al., 2013). The exact mechanisms of immune activation are unknown, however, microbial translocation from the gut and HIV antigen exposition itself have been proposed (Brenchley et al., 2006; Lederman et al., 2000). The increase in immune activation predicts disease progression (Giorgi et al., 1993; Zangerle et al., 1992) and drives CD4+ T cells depletion (Lederman et al., 2000). In addition, the cytopathic effects of HIV itself contribute to CD4+ decline. Budding of HIV virions disrupts cell membranes and compromises single cells, and HIV causes the formation of multinucleated cells using uninfected CD4+ T cells (Alimonti et al., 2003; Fauci, 1988; Levy, 1993; Lifson et al., 1986). All these events eventually lead to cell death, an increase in HIV viral load, and AIDS.
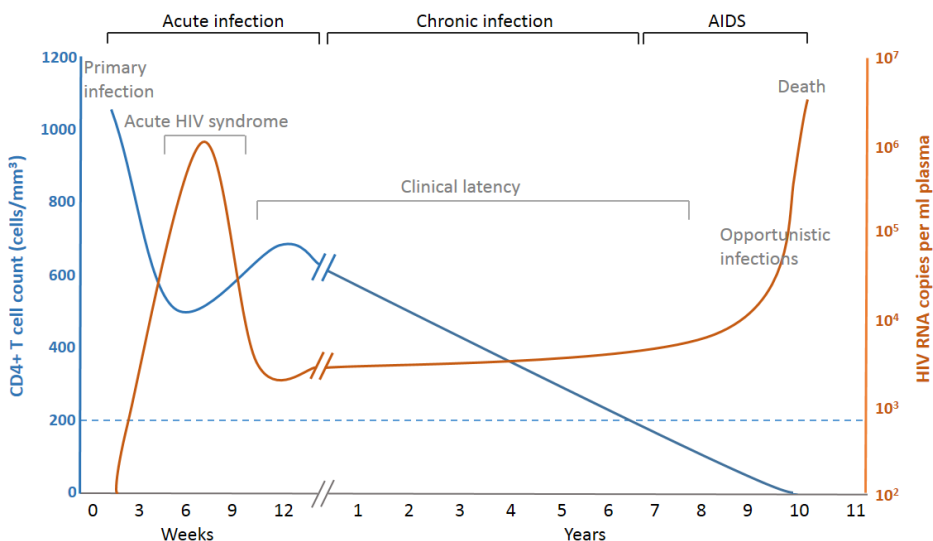

Figure 3. HIV pathogenesis. The CD4+ T cell count is presented with blue and the HIV RNA load with red (modified from Rowland-Jones, 2003).

The aforementioned pathogenic processes can be delayed using antiretroviral treatments. After the first antiviral treatment regimens were introduced during the 1980s and highly active antiretroviral therapy (HAART) during the 1990s,

the number of AIDS-related deaths decreased (Hammer et al., 1997). HAART enabled suppression of viral replication below the limit of detection (Opravil et al., 2000). The treatment stabilizes CD4+ cell counts and reduces the risk of opportunistic infections. The newer HAART regimens, especially those including integrase inhibitors, have increased the lifespan of HIV positive individuals (Deeks et al., 2013).

Multiple problems regarding HIV infection and pathogenesis are related to co-infections. As mentioned, two of the most important viral co-infections among HIV positive PWID are HBV and HCV. In addition to these, cytomegalovirus (CMV), GB virus type C (GBVC), human T-lymphotropic virus (HTLV), and multiple other viruses affect susceptibility to HIV and or its progression (Brites et al., 2009; Ernst et al., 2014; Gudo et al., 2009; Hodowanec et al., 2013; Jõgeda et al., 2016).

2.3.2. Viral factors influencing susceptibility to HIV

HIV-1 can be divided into three groups: M (major); O (outlier); and N (non-M/non-O). The most divergent group M, is further divided into nine subtypes (A, B, C, D, F, G, H, J, and K) and recombinant forms. To date, intra-subtype differences have not been related to increased/decreased susceptibility to HIV and or the disease's progression. Regarding susceptibility, some HIV inter-subtype differences have been described, however, the differences mostly reflect the socio-economic status of the study groups (Campbell, 2006). Differences between subtypes A, C, and D have been found among vertically infected subjects, where mothers infected with subtype D were less likely to transmit the infection to their children (Renjifo et al., 2001; Renjifo et al., 2004). Similarly, multiple other studies have shown higher subtype C transmission risks compared to non-C (Alcântara et al., 2013; John-Stewart et al., 2005). However, these results have not been confirmed by larger multinational studies (Kahle et al., 2014). Additional subtype effects have been proposed among PWID in Thailand, where subtype E resulted in increased transmission compared to subtype B (Hudgens et al., 2002). However, many other epidemiologic and host factors could have influenced this difference.

2.3.3. Host factors influencing susceptibility to HIV

There are multiple host factors that influence susceptibility to infections. The main host factors can be divided into genetic (single nucleotide polymorphisms, and copy number variations) and immunologic factors (e.g. levels of cytokines, cytokine balances, chemokine receptor expression, T cell distribution).

2.3.3.1. Host genetic factors associated with HIV infection

Host genetics plays a major role in susceptibility to HIV and progression of the disease. A lot of effort has been put into determining the host genes associated with resistance to HIV and its progression. The main focus of these studies has been on genes encoding HIV co-receptors and their ligands, human leukocyte antigen (HLA) encoding genes, and other genes related to immune response (Chatterjee, 2010).

CCR5 and CXCR4 are the main HIV co-receptors and polymorphisms in these genes directly affect their gene expression, together with co-receptor cell surface expression and thereafter viral entry to target cells (Alkhatib et al., 1996; Feng et al., 1996). The most well-known polymorphism related to HIV host genetics is the CCR5 gene 32 base pair deletion (CCR5 Δ32), which in a homozygous state provides complete protection against HIV R5-tropic infection (Dean et al., 1996; Samson et al., 1996). The deletion results in a shortened version of the receptor molecule, which is not transported to the cell surface and therefore the cell lacks the co-receptor necessary for viral entry (Benkirane et al., 1997). Although individuals carrying the CCR5 Δ32 mutation are resistant to CCR5 using viruses (R5 tropic), they are susceptible to CXCR4 using viruses (Balotta et al., 1997; Gorry et al., 2002). Data regarding Δ32 heterozygote individuals is not conclusive, as differences in HIV susceptibility have been found in some study groups.

In addition to the CCR5 Δ32 mutation, multiple other genetic polymorphisms – mainly in chemokine genes, chemokine receptor genes, and HLA genes – influence susceptibility to HIV and disease's progression (Table 1).

Table 1. Associations between human genetic polymorphisms and HIV infection

Gene	Polymorphism	Effect	Selected references
CCR5	Δ32/Δ32	Resistance to HIV CCR5 tropic strains	(Liu et al., 1996) (Samson et al., 1996)
	Δ32/wt	Protective against HIV, slower progression to AIDS	(Zimmerman et al., 1997) (Marmor et al., 2001)
	–2459A	Faster progression to AIDS	(McDermott et al., 1998)
CCR2	V64I	Protective against HIV, slower progression to AIDS	(Singh et al., 2003) (Zapata et al., 2013)
CCL3L1	Low copy number	Protective against HIV	(Huik et al., 2010)
CCL5	–28G	Slower progression to AIDS Faster progression to AIDS	(Liu et al., 1999) (Zhao et al., 2004)
	–403A	Protective against HIV, slower progression to AIDS	(Liu et al., 1999) (Zhao et al., 2004)
	1.1T	Slower progression to AIDS	(Liu et al., 1999)
SDF1	801A	Slower progression to AIDS	(Winkler et al., 1998)
HLA	B*27	Slower progression to AIDS	(Gao et al., 2005)
	B*35	Faster progression to AIDS	(Carrington et al., 1999)
	B*57	Slower progression to AIDS	(Altfeld et al., 2003)
	Cw*04	Faster progression to AIDS	(Carrington et al., 1999)

Another group of genes regarded as important in HIV transmission and progression are the genes that code cytokines, cytokine receptors, and other immune response genes. Cytokines alter immune response and have an inhibitory or stimulatory effect on immune reactions (Hogan and Hammer, 2001). The role of cytokines in HIV infection is presented in Figure 4.
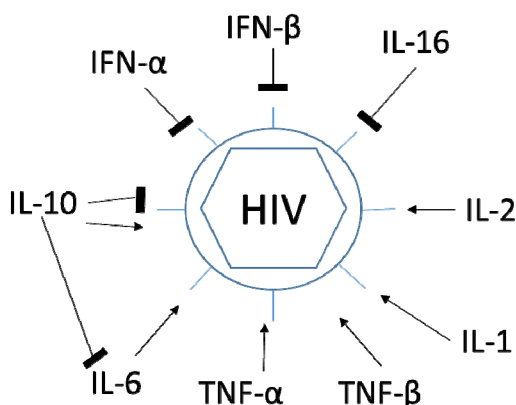
Figure 4. Cytokines influencing HIV infection. The arrows indicate a stimulative and the blocked arrows an inhibitory effect on HIV infection. (modified from Hogan and Hammer, 2001)

Levels of cytokines are influenced by multiple factors, such as their genetic polymorphisms. A list of cytokine related gene polymorphisms related to HIV infection is presented in Table 2.

Table 2. Associations between human genetic polymorphisms and HIV infection

Gene	Poly-morphism	Effect	Study population	Reference
IL-2	166T	Protective against HIV	Hispanic	(Wang et al., 2004)
	3896A	Protective against HIV	ALIVE cohort	(Shrestha et al., 2006) (Vlahov et al., 1991)
IL-4	−590T	Protective against HIV	REACH cohort	(Wang et al., 2004) (Wilson et al., 2001)
IL-6	174C	Trend towards faster progression to AIDS	Indian	(Sobti et al., 2010)
IL-10	−592A	Higher risk of acquiring HIV, faster progression to AIDS	Multiple	(Wang et al., 2004) (Shin et al., 2000) (Naicker et al., 2012)
	−1082G	Higher risk of acquiring HIV, faster progression to AIDS	Multiple	(Wang et al., 2004)
TNFα	−238A	Slower progression to AIDS	Caucasian	(Nasi et al., 2013)
TGFβ	−509T	Higher plasma viral load	Hispanic	(Freitas et al., 2015)

One important cytokine in the context of HIV is IL-10. It is an immuno-regulatory cytokine secreted by macrophages, monocytes, and T-helper cells (Moore et al., 2001). IL-10 inhibits the synthesis of proinflammatory cytokines and antigen presentation (Cassatella et al., 1993; de Waal Malefyt et al., 1991). It inhibits the production of multiple interleukins, TNF, and chemokines, affecting T cell responses (Andrew et al., 1998; Sozzani et al., 1998).

The IL-10 gene and promoter area possesses multiple genetic polymorphisms, of which −592C/A, −819C/T, and −1082G/A are the most studied (Eskdale and Gallagher, 1995; Eskdale et al., 1999; Eskdale et al., 1996; Eskdale et al., 1997; Lazarus et al., 1997; Turner et al., 1997). The −592 and −819 polymorphisms are in complete linkage disequilibrium and with −1082 form three haplotypes (GCC, ACC, and ATA) seen among Caucasian individuals (Edwards-Smith et al., 1999; Lazarus et al., 2002; Turner et al., 1997). In some populations, additional haplotypes (ATC and GTA) are present at low frequencies (Ramaseri Sunder et al., 2012). These three polymorphisms have been associated with susceptibility to HIV and its progression to AIDS (Oleksyk et al., 2009; Shin et al., 2000; Wang et al., 2004). Multiple studies have shown that −592A increases suscepti-bility to HIV infection and predisposes individuals to a faster progression to AIDS (Chatterjee et al., 2009; Naicker et al., 2012; Naicker et al., 2009; Oleksyk et al., 2009; Wang et al., 2004). −1082G carriers are more likely to acquire a HIV infection than −1082A carriers (Sun et al., 2013; Wang et al., 2004). Both polymorphisms have been associated with IL-10 production: −592A has been associated with low and −1082G with high IL-10 expression (Crawley et al., 1999; Edwards-Smith et al., 1999; Larsson et al., 2010; Reuss et al., 2002). However, the effects of these two polymorphisms in terms of susceptibility to HIV requires further investigation, because the effects of IL-10 production levels as a consequence of these two alleles seem to be opposite.

2.3.3.2. T cell factors associated with susceptibility to HIV infection

In addition to genetic factors, susceptibility to HIV is influenced by host immuno-logical factors, including T cell distribution and cell surface marker expression. Among HIV positive individuals, alterations in T cell subtypes and cell surface marker expressions have been described. These changes include loss of CD4+ T cells, an increase in immune activation, and alterations in multiple other immune functions. Similar changes, such as decline in CD4+ and an increase in immune activation, have been described among multiple cohorts of HIV exposed sero-negative individuals (ESN) (Biasin et al., 2000; Killian et al., 2004; Suy et al., 2007; Tran et al., 2006; Yang et al., 2002). Two hypotheses regarding changes in the immune system of ESN have been proposed: (a) these changes are a consequence of HIV exposure; or (b) they are constitutive characteristics of an individual that result in low susceptibility to HIV (Suy et al., 2007).

2.3.3.2.1. T lymphocyte classification

All human blood cells, including T lymphocytes, are derived from hematopoietic stem cells (Figure 5). Multiple classifications based on cell surface marker expression have been used to describe T lymphocytes. CD3+ cells are divided into CD4+ and CD8+ T cells, and thereafter both cell types are categorized into naïve and memory cell subsets. An additional group of CD4+ T cells are T regulatory cells (T_{reg}), which express CD4, FOXP3, and CD25.
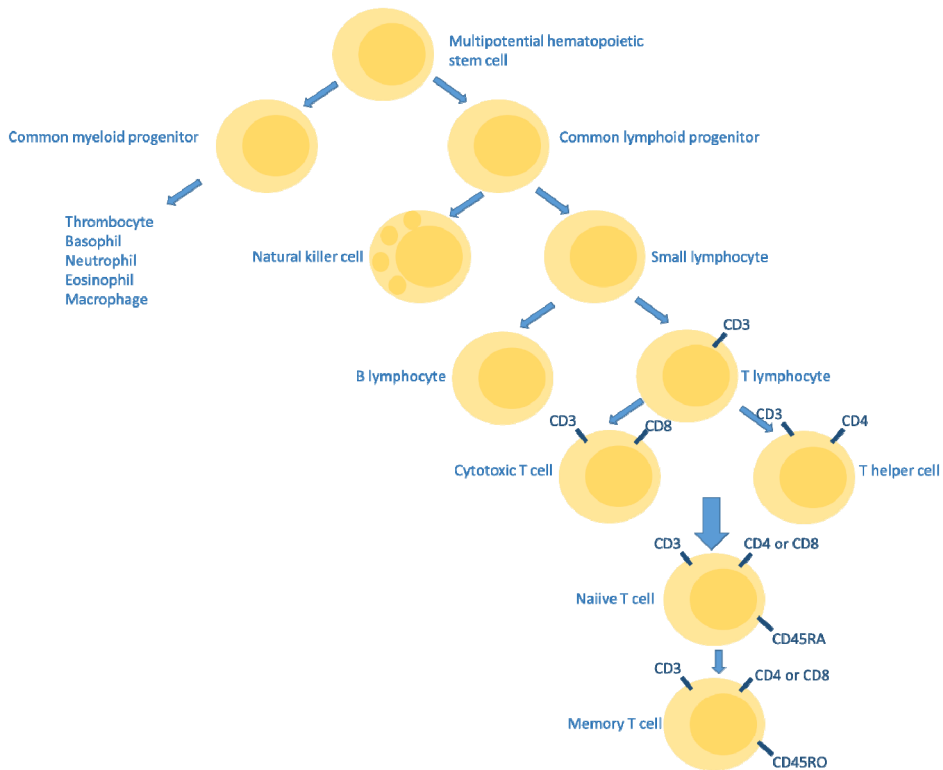
Figure 5. Blood cell development. All blood cells derive from multipotential hemato-poietic stem cells and through multiple stages of differentiation CD3+ T lymphocytes are formed. T cells are further divided into CD4+ T helper cells and CD8+ cytotoxic T cells, and then into naïve and memory cells.

Multiple classification methods have been used to divide CD4+ and CD8+ T cells into memory subtypes. Two widely used classifications are based on expression of CD45RA and CD45RO or CD45RA and CCR7 on cell surface. The simpler classification divides the cells using CD45RA and CD45RO into naïve (CD45RA+) and memory cells (CD45RO+). During transition from naïve to memory cells, there also exists a fraction of double positive CD45RA+RO+ cells, however, whether this cell type possesses distinct functions is unknown (Hamann et al., 1996).

The second classification divides T cells into four groups based on the expression of CD45RA and CCR7: naïve (T_N; CD45RA+ CCR7+); central memory (T_{CM}; CD45RA– CCR7+); effector memory (T_{EM}; CD45RA– CCR7–); terminally differentiated (T_{TEMRA}; CD45RA+ CCR7–) (Mahnke et al., 2013). When simply modelled, these cell types have a progenitor–product relationship (Figure 6) and distinct immunological functions. After antigen exposition, T cells evolve into T_{CM} cells and thereafter T_{EM} cells with immediate effector functions (Hamann et al., 1997; Sallusto et al., 1999). The final step in differentiation are T_{TEMRA} cells, which have low functional and reproductive capabilities (Geginat et al., 2003).

In addition to markers that divide cells into memory subtypes, T cells express immune activation markers such as CD38 and HLA-DR, and viral co-receptors such as CXCR4 and CCR5. These immune activation markers are mostly expressed on memory cells and HIV-specific T cells (Douek et al., 2002; Mahnke et al., 2013; Meditz et al., 2011). Both HIV co-receptors CCR5 and CXCR4 are expressed on T cells, however, CCR5 is mainly expressed on activated/memory cells and CXCR4 on naïve cells (Bleul et al., 1997; de Roda Husman et al., 1999). Therefore, CCR5-tropic HIV strains mainly infect memory CD4+ T cells (Schnittman et al., 1990); however, some CCR5+ memory cells, for example some types of T_{TEMRA}, can be resistant to HIV infection (Oswald-Richter et al., 2007).
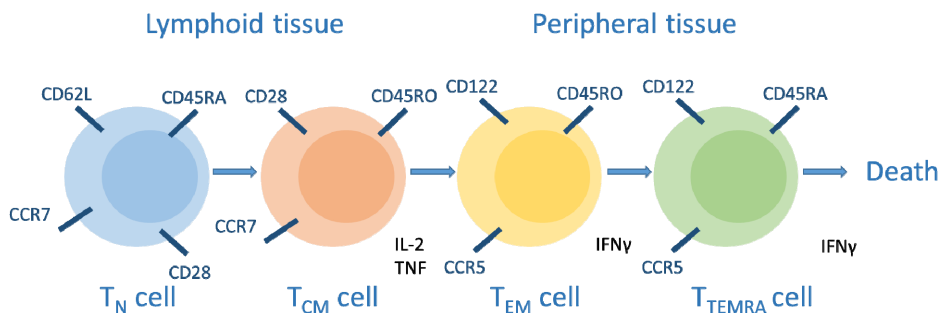
Figure 6. T cell differentiation. T_N = naïve; T_{CM} = central memory; T_{EM} = effector memory; T_{TEMRA} = terminally differentiated cells. Each cell type has different cell surface markers and produces different soluble substances (modified from Farber et al., 2014).

2.3.3.2.2. Changes in T cell populations and cell surface marker expression during exposure to and infection by HIV

Changes in all the aforementioned T cell populations are eminent among HIV positive individuals. These individuals have decreased numbers of CD4+ T cells and an increased percentage of CD8+ memory cell types. For example, HIV positive individuals have increased T_{EM} cell types and a T cell distribution

skewed towards terminally differentiated cells compared to healthy controls (Appay et al., 2002; Serrano-Villar et al., 2014). However, a higher proportion of T_{TEMRA} cells is protective against HIV progressing to AIDS (Northfield et al., 2007; Oswald-Richter et al., 2007). An important cell type in controlling HIV infections is T_{reg}, which reduce chronic immune activation, but conversely, can decrease direct anti-viral responses (Munier et al., 2013). As T_{reg} cells express the HIV co-receptor CCR5, they are possible targets for HIV infection (Schulze Zur Wiesch et al., 2011). The role and dynamics of this cell type during HIV infection is not yet clear, however, their levels can both increase and decrease during the persistent infection stage (Schulze Zur Wiesch et al., 2011; Simonetta et al., 2012).

Similar trends have been noticed among ESN, whereas their immune system changes in similar directions to HIV positive individuals, however, these alterations can depend on the route and level – for example their partner's viral load – of exposure (Biasin et al., 2000; Camara et al., 2010; Hasselrot et al., 2010; Jennes et al., 2003; Lo Caputo et al., 2003; Restrepo et al., 2010; Suy et al., 2007; Tran et al., 2006). Multiple studies have shown that ESN have skewed levels of CD4+/CD8+ T cells and or memory cell subpopulations, and in most cases increased levels of effector cells (Biasin et al., 2000; Jennes et al., 2003; Lo Caputo et al., 2003; Restrepo et al., 2010; Suy et al., 2007). These findings are summarized in Table 3. In addition to T cell distribution changes, both HIV positive individuals and ESN have altered expression of immune activation markers and HIV co-receptors. During HIV progression, the level of cells expressing immune activation markers increases (Meditz et al., 2011; Tiba et al., 2011).

The third T cell property extensively studied among sexually exposed subjects is the expression of the HIV co-receptor CCR5, and to a lesser extent CXCR4. As mentioned, CCR5 expression is dependent on multiple genetic and epigenetic polymorphisms in the CCR5 promoter region (Gornalusse et al., 2015; Thomas et al., 2006). However, during HIV infection CCR5 levels increase and correlate with a faster progression to AIDS (Bleul et al., 1997; Ostrowski et al., 1998; Yang et al., 2012). CCR5 levels also correlate with immune activation, because CCR5 is mainly expressed on activated cells (Meditz et al., 2011; Pierson et al., 2000). The situation among ESN is not as clear. Depending on the study population, CCR5 increased/decreased on CD4+ and or CD8+ T cells. For example, Tran et al. (2006) have shown decreased CCR5 expression among PWID, however, Suy et al. (2007) showed increased CCR5 expression among heterosexually exposed individuals. Regarding CXCR4, similarly contradictory results have been published showing both increased and decreased receptor expression during HIV infection (Jennes et al., 2003; Messele et al., 2001).

Table 3. Differences in T cell populations of HIV exposed seronegative individual (ESN) compared to controls

T cell subset	Direction	Route of exposure	References
CD4+	↓	Heterosexual	(Lo Caputo et al., 2003) (Suy et al., 2007)
CD4+CD45RA+	↑	Heterosexual	(Jennes et al., 2003) (Lo Caputo et al., 2003)
	↓		(Suy et al., 2007) (Biasin et al., 2000)
CD4+CD45RA+RO+	↑	Heterosexual	(Suy et al., 2007)
CD45RO+	↑	Heterosexual	(Biasin et al., 2000)
CD4+HLA-DR+	↑	Heterosexual	(Suy et al., 2007)
CD4+CD38+	↑	Heterosexual	(Suy et al., 2007) (Tran et al., 2006)
CD4+CCR5+	↑	Heterosexual Parenteral	(Suy et al., 2007) (Truong et al., 2003)
CD4+CXCR4+	↓	Heterosexual Parenteral	(Jennes et al., 2003) (Tran et al., 2006)
CD8+	↑	Heterosexual	(Killian et al., 2004) (Lo Caputo et al., 2003)
		Parenteral Homosexual	(Tran et al., 2006) (Yang et al., 2002)
CD8+CD28+	↓	Heterosexual Parenteral	(Suy et al., 2007) (Tran et al., 2006)
CD8+HLA-DR+	↑	Heterosexual	(Suy et al., 2007)
CD8+CD38+	↑	Heterosexual	(Biasin et al., 2000) (Suy et al., 2007) (Tran et al., 2006)
CD8+CCR5+	↓	Parenteral	(Tran et al., 2006)
CD8+CXCR4+	↓	Parenteral	(Tran et al., 2006)

2.4. Factors influencing susceptibility and progression of HBV and HCV

HIV, HBV, and HCV share routes of infection and co-infections are common. However, compared to HIV, both HBV and HCV can cause acute infections leading to chronic illness. The factors influencing hepatitis susceptibility and progression to chronic illness depend on both viral and host factors.

2.4.1. HBV and HCV pathogenesis

HBV infection occurs via multiple routes, for example from contaminated blood products, intravenous drug use (IVDU), mother-to-child transmission (MTCT), or unprotected sexual intercourse, especially among MSM (Remis et al., 2016). During acute infection, hepatocytes are mainly infected (Seeger and Mason, 2015; Summers et al., 2003). In 90% of adults, acute infections are resolved with the help of multiple cytokines, such as IFNα and IFNγ, and a vigorous CD4 and CD8 response (Ferrari et al., 1990; Liaw and Chu, 2009; Lucifora et al., 2014; Rehermann et al., 1995; Wieland et al., 2004). During chronic infection, HBV replication and accumulation of HBV viral proteins can lead to liver injury (Bertoletti et al., 2003; Chisari, 1997; Pugh and Summers, 1989). The reasons for chronic infection might be related to inhibition of antibody and T cell response by viral proteins, and the size of viral inoculum (Asabe et al., 2009; Chen et al., 2004; Chisari et al., 2010).

HCV is transmitted mainly through parenteral exposure (contaminated blood products or IVDU), and to a lesser extent MTCT (MacDonald et al., 2000). Similar to HIV, some HCV-exposed individuals remain uninfected, however, the mechanisms of this protection are unknown (Thurairajah et al., 2008; Warshow et al., 2012). An adequate CD8+ T cell response is thought to be the key factor in self-limiting infection. In the absence of an adequate cytotoxic T lymphocyte (CTL) response, acute HCV develops into a chronic infection (Chang et al., 2001; Grüner et al., 2000; Thimme et al., 2001).

2.4.2. Host factors associated with HBV and HCV infections

Similar to HIV, host factors influence susceptibility to hepatitis and its progression. Host factors can be divided into genetic and immunological. These host factors can in turn be divided into factors associated with acute and or chronic hepatitis infection.

2.4.2.1. Host genetic factors associated with HBV and HCV infections

Multiple studies have focused on the influence of genetic factors upon the course chronic HBV and HCV infections. Less attention has been paid to the influence of polymorphisms upon susceptibility to hepatitis or spontaneous clearance. As

T cell function is crucial in clearing HBV, genetic factors that determine immune responses to viruses have received the most attention (Table 4). For example, multiple polymorphisms in JAK/STAT pathway genes have been associated with chronic hepatitis B (Lu et al., 2015; Zhu et al., 2005).

Table 4. Associations between human genetic polymorphisms and HBV infection

Gene	Poly-morphism	Effect	Study population	Selected references
IL-6	−572G	Associated with HBV spontaneous clearance	Chinese	(Lu et al., 2014)
IL-10	−592A	Associated with HBV persistence Associated with asymptomatic HBV	Multiple	(Cheong et al., 2006) (Peng et al., 2006) (Ahmadabadi et al., 2012)
IL-18	−607A	Associated with HBV seronegativity		(Karra et al., 2015)
	−137G	Associated with HBV seronegativity		
IL-20	1807T	Decreased risk of chronic HBV	Caucasian	(Truelove et al., 2008)
BIM	rs3827537A	Elevated risk of chronic HBV	Han Chinese	(Peng et al., 2015)
TNFγ	−238A	Decreased susceptibility to HBV	Han Chinese	(Wang et al., 2012)
IFNγ	874A	Associated with intrauterine HBV	Chinese	(Zhu et al., 2005)
STAT4	rs7574865T, rs7282694C, rs11889341T, rs8179673C	Decreased risk of chronic HBV	Han Chinese	(Lu et al., 2015)

Host genetic factors play a major role in HCV pathogenesis, viral clearance, and treatment outcome. Studies among multiple ethnic populations have highlighted the role of interleukin genes in terms of susceptibility and spontaneous clearance (Table 5). For example, studies of IL-10 have shown that −592A and −1082C are associated with an increased risk of chronic HCV (Edwards-Smith et al., 1999; Knapp et al., 2003; Mangia et al., 2004; Sun et al., 2013). Additional genetic polymorphisms that affect the outcome of HCV infection are listed in Table 5. Most of the studies were focused on HCV viral clearance or treatment outcome; fewer studies have been conducted regarding susceptibility to HCV.

Table 5. Associations between human genetic polymorphisms and HCV infection

Gene	Poly-morphism	Effect	Study population	Selected references
IL-2	–330T	Higher risk of chronic HCV and or HBV	Han Chinese	(Gao et al., 2009)
IL-4	33C	Decreased risk of chronic infection	Brazilian	(Ramos et al., 2012)
IL-10	–592A	Higher HCV spontaneous clearance	Multiple	(Sun et al., 2013) (Mangia et al., 2004) (Edwards-Smith et al., 1999)
	–1082G	Increased/decreased risk of chronic infection	HIV+/HCV + Caucasian Brazilian	(Sun et al., 2013) (Persico et al., 2006) (Ramos et al., 2012)
IL28B	rs 12979860C rs 8099917T	Decreased risk of chronic infection	Brazilian	(Ramos et al., 2012) (Kurbanov et al., 2011)
TNFα	–238G	Increased risk of liver cirrhosis	Caucasian	(Corchado et al., 2013)
IFNγ	874A	Higher risk of chronic HCV	Han Chinese	(Gao et al., 2009)
	rs2069707G	Asociated with spontaneous clearance of HCV		(Azam et al., 2015)
TLR9	rs187084C	Associated with spontaneous clearance in women	Swiss	(Fischer et al., 2016)

2.4.2.2. T cell factors associated with HBV and HCV infections

Multiple studies investigating chronic HBV and HCV infections have been conducted, however, studies investigating factors related to hepatitis sero-positivity are lacking. In both self-limiting and chronic infections, CD4+ and CD8+ cell response determines the course of infection (Grakoui et al., 2003; Schmidt et al., 2013). A lack of adequate T cell response correlates with chronic infection (Ferrari et al., 1990; Grakoui et al., 2003; Rehermann et al., 1995; Thimme et al., 2001).

At the beginning of acute HBV infection, T-helper 1 response, together with IFNγ production, is triggered (Wieland et al., 2004). Thereafter, a HBV-specific CD8+ response is responsible for virus eradication (Thimme et al., 2003). During the acute infection phase, the level of T_{CM} cells increases, which is replaced by an increase in T_{EM} cells during the chronic infection phase (Carotenuto et al., 2011; Sobao et al., 2002).

During the acute HCV infection phase, CD8+CD38+ T cell response, together with IFNγ and CD4+ responses, determine the outcome of the infection. A lack of adequate T cell response results in chronic HCV (Thimme et al., 2001). These HCV-specific T cells responses are detectable – in the liver, where the cells mostly reside, not in peripheral blood – decades after the initial infection and help clear re-infections (He et al., 1999; Osburn et al., 2010; Takaki et al., 2000). One important aspect of HCV clearance is the ability to produce IFNγ to suppress viral replication (Gruener et al., 2001; Jo et al., 2009). However, high levels of immunosuppressive IL-10 are associated with progression to chronic infection (Flynn et al., 2011). During HCV infection, high numbers of HCV-specific T_{EM} and T_{TEMRA} cells are produced, and immune activation occurs (Appay et al., 2002; Shen et al., 2010; Urbani et al., 2002).

2.5. IVDU and its relationship to T cell distribution and HIV, HBV, and HCV susceptibility

According to the United Nations Office of Drugs and Crime (UNODC), about 1 in 20 adults worldwide used at least one drug in 2014, and over 29 million people have drug use disorders (UNODC, 2016). There were approximately 12 million intravenous drug users in 2013, all of whom have an elevated risk of death due to overdose or HIV infection (Mathers et al., 2013). Worldwide, about 14% of PWID are HIV positive (EMCDDA, 2015; UNODC, 2016). The overall trend in the EU indicates that IVDU account for about 8% of new HIV cases, however, the level is up to 22 times higher in the Baltic region (EMCDDA, 2015; UNAIDS, 2014). In addition to HIV, PWID are also exposed to HCV and HBV (Estrada, 2002; Rahimi-Movaghar et al., 2010; Zhou et al., 2012). In the EU, HCV antibody prevalence among PWID ranges from 14–84% and worldwide, about 6 million PWID are anti-HBc positive (EMCDDA, 2015; Nelson et al., 2011).

The effects of drug use on the immune system depend on the substance and dose consumed (Peterson et al., 2004). Experiments regarding opioids have mainly been conducted on animal models or cell cultures; *in vivo* data are limited. Most studies have focused on morphine, which inhibits IL-2 transcription and increases IL-4 and IL-12 production, which directs T cells into the T-helper 2 lineage (Börner et al., 2009; Messmer et al., 2006; Roy et al., 2004; Sacerdote et al., 2003; Wang et al., 2003). Through the suppression of IFNα and IFNγ, morphine decreases CD8+ T cell responses (Wang et al., 2005). In addition, morphine up-regulates HIV co-receptor CCR5 expression, increasing the risk of HIV infection (Mahajan et al., 2005). Similar effects with CCR5 increase or T cell permissiveness to HIV infections have been noted with cocaine, heroin, and methadone (Baum et al., 2009; Friedman et al., 2003; Kim et al., 2013; Li et al., 2002; Steele et al., 2003; Suzuki et al., 2002).

2.6. Summary of the literature

Some individuals remain HIV-uninfected despite repeated exposure. Most of the studies investigating these individuals have focused on men having sex with men, serodiscordant couples, and commercial sex workers (Horton et al., 2010). Data regarding persons who inject drugs remains limited.

Most studies have focused on genetic polymorphisms and their effect upon susceptibility to HIV and its progression. In addition to the HIV co-receptor CCR5, researchers have focused on the HLA region and the effect of interleukin polymorphisms on viral susceptibility. For example, two polymorphisms in the IL-10 promoter region (–1082 and –592) have been associated with susceptibility to HIV and its disease progression. However, the results of these studies have depended on the study groups involved; information about persons who inject drugs remains limited.

In addition to genetic polymorphisms, immunological responses to HIV infection have been investigated. Multiple studies have shown that HIV exposed seronegative individuals have an altered T cell distribution (increased numbers of memory T cells), higher levels of immune activation, and differences in CCR5 expression, compared to healthy volunteers. As with genetic studies, analysis has focused mainly on sexually exposed subjects. However, the defense mechanisms against HIV might be different during parenteral exposure, because in this case there is no mucosal barrier.

When investigating PWID populations, other blood borne infections, such as HBV and HCV, must be taken into account. Both genetic and immunological factors influence HBV and HCV clearance and the course of chronic infections. The main focus of studies has been on the course of infections, not susceptibility, because it is more difficult to recruit groups to assess the factors that affect susceptibility or hepatitis spontaneous clearance.

The Estonian PWID population consists mainly of young male Caucasian individuals infected with monophyletic HIV-1 CRF06_cpx viruses (Adojaan et al., 2005; Avi et al., 2011; Avi et al., 2010). This provides an opportunity to analyze the effects of genetic polymorphisms upon susceptibility to HIV among a homogenous population, without taking into account the potential effects of ethnic and viral variation. There are high rates of opioid use, especially fentanyl, among Estonian PWID (Uusküla et al., 2007; Uusküla et al., 2015b). Similar to other PWID populations, Estonian PWID are often co-infected with HIV, HBV, and or HCV viruses, making it possible to analyze the effects of co-infections on immunological factors (Soodla et al., 2015; Uusküla et al., 2007).

In conclusion, genetic polymorphisms, immunological factors, and IVDU play an important role in HIV, HBV, and HCV infections. This thesis focuses on two IL-10 polymorphisms (–1082C/A and –592G/A) and T cell distribution in the seropositivity of HIV, HBV, and HCV infections and their co-infections among persons who inject drugs.

3. AIMS OF THE STUDY

The general aim of this thesis was to assess the effect of IL-10 polymorphisms on susceptibility to HIV, HBV, and or HCV, and to determine how exposure and seropositivity to these viruses influences T cell distribution among a Caucasian PWID population.

The study had the following specific objectives:

1. To investigate whether and how the IL-10 genetic polymorphisms −1082 and −592 affect susceptibility to HIV, HBV, and or HCV infections in PWID
2. To examine the associations between HIV exposure, CCR5 expression, immune activation (the number of HLA-DR+ cells) and T cell distribution among PWID
3. To investigate the associations between HIV co-infections (HIV/HCV or HIV/HBV or HIV/HBV/HCV) and CCR5 expression, immune activation, and T cell distribution among PWID
4. To investigate whether and how intravenous drug use influences CCR5 expression, immune activation, and T cell distribution among PWID

4. MATERIALS AND METHODS

4.1. Study design and population

All three studies were conducted in Caucasian PWID (formerly regarded as intravenous drug users – IDU) recruited in 2011 Tallinn, Estonia. A total of 345 subjects were enrolled from syringe exchange program using respondent-driven sampling (Malekinejad et al., 2008) by the Estonian National Institute for Health Development and the Institute of Family Medicine and Public Health of University of Tartu. Venous blood samples and interviewer administered questionnaire was obtained from all study subjects. The questionnaire included questions regarding demographical data such as date of birth, gender, nationality, duration of IVDU, and other risk behaviors. Of all the PWID (Figure 7), 173 were HIV negative. From these HIV negative individuals, 20 reported syringe/needle sharing at least once a month during the six months prior to the interview. They were assigned as heavily exposed HIV seronegative subjects (HESN). In addition, 47 HIV negative PWID reported syringe/needle sharing at least once within the previous six months and they were classified as exposed HIV seronegative subjects (ESN). Similar categorization for HBV and HCV was used. From the 345 PWID, 13 highly exposed subjects were HBV and 4 HCV negative.
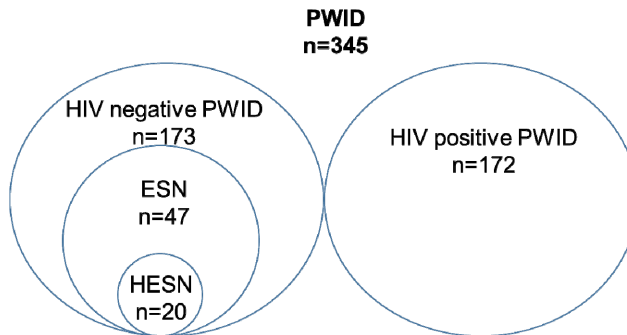
Figure 7. The classification of persons who inject drugs (PWID). HESN – heavily exposed HIV seronegative subjects, ESN – exposed seronegative subjects.

The main characteristics of the studies are presented in Table 6. The studies included two different control groups. The first group consisted of 496 blood donors collected in Tallinn and Ida-Viru County in 2010. The demographic data of these controls was not available. The second control group consisted of 47 HIV, HBV, HCV negative healthy volunteers matched by age- and sex to the ESN group. They were recruited in 2012–2013 in Tartu, Estonia.

To determine, the associations between frequent HIV exposure, HESN were selected as the study group in IL-10 study. Because these individuals are rare and difficult to recruit, a bigger sample size of ESN was chosen for the flow cytometry studies, to increase the power of the study.

Table 6. Studies that form basis of this thesis

Study name	Study populations	Primary aim	Publi- cation
IL-10 study	**PWID** (n = 345) ↳HIV+ (n = 172) ↳HIV– (n = 173) ↳HESN (n = 20) **Blood donors** (n = 496)	To determine the associations between IL-10 –1082 and –592 polymorphisms and HIV, HBV, and HCV seropositivity	1
T cell HIV study	**PWID** (n = 88) ↳HIV+ (n = 41) ↳ESN (n = 47) **Controls** (n = 47)	To determine the associations between HIV seropositivity, CCR5 expression, immune activation, and T cell distribution	2
T cell co-infection study		To determine the associations between HIV, HBV and HCV co-infections, IVDU CCR5 expression, immune activation, and T cell distribution	3

Note: PWID – persons who inject drugs, HESN – heavily exposed HIV seronegative subjects, ESN – exposed seronegative subjects

4.2. Ethical considerations

All studies were approved by the Research Ethics Committee of the University of Tartu (Ethics Review Committee approval no 204/T-13 in 08.06.2011, 209T-16 in 11.12.2011, and 216/T-18 in 25.06.2012). All subjects signed informed consent and blood donors agreed with using leftover blood for research.

4.3. Sample collection and processing

For IL-10 polymorphisms analyses, leftover samples of blood donors were collected into EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and stored at +4 °C. After HIV, HCV and HBV testing, confirmed seronegative blood samples were sent to the laboratory of University of Tartu, Department of Microbiology. The blood samples were stored at –80 °C for DNA extraction.

From each PWID and flow cytometry control subject, 16 ml venous blood was collected into EDTA tubes (VACUETTE®, Greiner Bio-One GmbH, Fricken-hausen, Germany) and transported to the laboratory of the University of Tartu, Department of Microbiology within 24 hours. Thereafter, peripheral blood mononuclear cells (PBMC) were separated using Ficoll gradient and stored at –80 °C in freezing media (90% fetal bovine serum and 10% dimethyl sulfoxide).

4.4. HIV, HBV and HCV antibody testing

The serostatus testing for HIV was performed in the Estonian Central HIV Reference Laboratory using a fourth generation enzyme-linked immunoassay (Vironistica HIV Uniform II Ag/Ab, BioMerieux, Marcy Etoile, France) as previously described by Huik *et al*. (Huik et al., 2013). The results were confirmed by using immunoblotting (INNO LIA HIV I/II Score Westernblot, Microgen Bioproducts Ltd, Surrey, UK). The HBV and HCV serostatuses were defined in the laboratory of the Estonian National Institute for Health Development. The HBV antibodies and antigens were detected with ETI-MAK-4 HBsAg and ETI-AB-COREK Plus (both DiaSorin, Saluggia, Italy). Vaccination status against HBV was defined based on the HBs Ab. The seropositivity of HCV was tested with the ETI-AB-HCVK-3 test (DiaSorin, Vercelli, Italy).

4.5. Determination of IL-10 promoter polymorphisms

For the detection of IL-10 promoter polymorphisms, genomic DNA was extracted from blood donors' whole blood samples and PBMC samples of PWID using PureLink® Genomic DNA Kit (Invitrogen, Applied Biosystems Foster City, CA, USA) according to the manufacturer's instructions and thereafter stored at − 80 °C. IL-10 polymorphisms −1082G/A and −592C/A (rs1800896 and rs1800872, respectively) were analyzed using 7900HT Fast Real-Time PCR System and TaqMan allelic discrimination assays (both Applied Biosystems Foster City, CA, USA) (De La Vega et al., 2002).

4.6. T cell distribution analysis

The T cell subsets, immune activation (HLA-DR expression), and CCR5 expression were determined using multi-color flow cytometry. The thawed PBMC were diluted using 1x Dulbecco's phosphate-buffered saline (DPBS, Sigma Life Science, St. Louis, MO, USA) and stained with titrated fluorochrome-conjugated monoclonal antibodies (Table 7) according to antibody's manufacturer's instructions.

Table 7. Monoclonal antibodies used for the experiments

Cell surface marker	Fluorochrome	Manufacturer
CD3	PerCP-Cy5.5	Biolegend, San Diego, CA, USA
CD4	FITC	Biolegend, San Diego, CA, USA
CD8	PE	Biolegend, San Diego, CA, USA
CD45RA	PE-Cy7	Biolegend, San Diego, CA, USA
CD45RO	BV711	Biolegend, San Diego, CA, USA
CCR7 (CD197)	PE-CF594	Biolegend, San Diego, CA, USA
HLA-DR	BV510	Biolegend, San Diego, CA, USA
CCR5 (CD195)	APC	Becton Dickinson, Franklin Lakes, NJ, USA

Thereafter cells were fixed with 1x FACS lysing solution (Becton Dickinson, Franklin Lakes, NJ, USA) and washed twice with 1x DPBS (Sigma Life Science, St. Louis, MO, USA). Next, cells were dissolved in 500 µl of 1% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and analyzed within two hours on a LSR Fortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The flow cytometer configuration is presented in Table 8. For the flow cytometry data analyses FACS Diva software version 6.2 (Becton Dickinson, Franklin Lakes, NJ, USA), automatic compensation calculations, and fluorescence minus one (FMO) controls for CCR5 and HLA-DR were used. All data analyses were performed in a blinded manner. The data are presented as percentages from the parent cell populations. Additionally to percentages, the CCR5 cell surface density was analyzed as median fluorescence intensities (MFI-s).

For the HIV susceptibility study, the T cells were hierarchically divided into CD4+ and CD8+ T cells and thereafter in to naïve (CD45RA+CD45RO–) and memory cells (CD45RA–CD45RO+). For the co-infection study, a more complex allocation of memory cells was used. Both CD4+ and CD8+ T cells were divided into naïve (T_N; CD45RA+ CCR7+), central memory (T_{CM}; CD45RA– CCR7+), effector memory (T_{EM}; CD45RA– CCR7–), and terminally differen-tiated (T_{TEMRA}; CD45RA+ CCR7–) cells based on CD45RA and CCR7 expres-sion. In addition, in both studies the percentages of activated cells (HLA-DR+) and CCR5+ cells were measured. To avoid spectral overlap in the CCR5 analyses, the CCR5-APC was the only fluorochrome chosen for the red laser.

Table 8. LSR Fortessa configuration

Laser	Wavelength	Mirror	Filter	Parameter
Violet	405	670 LP	710/40 BP	BV711
		505 LP	525/50 BP	BV510
Blue	488	685 LP	710/50 BP	PerCP-Cy5.5
		505 LP	530/30 BP	FITC
		–	488/10 BP	SSC
Yellow-Green	561	750 LP	780/60 BP	PE-Cy7
		600 LP	610/20 BP	PE-CF594
		–	586/15 BP	PE
Red	640	–	670/14 BP	APC

Note: SSC – side scatter, LP – longpass, BP – bandpass

4.7. CCR5 Δ32 analyses

To analyze the effect of CCR5 Δ32 mutation on the CCR5 expression, DNA was extracted from PBMC samples of flow cytometry study subjects (47 controls, 41 ART naïve HIV positive PWID, and 47 ESN) using PureLink® Genomic DNA Kit (Invitrogen, Foster City, CA, USA). The CCR5 Δ32 was analyzed using PCR at the laboratory of the University of Tartu, Department of Microbiology as described previously by Samson *et al*. (Samson et al., 1996).

4.8. Statistical analysis

In all studies R version 2.13 was used for statistical analyses and p-values < 0.05 were considered statistically significant. The p-values in IL-10 analyses were not corrected for multiple testing.

Fisher's exact test was used for the IL-10 polymorphism allele frequencies and genotype pairs' analyses. Uni- and multivariate logistic regression was used for determining the associations between IL-10 polymorphisms and HIV and HBV serostatuses. Two different models were used for the comparisons: allele and genotype model. In both models the allele/genotype of interest was compared to the sum of all other alleles/genotypes. In addition, Hardy-Weinberg Equilibrium was tested in all alleles (Rodriguez et al., 2009).

The differences in the T cell subset analyses were determined using Mann-Whitney-Wilcoxon test and the frequency of CCR5 Δ32 mutation with Fisher's exact test. Holm–Bonferroni method was used for adjusting the results for multiple comparisons.

5. RESULTS AND DISCUSSION

5.1. The effect of IL-10 polymorphisms on HIV, HBV, and HCV serostatus (Paper I)

Paper I describes the influence of IL-10 polymorphisms in positions −592 and −1082 on the HIV, HBV, and HCV serostatus of Caucasian PWID. This is the first study to evaluate IL-10 −1082 and −592 alleles, genotypes, and genotype pairs among Eastern-European Caucasian PWID.

5.1.1. Population characteristics in the IL-10 study

Detailed characteristics of the study population are summarized in Table 9. The median duration of IVDU was 11 years, and the PWID subjects had high percentages of HCV and HBV infections (89% and 67%, respectively). Demographical and HBV vaccination data of the blood donors was not available.

Table 9. Characteristics of the PWID

	PWID (n = 345)
Males, n (%)	272 (79%)
Age in years, median (IQR)	30 (25–34)
Duration of IVDU (years), median (IQR)	11 (7–14)
HIV seropositive, n (%)	172 (50%)
HCV seropositive, n (%)	306 (89%)
HBV seropositive, n (%)	232 (67%)
HBV vaccinated, n (%)	43 (12%)

Note: IQR = interquartile range

5.1.2. IL-10 allelic frequencies and HIV serostatus

The allelic frequencies of IL-10 −1082 and −592 among the study groups are presented in Table 10. The −1082A allele and the −592C allele were the most frequent in all groups (Table 10).

Table 10. Allelic frequencies in study populations

	−1082		−592	
	A	G	C	A
PWID (n = 345)	0.57	0.43	0.78	0.22
↳**HIV negative PWID** (n = 173)	0.55	0.45	0.76	0.24
↳**HESN** (n = 20)	0.70	0.30	0.70	0.30
↳**HIV positive PWID** (n = 172)	0.59	0.41	0.80	0.20
Blood donors (n = 496)	0.54	0.46	0.79	0.21

Note: PWID = persons who inject drugs, HESN = heavily exposed HIV seronegative subjects

Regarding HIV infection, differences in both –1082 and –592 polymorphisms were observed between HESN and blood donors. The allelic frequencies of IL-10 –1082 and –592 were similar between PWID and blood donors. However, all HESN had at least one –1082A allele compared to 81.4% of HIV positive PWID (p=0.029) and 79.0% of blood donors (p=0.019).

When investigating position –592, HESN had more A carriers than HIV positive PWID (60% and 34.3%, p=0.029). In univariate analyses, –592A was associated with decreased odds of HIV infection (OR 0.35; 95% CI 0.13–0.90; p=0.029), which remained significant after adjustment to HCV, HBV serostatus, and IVDU (OR 0.28; 95% CI 0.10–0.81; p=0.019).

To sum up, IL-10 promoter polymorphisms –1082A and –592A were over-represented among HESN compared to HIV positive PWID.

Multiple studies have analyzed the effects of IL-10 polymorphisms –1082 and –592 on susceptibility to HIV and its progression. However, studies among PWID have been limited and they have not been analyzed separately, but in pools with sexually exposed individuals (Shin et al., 2000; Wang et al., 2004). More data is available about the –592C/A polymorphism in relation to suscepti-bility to HIV, where the –592A has been associated with increased suscepti-bility to HIV among multiple cohorts of different ethnicities and exposure routes (Chatterjee et al., 2009; Naicker et al., 2009; Shin et al., 2000; Shrestha et al., 2006). These results are opposite to those of the current study; only a small study has observed a higher –592A frequency among HIV/tuberculosis co-infected patients compared to controls (Ramaseri Sunder et al., 2012). The reasons for this discrepancy with larger studies are unknown, but might be related to the type of study subjects. Most studies have investigated mixed ethnic and exposure groups, neither focusing solely on Caucasian nor parenterally exposed subjects (Chatterjee, 2010; Naicker et al., 2009; Shin et al., 2000; Wang et al., 2004). In addition, none of these studies analyzed individuals with high expose to HIV, which could account for the differences between this and previous studies. Another reason for dissimilarities might be other poly-morphisms of the IL-10 gene and promoter region, or combinations of different interleukin gene polymorphisms (for example IL-10 together with IL-6) not detected during this study that may have affected IL-10 production and inter-leukin balance, and therefore susceptibility to HIV (Eskdale et al., 1999; Eskdale et al., 1996; Eskdale et al., 1997; Gibson et al., 2001; Sobti et al., 2010). Less data are available about –1082A/G polymorphism, however, similarly to the results of the current study, –1082G has been associated with susceptibility to HIV among multiple study cohorts (Ramaseri Sunder et al., 2012; Wang et al., 2004).

5.1.3. IL-10 genotype frequencies and HIV serostatus

Next, the distribution of IL-10 genotypes was assessed (Table 11). As mentioned in the previous chapter, all HESN possessed at least one −1082A allele and therefore no −1082GG homozygotes were found, compared to 18.6% of HIV positive PWID and 21.0% of blood donors (p=0.029 and p=0.019, respectively). In addition, HESN had less −592CC homozygotes than HIV positive PWID (40.0% and 65.8%, respectively; p=0.029).

Table 11. Genotype frequencies among the different study populations, n (%)

	−1082			−592		
	AA	AG	GG	CC	AC	AA
PWID (n = 345)	117 (34)	159 (46)	69 (20)	212 (61)	114 (33)	19 (6)
HIV− PWID (n =173)	55 (32)	81 (47)	37 (21)	99 (57)	65 (38)	9 (5)
HESN (n = 20)	8 (40)	12 (60)	0 (0)	8 (40)	12 (60)	0 (0)
HIV+ PWID (n = 172)	62 (36)	78 (45)	32 (19)	113 (66)	49 (28)	10 (6)
Blood donors (n = 496)	141 (28)	251 (51)	104 (21)	306 (62)	167 (34)	23 (5)

Figure 8 shows, that the percentage of −592AC heterozygotes was higher among HESN than HIV positive PWID and blood donors (60.0% to 28.3% and 60.0% to 33.7%; p=0.009 and p=0.028, respectively). Persons possessing −592AC had decreased odds of HIV infection compared to those not carrying this genotype (OR 0.27; 95% CI 0.10–0.69; p=0.064). It remained significant after adjusting for HCV and HBV serostatus, and IVDU (OR 0.24 95% CI 0.08–0.69; p=0.008).
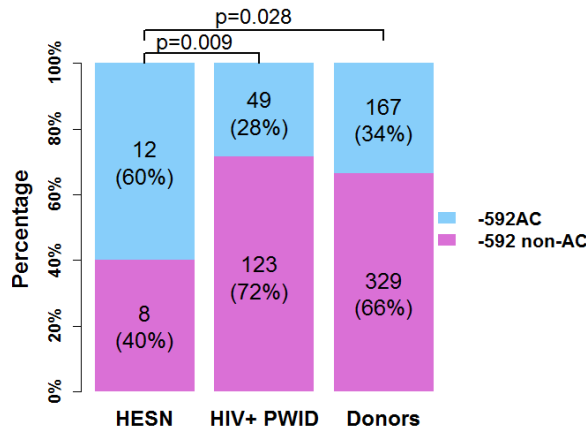

Figure 8. The frequency of the IL-10 −592AC genotype compared to HIV serostatus among heavily exposed HIV seronegative individuals (HESN), HIV positive persons who inject drugs (HIV+ PWID), and blood donors. Blue indicates those with and violet those without the genotype. Values in the columns represent the numbers and percentages of subjects carrying the genotype.

Although the –592A and –592A allele containing genotypes have been associated with increased susceptibility to HIV, opposite to the results of the current study, data regarding the heterozygote genotype is largely absent (Naicker et al., 2009; Shin et al., 2000). Only a small-scale study conducted among Indian HIV/tuberculosis patients has shown high –592AC genotype frequency among HIV positive individuals (Ramaseri Sunder et al., 2012). Based on these two studies, one might hypothesize that in addition to the –592A allele having a protective effect, the –592AC heterozygote genotype that is over-represented among HESN is protective against HIV. However, as the –592AA genotype was rare among the study groups (5–6% of subjects), and the HESN study group was very small, the potential protective effect of just –592A alleles against HIV infection cannot be excluded.

5.1.4. IL-10 genotype pair frequencies and HIV serostatus

Among the study subjects, six genotype pairs comprising of –1082 and –592 genotypes were present (Figure 9). Among all populations, the most common genotype pair was AG/CC (–1082/–592).
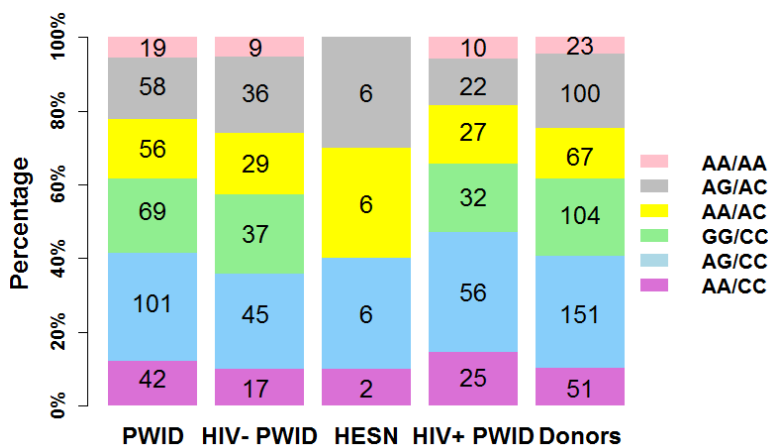
Figure 9. The frequency of IL-10 genotype pairs among persons who inject drugs (PWID), HIV negative PWID, heavily exposed HIV seronegative individuals (HESN), HIV positive PWID, and blood donors. Pink indicates those with AA/AA, grey those with AG/AC, yellow those with AA/AC, green those with GG/CC, blue those with AG/CC, and violet those with AA/CC genotype pairs. Values in the columns represent the number of subjects.

The HIV positive PWID group had less individuals with AG/AC genotype pairs compared to blood donors (12.8% and 20.2%, respectively; p=0.03) (Figure 10a). As all HESN lacked the –1082GG genotype, no one had a GG/CC genotype pair (Figure 10b). In addition, the HESN group had more individuals with the AA/AC genotype pair than the blood donor group (30.0% and 13.5% respectively; p=0.049) (Figure 10c).
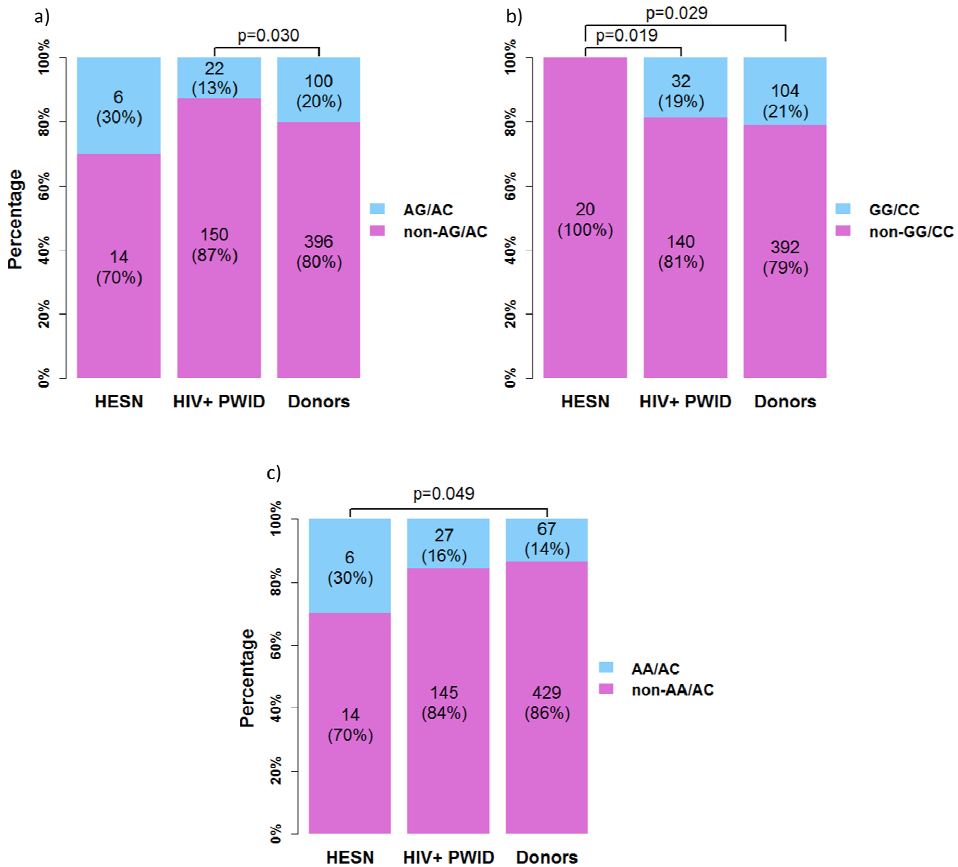
Figure 10. The frequency of IL-10 genotype pairs AG/AC, GG/CC, and AA/AC compared to HIV serostatus among heavily exposed HIV seronegative individuals (HESN), HIV positive persons who inject drugs (HIV+PWID), and blood donors. Blue indicates those with and violet those without the respective genotype pair among the HESN, HIV positive PWID, and blood donor groups. Values in the columns represent the number and percentage of subjects carrying the given allele.

Overall, HESN had overrepresentation of AG/AC and AA/AC genotype pairs, and underrepresentation of GG/CC genotype pairs, compared to controls.

At least 50% of IL-10 production is genetically determined and both $-592A$ and $-1082A$ have been associated with low IL-10 expression (Crawley et al., 1999; Reuss et al., 2002; Westendorp et al., 1997). Therefore, haplotypes comprised of $-1082A$ and $-592A$ have the lowest IL-10 expression and $-1082G$ and $-592C$ the highest (Crawley et al., 1999). Based on the current study, possessing a low IL-10 producing genetic variant is protective against HIV. Comparing the results of the current with previous studies, some differences are apparent, as the $-592A$ allele has mainly been associated with increased susceptibility to HIV (Naicker et al., 2009; Shin et al., 2000; Wang et al., 2004).

These differences could be explained by the analyzing of additional poly-morphisms in the IL-10 promoter region during other studies. Wang et al. (2004) investigated the presence of multiple IL-10 polymorphisms in large HIV study cohorts and found that −1082G is protective against HIV and −592A increases susceptibility to HIV. However, when the same study analyzed IL-10 haplotypes comprised of 5 SNPs in the region, the results were somewhat opposite to the allele frequency results. More specifically, −1082G/−592C con-taining genetic variants were associated with increased and −1082A/−592A with decreased susceptibility to HIV, which is in accordance with the results of the current study. Overall, this shows that not only alleles, but their combinations, must be taken into account when analyzing IL-10 polymorphisms, and that low IL-10 producing genetic variants are protective against HIV.

The protective effect of low IL-10 producing variants against HIV might be related to CCR5 expression. Multiple studies have shown that IL-10 up-regulates HIV co-receptor CCR5 expression and promotes HIV infection (Alfano and Poli, 2005; Juffermans et al., 2000; Sozzani et al., 1998; Wang et al., 2002). In addition, an IL-10 deficiency enhances HIV viral clearance and functional T-cell response (Brooks et al., 2006). Based on these mechanisms, lower IL-10 producing genetic variants could be protective against HIV.

5.1.5. IL-10 polymorphisms and HCV and HBV serostatus

The allelic frequencies between HBV-seropositive and -seronegative PWID and HCV-seropositive and -seronegative PWID were similar. Only one significant difference was observed with HCV: HCV negative PWID were less likely to have a AG/CC genotype pair than blood donors (15.4% and 30.4%, respec-tively; p=0.046).

Of the HBV negative PWID, 13 were highly exposed to the virus – reported syringe/needle sharing at least once a month during the previous six months and were not vaccinated against HBV – yet seronegative. Similar to the HIV results, highly exposed HBV seronegative PWID had more −592AC heterozygotes than HBV positive PWID (61.5% and 33.7%, respectively; p=0.03) (Figure 11a). Presence of −592AC was associated with decreased odds of HBV infection (OR 0.28; 95% CI 0.09–0.87; p=0.028), which remained significant after adjustment for HCV and HIV serostatus, and the duration of intravenous drug use (OR 0.29; 95% CI 0.09–1.00; p=0.05). Due to the increased number of −592AC among HBV exposed seronegative PWID, they also had more AG/AC genotype pairs than HBV positive PWID and blood donors (46.2% to 14.2% and 46.2% to 20.2%; p=0.008 and p=0.034, respectively) (Figure 11b). Persons not carrying AG/AC had approximately five times lower odds of being HBV positive compared to AG/AC carriers (OR 0.19; 95% CI 0.06–0.61; p=0.052); this remained significant in multivariate analyses (OR 0.20; 95% CI 0.06–0.70; p=0.012).
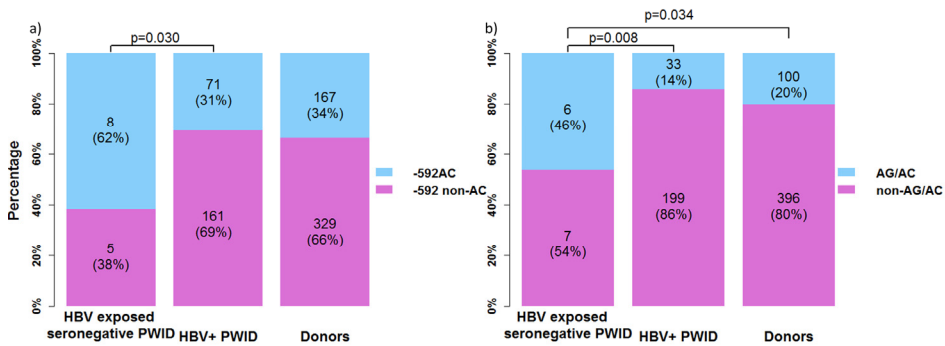
Figure 11. IL-10 –592AC and the genotype pair AG/AC among HBV seronegative exposed persons who inject drugs (PWID), HBV positive PWID, and blood donors. Blue bars indicate those with and violet bars without the given genotype (panel a) or genotype pair (panel b). Values in the columns represent the number and percentages of subjects.

Multiple studies have evaluated the effect of IL-10 genetic polymorphisms in the context of HBV spontaneous clearance or treatment outcome. There are no studies that have investigated IL-10 polymorphisms and HBV susceptibility among PWID. Owing to the different outcomes of studies (HBV clearance or treatment outcome) and variable study populations (ethnicity, exposure route, and co-infections), results have been inconclusive, demonstrating both positive and negative effects of –592A during chronic HBV infection (Cheong et al., 2006; Gao et al., 2009; Miyazoe et al., 2002; Peng et al., 2006). Some researchers have hypothesized that higher IL-10 levels might decrease effective immune responses against HBV infection, leading to chronic or occult HBV infection, and that the carriers of –592A do not clear the virus spontaneously (Ramezani et al., 2012; Shin et al., 2003; Truelove et al., 2008). The results of the current study imply that similar to HIV infection and HBV progression, high IL-10 producing genetic variants appear to have higher susceptibility to HBV. However, based only on the results of the current study, it is not possible to draw generalized conclusions regarding the role of IL-10 polymorphisms in susceptibility to HBV, thus additional studies of larger populations are needed to clarify the effects of IL-10.

5.2. T cell distribution and CCR5 expression in PWID (Papers II and III)

Papers II and III describe the differences in T cell distribution and CCR5 expression among multiple groups of PWID and controls. PWID were categorized based on their HIV status and then according to the presence or absence of HBV and or HCV co-infections.

5.2.1. Population characteristics in the T cell study

The characteristics of the study population are presented in Table 12. In terms of age and gender, PWID and controls were similar. Less PWID were vaccinated against HBV than controls (13% and 49%, respectively; p<0.001).

Table 12. Characteristics of the study population

	PWID (n = 88)	**Controls** (n = 47)
Gender Male, n (%)	72 (82%)	39 (83%)
Age Years, median (IQR)	30 (25–33)	29 (25–33)
Duration of intravenous drug use Years, median (IQR)	11 (7–14)	NA
Receptive syringe sharing During last six months, n (%)	83 (94%)	NA
HBV vaccination n (%)	11 (13%)	23 (49%)

Note: PWID = persons who inject drugs; controls = unexposed seronegative subjects; IQR = interquartile range.

The PWID population was characterized by high levels of co-infections. In total, half of PWID were HIV positive, most of them HCV positive, and 55 (63%) HBV seropositive (Figure 12). Of PWID, 35 (40%) had a triple infection (HIV+ HBV+HCV+) and only 11 (8%) were seronegative of all three viruses.
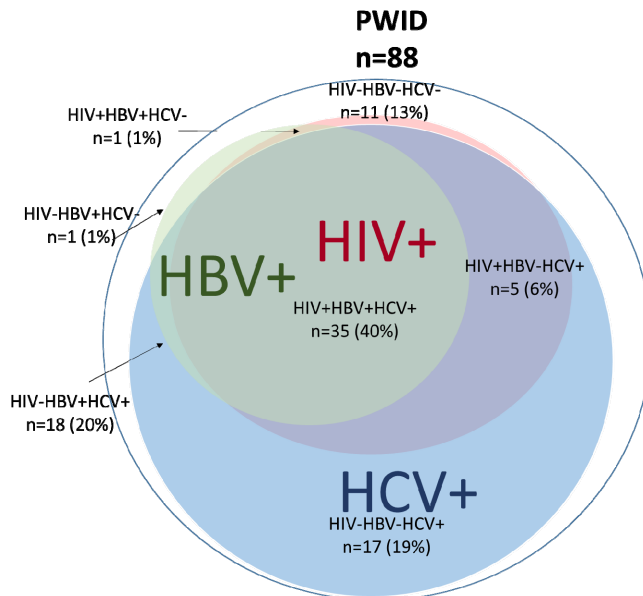

Figure 12. HIV, HCV, and HBV serostatus among the PWID. The white circle represents all PWID, the blue circle HCV+ PWID, the green circle HBV+ PWID, the red circle HIV+ PWID, and the overlapping areas co-infected subjects. HCV serostatus was based on HCV Ab+ and HBV serostatus on anti-HBc or HBsAg+.

The 135 study subjects were divided into three groups: (a) 41 HIV positive PWID; (b) 47 HIV exposed seronegative PWID (ESN); (c) 47 controls matched to the ESN by gender and age.

5.2.2. T cell distribution in relation to HIV susceptibility

One subject's flow cytometry data analyses is presented on Figure 13. The used flow cytometry panel gives a good overview of the whole CD4+ and CD8+ T cell distribution among the study subjects, because of the use of eight colors simultaneously enabling the allocation of the cells into many subtypes. In addition, the CCR5 APC is the only fluorochrome in the red spectral area enabling exact measurements of HIV co-receptor CCR5 signal.

The main results of the T cell analyses in relation to HIV serostatus are presented in Table 13. HIV positive individuals showed typical changes in their cell percentages. As expected, HIV positive PWID had lower percentages of CD4+ T cells and more immune activation (higher percentages of HLA-DR+ cells) compared to ESN. In addition, HIV-positive PWID showed increased percentages of both CD4+CD45RA+RO+ and CD8+CD45RA+RO+ double-positive cells compared to ESN.

When comparing the two HIV negative groups (ESN and controls), three main differences were observed. First, ESN had higher percentages of CD4+ and CD8+ CD45RA+CD45RO+ cells compared to controls (0.8% to 0.5% and 0.8% to 0.3%, respectively; p=0.016 in both cases). Similar trends have been described by Suy et al. (2007) among sexually exposed individuals. Thus, an increase in double positive cells occurs among sexually and parenterally exposed individuals, and being similar in two exposure routes is rather caused by HIV-exposure and not intravenous drug use. The role of these double positive cells during infections, including HIV, is unknown, however, their number increases during various infections, including HIV disease progression (de Roda Husman et al., 1999; Palacios-Martínez et al., 2012; Rentenaar et al., 2000). Therefore, these cells might just reflect an overall T cell response during HIV exposure and or infection, or possess a specific function in susceptibility to HIV and its progression not yet fully understood.

The second main difference between ESN and controls was the higher percentage of immune activated cells among ESN (Table 13). ESN had higher percentages of HLA-DR+ cells of all the studied CD4+ and CD8+ T cell subtypes (CD45RA+, CD45RA+RO+, and CD45RO+) compared to controls. These results are similar to those of previous studies among sexually exposed individuals, where higher immune activation, likely caused by extensive viral exposure, has been demonstrated (Biasin et al., 2000; Lo Caputo et al., 2003; Restrepo et al., 2010; Suy et al., 2007). Only a few studies have investigated the effects of HIV exposure among PWID ESN (Makedonas et al., 2002; Tran et al., 2006).

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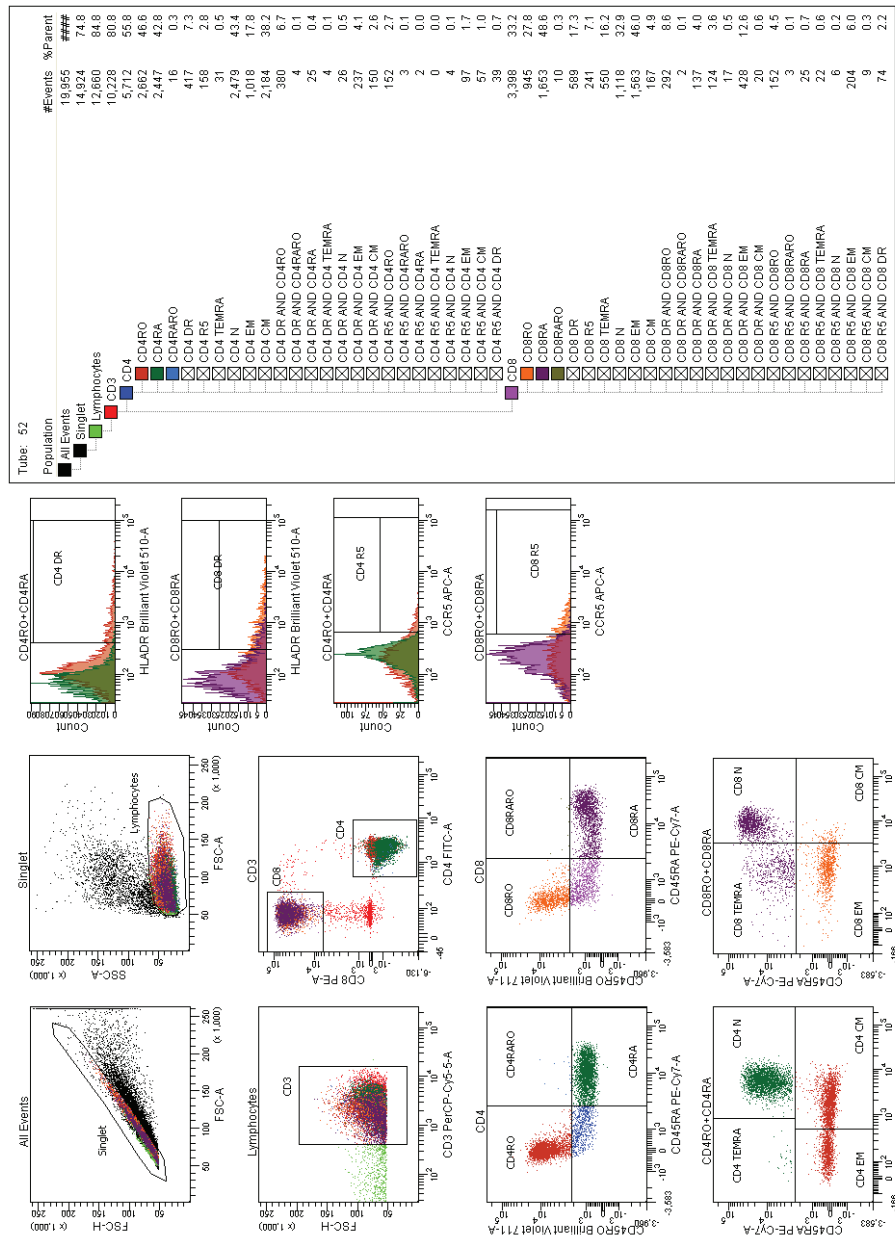
Figure 13. Example of the flow cytometry gating strategy. The initial analysis used forward scatter (FSC-A versus FSC-H) to identify single cells, thereafter forward scatter and side scatter (FSC-A and SSC-A) was used to identify lymphocytes. T lymphocytes were analyzed using sequential gating of CD3, then CD4 or CD8, and CD45RA and CD45RO or CD45RA and CCR7. The cells were thereafter categorized as HLA-DR positive/ negative and CCR5 positive/negative.

Tube: 52

Population	#Events	%Parent
All Events	19,955	####
Singlet	14,924	74.8
Lymphocytes	12,660	84.8
CD3	10,228	80.8
CD4	5,712	55.8
CD4RO	2,662	46.6
CD4RA	2,447	42.8
CD4RARO	16	0.3
CD4 DR	417	7.3
CD4 R5	158	2.8
CD4 TEMRA	31	0.5
CD4 N	2,479	43.4
CD4 EM	1,018	17.8
CD4 CM	2,184	38.2
CD4 DR AND CD4RO	380	6.7
CD4 DR AND CD4RARO	4	0.1
CD4 DR AND CD4RA	25	0.4
CD4 DR AND CD4 TEMRA	4	0.1
CD4 DR AND CD4 N	26	0.5
CD4 DR AND CD4 EM	237	4.1
CD4 DR AND CD4 CM	150	2.6
CD4 R5 AND CD4RO	152	2.7
CD4 R5 AND CD4RARO	2	0.1
CD4 R5 AND CD4RA	2	0.0
CD4 R5 AND CD4 TEMRA	0	0.0
CD4 R5 AND CD4 N	4	0.1
CD4 R5 AND CD4 EM	97	1.7
CD4 R5 AND CD4 CM	57	1.0
CD4 R5 AND CD4 DR	39	0.7
CD8	3,398	33.2
CD8RO	945	27.8
CD8RA	1,653	48.6
CD8RARO	10	0.3
CD8 R5	589	17.3
CD8 TEMRA	241	7.1
CD8 N	550	16.2
CD8 CM	1,118	32.9
CD8 EM	1,563	46.0
CD8 DR AND CD8RO	167	4.9
CD8 DR AND CD8RARO	292	8.6
CD8 DR AND CD8RA	2	0.1
CD8 DR AND CD8 TEMRA	137	4.0
CD8 DR AND CD8 N	124	3.6
CD8 DR AND CD8 EM	17	0.5
CD8 DR AND CD8 CM	428	12.6
CD8 R5 AND CD8RO	20	0.6
CD8 R5 AND CD8RARO	152	4.5
CD8 R5 AND CD8RA	3	0.1
CD8 R5 AND CD8 TEMRA	25	0.7
CD8 R5 AND CD8 N	22	0.6
CD8 R5 AND CD8 CM	6	0.2
CD8 R5 AND CD8 EM	204	6.0
CD8 R5 AND CD8 DR	74	2.2

Table 13. Percentages and interquartile ranges of the lymphocyte subsets from CD4+ or CD8+ parent cell populations among the study groups. Statistically significant comparisons (p<0.05) are marked in bold

Subset	HIV+ PWID (n = 41)	ESN (n = 47)	Controls (n = 47)	ESN to HIV+ PWID	ESN to Controls
CD4$^+$	20.9 (12.5–29.9)	55.8 (49.9–61.1)	55.9 (49.5–63.3)	**<0.001**	0.787
CD45RA$^+$	41.2 (30.1–53.8)	37.3 (29.7–45.8)	30.6 (24.1–37.5)	0.270	**0.010**
CD45RA$^+$HLA-DR$^+$	1.9 (1.0–2.9)	1.2 (0.8–1.5)	1.3 (0.8–1.7)	**0.040**	0.537
CD45RA$^+$RO$^+$	1.2 (0.6–2.8)	0.8 (0.4–1.3)	0.5 (0.2–0.7)	**0.016**	**0.016**
CCD45RA$^+$RO$^+$HLA-DR$^+$	12.5 (5.6–24.1)	12.9 (7.7–19.1)	25.4 (17.6–40.0)	0.679	**<0.001**
CD45RA$^+$RO$^+$CCR5$^+$	5.7 (0.0–13.5)	10.3 (6.7–17.6)	13.3 (8.1–19.2)	**0.028**	0.278
CD45RO$^+$HLA-DR$^+$	19.8 (12.9–31.5)	8.6 (6.6–10.7)	7.4 (5.9–8.6)	**<0.001**	**0.026**
HLA-DR$^+$	10.6 (6.6–18.7)	5.4 (4.1–6.7)	4.6 (3.8–5.4)	**<0.001**	0.101
CD8$^+$	70.6 (61.5–80.9)	33.9 (27.7–37.7)	32.3 (28.2–41.2)	**<0.001**	0.982
CD45RA$^+$	37.1 (29.4–49.1)	60.2 (49.0–65.0)	53.2 (41.4–65.4)	**<0.001**	0.213
CD45RA$^+$HLA-DR$^+$	29.1 (20.6–39.3)	10.6 (6.9–16.6)	6.4 (4.6–9.7)	**<0.001**	**<0.001**
CD45RA$^+$RO$^+$	1.5 (0.9–2.5)	0.8 (0.3–1.5)	0.3 (0.2–0.7)	**0.016**	**0.006**
CD45RA$^+$RO$^+$HLA-DR$^+$	44.8 (33.9–53.6)	26.2 (19.1–40.8)	30.0 (16.7–50.0)	**<0.001**	0.443
CD45RA$^+$RO$^+$CCR5$^+$	14.6 (9.9–20.7)	20.0 (10.8–28.1)	30.0 (16.7–42.5)	0.084	**0.006**
CD45RO$^+$	44.8 (36.7–58.9)	24.2 (21.8–36.3)	23.5 (17.4–38.4)	**<0.001**	0.346
CD45RO$^+$HLA-DR$^+$	56.4 (52.4–68.3)	30.4 (19.6–38.4)	20.0 (14.9–29.5)	**<0.001**	**<0.001**
HLA-DR$^+$	45.8 (36.8–53.7)	16.7 (11.9–24.8)	10.5 (8.2–15.9)	**<0.001**	**<0.001**
CCR5$^+$	5.4 (1.8–10.0)	1.9 (1.0–3.8)	1.3 (0.8–1.8)	**<0.001**	**0.011**

Note: HIV+ PWID = treatment naïve HIV positive persons who inject drugs; ESN = HIV exposed seronegative persons who inject drugs; controls = unexposed seronegative subjects. Only rows with p-values <0.05 are presented in the table.

These studies also found higher immune activation among ESN compared to controls. Overall, with regard to the current and previous studies (Biasin et al., 2000; Jennes et al., 2003; Restrepo et al., 2010; Suy et al., 2007), one can conclude that immune activation in exposed individuals occurs and that the mechanism of immune activation among ESN is independent of the route of infection. The effect of immune activation on susceptibility to HIV in not fully understood. Although HIV exposure increases the expression of CCR5 and therefore the number of cells susceptible to HIV, an effective immune response together with immune activation is necessary for prevention of HIV infection (Makedonas et al., 2002; Ostrowski et al., 1998; Restrepo et al., 2010).

5.2.3. CCR5 expression in relation to susceptibility to HIV

The third major aspect analyzed during the T cell study, was the number of CCR5+ cells and expression of CCR5. Prior to CCR5 flow cytometry analysis, CCR5 32 base-pair deletion (Δ32) was examined to assess its effects on CCR5 expression. The proportion of Δ32 heterozygotes was 17% in ESN, 27% in HIV positive PWID, and 15% in controls. The percentages were not significantly different between the three groups (ESN to HIV+ PWID $p=0.331$; HIV+PWID to controls $p=0.193$, and ESN to controls $p=0.785$). The percentages of Δ32 heterozygotes were in similar ranges to those of previous studies in Estonia (Adojaan et al., 2007). In addition, one ESN was homozygous of the mutation and had no CCR5 expression of T cells, which verified the flow cytometry data. Excluding individuals with at least one Δ32 mutation (Δ32/wt and Δ32/ Δ32) did not change the results of comparisons between ESN and controls of T cell subset analyses. As seen in Figure 14, the CCR5 wild type homozygotes had higher percentages of CCR5+ cells and higher CCR5 MFI values compared to carriers of one Δ32 allele (wt/Δ32 heterozygote), indicating lower CCR5 expression among CCR5 heterozygote individuals.
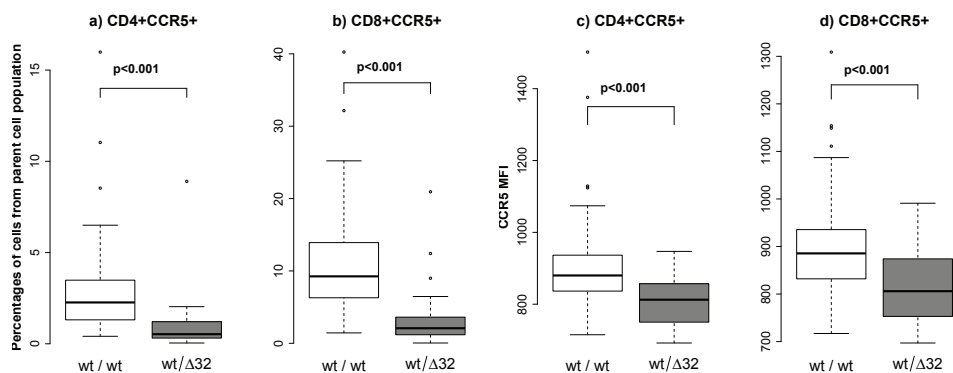

Figure 14. The ratio (median %) of CCR5+ lymphocyte subsets from the parent cell population (a and b), CCR5 median fluorescent intensities (MFIs; c and d), and interquartile ranges of all study subjects. White represents wt/wt homozygotes and grey wt/Δ32 heterozygotes.

HIV positive PWID had higher percentages of CD4+CD45RA+RO+CCR5+ cells than ESN and controls (5.7% to 10.3% and 5.7 to 13.3%; p=0.028 and p=0.015, respectively) and higher percentages of CD8+CD45RA+RO+CCR5+ cells than controls (20.0% to 30.0%; p=0.006) (Table 13). In terms of CCR5 expression level (MFI), ESN had lower CCR5 surface density of CD4+CCR5+ and CD8+CCR5+ cells (Figure 15) than HIV positive PWID. CCR5 expression did not differ between the HIV positive and HIV negative PWID groups, regardless of their HIV serostatus.

These results were somewhat surprising, showing that CCR5 expression on CD4+ T cells was higher among controls than HIV negative and HIV positive PWID. These results contrast with previous studies, which have showed increased percentages of CCR5 expression during exposure to HIV (half of the PWID population was ESN) and in vitro opioid exposure (Li et al., 2002; Steele et al., 2003; Suy et al., 2007). Multiple opioids, such as methadone and morphine, increase the expression of CCR5 via the μ-opioid receptor (Li et al., 2002; Steele et al., 2003). Similarly to the current study, Tran et al (2006) showed decreased percentages of CD8+CCR5+ cells among Vietnamese PWID. The higher and lower CCR5 expression reported in the literature could be explained by genetic polymorphisms other than Δ32 deletion in the CCR5 gene and promoter region, epigenetic modifications, or by multiple other factors such as co-infections (de Roda Husman et al., 1999; Gornalusse et al., 2015). However, the CCR5 expression similarities between PWID groups suggests that CCR5 expression may be related to IVDU, regardless of HIV serostatus.
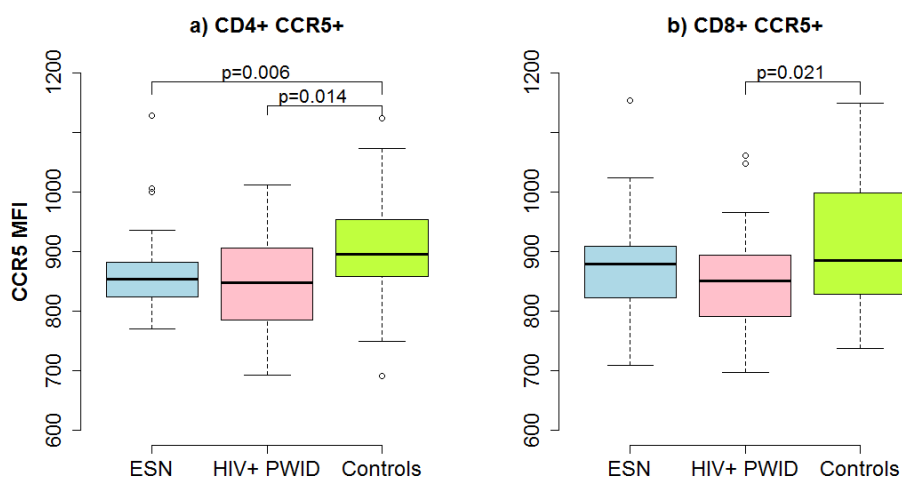

Figure 15. CCR5 median fluorescent intensities (MFIs) and interquartile ranges of CCR5+ cell numbers of the study groups. Blue indicates HIV exposed seronegative subjects (ESN), pink indicates HIV positive persons who inject drugs (PWID), and green indicates controls (unexposed seronegative subjects). Panel (a) indicates total CD4+ CCR5+ and (b) total CD8+ CCR5+ cells.

5.2.4. T cell distribution in relation to HIV, HCV, and HBV seropositivity

Multiple studies have analyzed the effects of HIV, HBV, and HCV mono-infections on T cell distribution, however, data regarding co-infections, especially among PWID, is limited. To analyze the effect of HIV, HCV, and HBV co-infections, flow cytometry study subjects were divided into five groups:

Group (a) HIV+HCV+HBV+ PWID (n = 35);
Group (b) HBV+HCV+ dual positive PWID (n = 18);
Group (c) HIV–HBV–HCV+ PWID (n = 17);
Group (d) Triple negative PWID (n = 11);
Group (e) Controls (n = 47).

Multiple T cell subsets differed between HIV+HBV+HCV+ PWID and HIV negative PWID (HIV–HBV+HCV+, HIV–HBV–HCV+, and triple negative). In addition to lower percentages of CD4+ T cells and higher percentages of CD8+ T cells from the parent cell population, HIV+ individuals had lower percentages of CD4+ T_{CM} and higher percentages of CD4+ T_{TEMRA} cells than HIV negative but HBV+HCV+ PWID (Figure 16b, c). In addition, HIV+HBV+HCV+ PWID had lower percentages of CD8+ T_N and CD8+ T_{CM}, and higher percentages of CD8+T_{EM} cells, than other PWID groups (Figure 16). HIV+HBV+HCV+ had higher percentages of HLA-DR+ cells compared to HIV–HBV+HCV+ PWID and HIV–HBV–HCV+ PWID (Figure 17). In terms of CCR5, triple infected PWID had higher levels of CD8+ T_N CCR5+ and CD8+ T_{CM} CCR5+ cells than PWID with HBV/HCV dual- or HCV mono-infection (Figure 18a–c). Overall, most of the observed differences were between HIV positive and HIV negative PWID groups.

Although this study did not analyze HIV mono-infected subjects, but co-infected subjects, the results are similar to those found among HIV mono-infected individuals (Killian et al., 2004; Suy et al., 2007; Yang et al., 2002). The changes among HIV infected individuals included a decrease in percentages of CD4+ T cells, an increase in percentages of memory cell subpopulations, and an increase in immune activation. These results indicate that T cells distribution changes among HIV+HBV+HCV+ (triple infected) PWID are driven by HIV infection, whereas seropositivity of hepatitis infections has minimal or no additional effects on T cell distribution.

Analyzing only HIV negative subjects, we found T cell distribution did not differ between HBV+ HCV+ dual-infected to HCV mono-infected patients and triple negative PWID to HCV mono-infected PWID (Table 14). HBV+ HCV+ co-infected PWID had lower percentages of bulk CD4+ T cells than triple negative PWID (52.1% and 58.6%, respectively; p=0.021; Figure 16a). Previous studies have shown that both HIV exposure and hepatitis infections may lead to CD4+ cell decline (Asabe et al., 2009; Grakoui et al., 2003; Yang et al., 2002).

Figure 16. The percentages (median %) of T cell subsets from the parent cell population among HIV+HBV+HCV+ persons who inject drugs (PWID)(●), HIV−HBV+HCV+ PWID (●), HIV−HBV− HCV+ PWID (●), and triple negative PWID (○). Panel (a) indicates CD4+, (b) CD4+ T_{CM}, (c) CD4+ T_{TEMRA}, (d) CD8+, (e) CD8+ T_N, (f) CD8+ T_{CM}, and (g) CD8+ T_{EM} cells. Holm-Bonferroni corrected p-values <0.05 were considered statistically significant and are presented on the figure.

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Figure 17. The percentages (median %) of HLA-DR+ T cell subsets from the parent cell population among HIV+HBV+HCV+ persons who inject drugs (PWID) (●), HIV–HBV+HCV+ PWID (●), HIV–HBV–HCV+ PWID (●), and triple negative PWID (○). Holm-Bonferroni corrected p-values <0.05 were considered statistically significantand are presented on the figure.
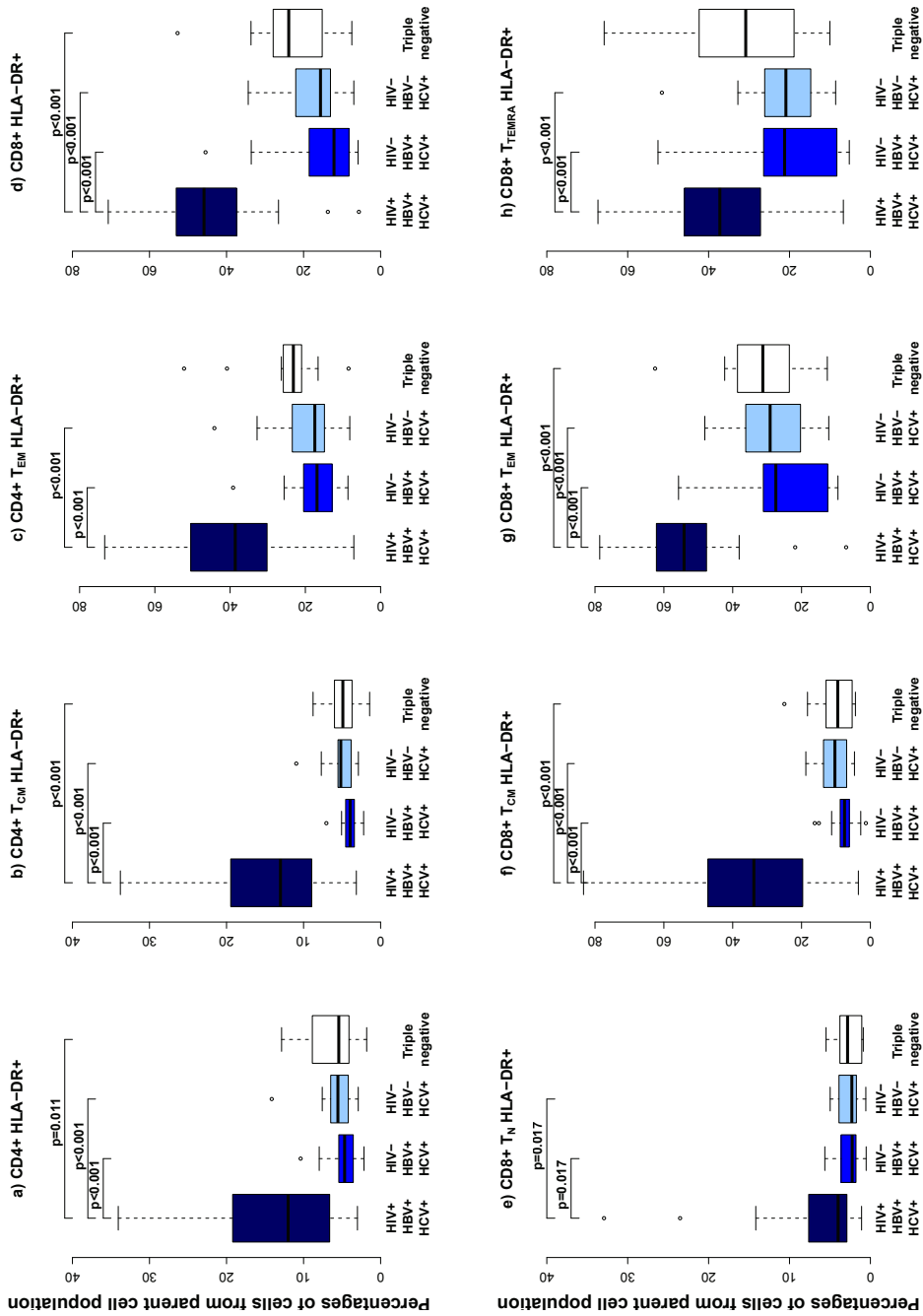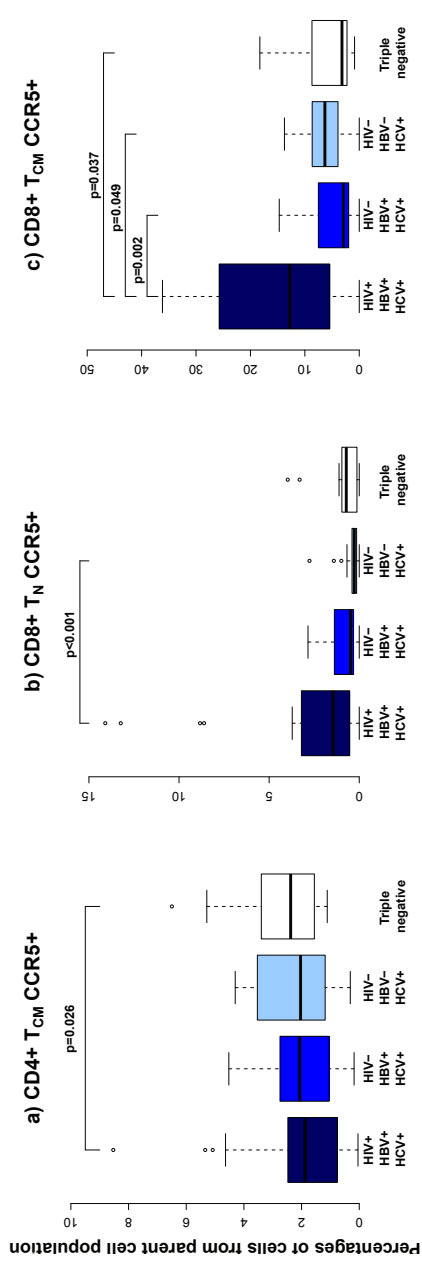
Figure 18. The percentages (median %) of CCR5+ T cell subsets from the parent cell population among HIV+HBV+HCV+ persons who inject drugs (PWID) (●), HIV–HBV+HCV+ PWID (●), HIV–HBV– HCV+ PWID (●), and triple negative PWID (○). Holm-Bonferroni corrected p-values <0.05 were considered statistically significant and are presented on the figure.

Table 14. The percentages (median %) of lymphocyte subsets of parent cell populations among persons who inject drugs (PWID)

Cell populations	HIV+HBV+HCV+ (n = 35)	HIV−HBV+HCV+ (n = 18)	HIV−HBV−HCV+ (n = 17)	Triple negative (n = 11)	P^a	P^b	P^c	P^d
CD4+	19.5 (12.7–25.7)	52.1 (47.3–56.6)	55.8 (49.1–61.8)	58.6 (56.6–64.9)	**<0.001**	**<0.001**	**<0.001**	**0.021**
T_N	44.0 (29.9–58.6)	31.4 (28.3–41.3)	41.3 (33.5–46.9)	36.4 (33.5–52.9)	0.342	1.0	1.0	1.0
T_N HLA-DR$^+$	1.3 (0.9–2.2)	1.2 (0.9–1.5)	1.2 (0.8–1.7)	1.5 (1.0–1.9)	1.0	1.0	1.0	1.0
T_N CCR5$^+$	0.4 (0.1–0.9)	0.5 (0.2–0.8)	0.2 (0.1–0.3)	0.3 (0.1–1.1)	1.0	1.0	1.0	1.0
T_{CM}	36.1 (28.6–46.7)	50.9 (46.3–56.5)	41.3 (39.6–51.0)	44.0 (37.0–53.9)	**0.001**	0.441	0.441	0.552
T_{CM} HLA-DR$^+$	13.0 (8.9–19.5)	4.0 (3.5–4.7)	5.2 (3.8–5.5)	4.9 (3.7–6.0)	**<0.001**	**<0.001**	**<0.001**	0.441
T_{CM} CCR5$^+$	1.3 (0.6–2.9)	1.6 (0.8–2.0)	1.8 (1.3–3.0)	2.3 (1.3–2.6)	1.0	0.917	0.923	0.917
T_{EM}	14.1 (9.3–21.6)	12.5 (10.2–15.1)	13.5 (9.6–16.2)	10.8 (8.9–15.4)	1.0	1.0	0.953	1.0
T_{EM} HLA-DR$^+$	38.6 (30.2–50.5)	18.0 (13.2–20.6)	17.5 (14.9–23.5)	23.2 (21.0–25.9)	**<0.001**	**<0.001**	0.050	0.050
T_{EM} CCR5$^+$	7.0 (2.3–9.3)	7.6 (4.1–10.0)	9.6 (6.0–12.0)	10.9 (8.2–12.6)	0.987	0.239	**0.026**	0.369
T_{TEMRA}	0.6 (0.3–1.1)	0.2 (0.1–0.3)	0.3 (0.1–1.2)	0.5 (0.1–2.2)	**0.002**	0.970	1.0	0.815
T_{TEMRA} HLA-DR$^+$	29.0 (6.1–43.8)	18.6 (13.0–26.3)	11.8 (2.5–18.8)	22.2 (16.9–37.5)	0.967	0.260	0.967	0.967
T_{TEMRA} CCR5$^+$	0.0 (0.0–3.4)	0.0 (0.0–0.6)	0.0 (0.0–1.8)	1.5 (0.0–8.7)	1.0	1.0	0.949	0.524
HLA-DR$^+$	12.0 (6.6–19.2)	4.9 (3.6–5.6)	5.5 (4.2–6.5)	5.4 (4.1–8.7)	**<0.001**	**<0.001**	**0.017**	0.818
CCR5+	1.9 (0.8–2.5)	1.9 (1.0–2.7)	2.0 (1.2–3.5)	2.4 (1.6–3.4)	1.0	1.0	1.0	1.0

Cell populations	HIV+HBV+HCV+ (n = 35)	HIV−HBV+HCV+ (n = 18)	HIV−HBV−HCV+ (n = 17)	Triple negative (n = 11)	P[a]	P[b]	P[c]	P[d]
CD8^{+}	71.9 (61.8–80.1)	36.8 (30.9–44.0)	32.6 (28.5–36.4)	31.5 (25.6–34.1)	**<0.001**	**<0.001**	**<0.001**	0.165
T_N	6.4 (3.2–11.6)	28.6 (14.5–37.0)	27.3 (20.9–39.2)	25.8 (16.8–39.2)	**<0.001**	**<0.001**	**<0.001**	1.0
T_N HLA-DR[+]	4.0 (2.9–7.6)	2.3 (1.8–3.7)	2.3 (1.7–3.9)	2.8 (1.1–3.8)	**0.017**	**0.017**	0.076	1.0
T_N CCR5^{+}	1.5 (0.5–3.2)	0.5 (0.3–1.4)	0.3 (0.1–0.4)	0.7 (0.1–1.0)	0.055	**<0.001**	0.162	0.875
T_{CM}	1.4 (0.8–2.1)	4.6 (3.1–5.6)	3.2 (2.6–5.7)	3.9 (3.1–5.4)	**<0.001**	**<0.001**	**<0.001**	1.000
T_{CM} HLA-DR[+]	33.9 (19.7–47.2)	7.6 (6.4–8.9)	10.3 (6.9–13.6)	9.5 (5.3–12.9)	**<0.001**	**<0.001**	**<0.001**	0.690
T_{CM} CCR5^{+}	12.8 (5.4–25.8)	2.8 (1.9–7.2)	6.3 (3.9–8.7)	3.2 (2.3–8.7)	**0.002**	**0.049**	**0.037**	0.669
T_{EM}	58.0 (48.4–63.3)	35.2 (29.4–45.8)	33.0 (28.4–42.3)	31.6 (27.4–40.3)	**<0.001**	**<0.001**	**<0.001**	1.000
T_{EM} HLA-DR[+]	54.1 (47.6–62.2)	23.0 (12.6–31.1)	29.1 (20.3–36.2)	31.3 (23.5–38.6)	**<0.001**	**<0.001**	**<0.001**	0.335
T_{EM} CCR5^{+}	14.6 (8.9–21.3)	11.6 (5.7–17.4)	19.2 (13.5–20.5)	16.4 (14.5–24.0)	0.880	0.880	0.880	0.341
T_{TEMRA}	31.8 (27.8–39.1)	29.3 (26.3–33.3)	22.7 (21.0–45.5)	29.2 (24.3–35.9)	1.0	1.0	1.0	1.0
T_{TEMRA} HLA-DR[+]	37.2 (27.2–46.0)	21.2 (9.2–26.1)	20.9 (14.7–26.1)	30.8 (18.9–42.3)	**<0.001**	**<0.001**	0.718	0.245
T_{TEMRA} CCR5^{+}	3.5 (1.8–5.6)	1.8 (1.3–4.9)	4.3 (2.0–8.0)	4.4 (3.4–9.5)	0.880	1.0	0.880	0.236
HLA-DR[+]	45.8 (37.4–53.1)	13.6 (8.5–18.5)	15.6 (13.0–22.0)	23.9 (15.2–27.9)	**<0.001**	**<0.001**	**<0.001**	0.277
CCR5[+]	9.9 (5.4–14.7)	4.5 (2.9–8.6)	8.3 (5.4–10.4)	8.7 (6.4–13.2)	0.094	1.0	1.0	0.287

P[a] = HIV+ HBV+ HCV+ compared to HIV− HBV+ HCV+; P[b] = HIV+ HBV+ HCV+ compared to HIV− HBV− HCV+; P[c] = HIV+ HBV+ HCV+ compared to triple negative; P[d] = HIV− HBV− HCV+ compared to triple negative.

However, during hepatitis infections, the majority of changes are localized in the liver and not observable in peripheral blood (Appay et al., 2002; He et al., 1999; Urbani et al., 2002). Thus, we speculate that the mild decrease in total CD4+ T cell populations among HIV negative HBV+/HCV+ PWID may have been caused by either HBV/HCV seropositivity and or exposure to HIV.

Multiple studies have investigated the effect of HIV/HBV or HIV/HCV co-infections on T cell distribution (Gras et al., 2015; Hodowanec et al., 2013; Kottilil et al., 2009; Kovacs et al., 2008; Rallón et al., 2011; Restrepo et al., 2010). The current study is the first to evaluate the effects of all three infections in a PWID population. Current study showed that independent of their co-infection status, PWID who have HIV exhibited the typical changes associated with HIV infection (CD4+ cell decline and increased immune activation). HBV/HCV co-infected PWID also had a mild decrease in CD4+ T cells compared to triple negative PWID.

A similar decrease in CD4+ T cells during HBV and HCV mono-infections has been demonstrated in primate studies and during fibrosis, but has not been studied in PWID populations (Asabe et al., 2009; Grakoui et al., 2003; Rashkin et al., 2010). As one might assume that HBV/HCV co-infected PWID are more frequently exposed to HIV than seronegative subjects, the reason for CD4+ decline might be associated with exposure to HIV or intravenous drug use per se.

It is interesting to note that the CCR5 analysis results differed somewhat between the two flow cytometry studies. In the first study, the percentages of CCR5 positive cells were similar between ESN and controls, but ESN had lower CCR5 MFI on multiple T cell subsets compared to controls. In the second study, triple negative PWID had increased percentages of CCR5+ cells compared to controls. In the first study, differences were seen in CCR MFI levels and in the second analysis in CCR5 percentages. We can only speculate what caused these differences; co-infections (absent in triple negative PWID) or some additional factors among ESN not yet known might have been responsible. Consequently, percentages of CCR5 cells and CCR5 expression levels are interesting topics for further research.

5.2.5. T cell distribution and intravenous drug use

To evaluate how IVDU affects the immune system, we compared triple negative PWID to controls. All the PWID had a history of syringe/needle sharing and there-fore potential exposure to infections. The results can be seen in Paper III Supple-mentary Table 2 and Figure 19. Of the memory cell subsets, two differed bet-ween triple negative PWID and controls. First, triple negative PWID showed lower percentages of CD4+ T_{EM} cells and higher percentages of CD8+ T_{CM} cells compared to controls (Figure 19a and Figure 19g). Additionally, triple negative PWID had higher percentages of immune activated cells and CCR5+ cells compared to controls (Figure 19b–f, h–k).
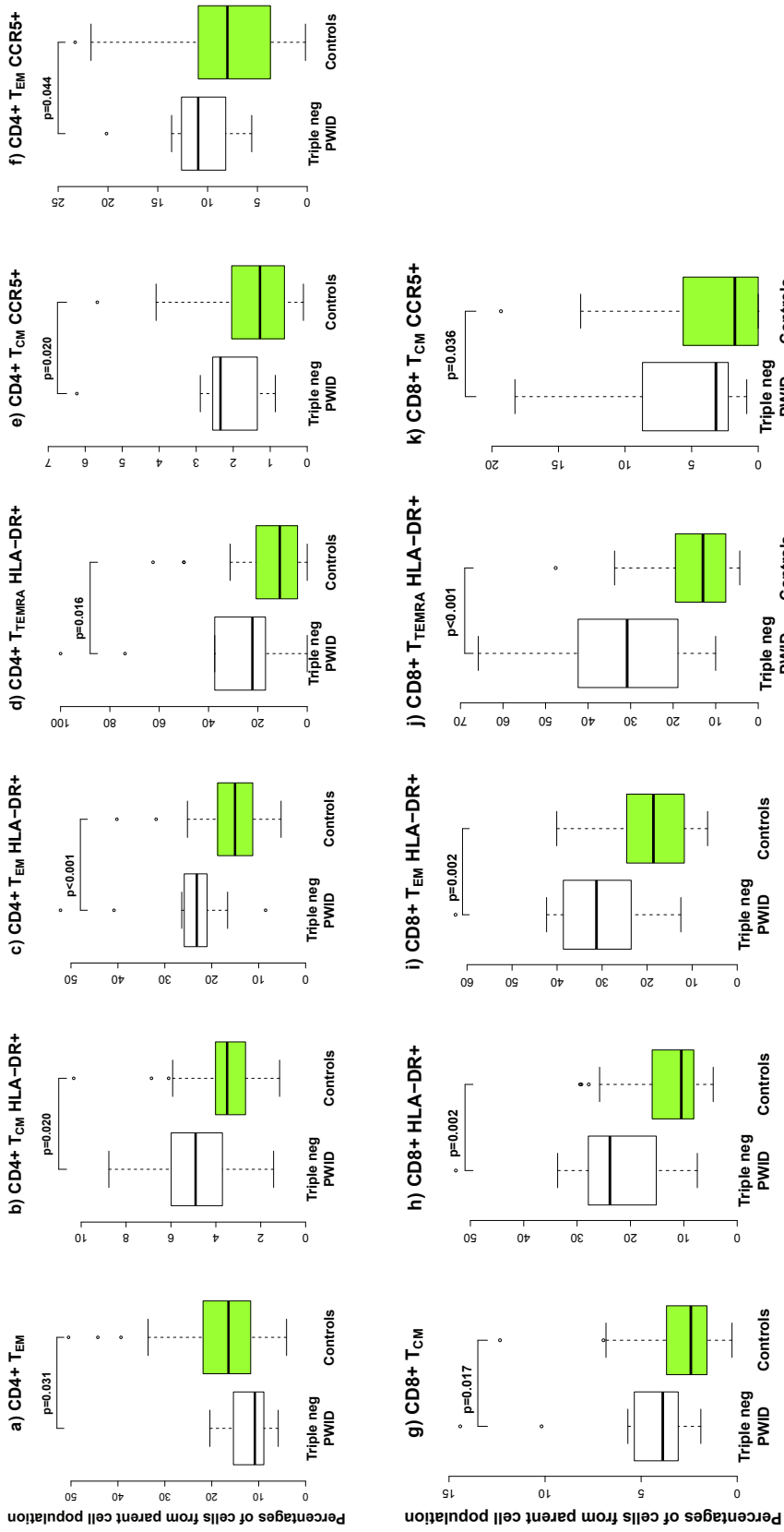
Figure 19. The percentages (median %) of lymphocyte subsets from the parent cell population among HIV, HCV, and HBV seronegative persons who inject drugs (triple negative PWID) (○) and controls (●). Panel (a) indicates T_{EM}, (b) T_{CM} HLA–DR+, (c) T_{EM} HLA–DR+, (d) T_{TEMRA} HLA–DR+, (e) T_{CM} CCR5+, (f) T_{EM} CCR5+, (g) T_{CM}, (h) HLA–DR+, (i) T_{EM} HLA–DR+, (j) T_{TEMRA} HLA–DR+, and (k) T_{CM} CCR5+ cells.

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To the best of our knowledge, this is the first study to investigate T cell distribution, immune activation, and the number of CCR5+ cells among triple negative PWID. The results complement earlier *in vitro* and animal studies, which showed that opioids increase immune activation and CCR5 expression (Bayer et al., 1990; Steele et al., 2003; Wang et al., 2007; Wang et al., 2011). In addition, higher percentages of CD8+ T_{CM} cells and lower percentages of CD4+ T_{EM} cells among triple negative PWID compared to controls indicate that the immune system is already compromised by IVDU, despite individuals remaining seronegative of HIV, HBV, and HCV.

6. GENERAL DISCUSSION

HIV, HBV, and HCV share routes of infection and factors that influence an individual's susceptibility to them. Despite numerous studies investigating genetic and immunological aspects of these three infections, studies among PWID have so far been limited. The current study is the first to evaluate associations between (a) IL-10 gene polymorphisms, HIV and HCV infections, and T cell distribution and (b) HIV, HBV, and HCV infections, among Caucasian PWID.

6.1. The importance of conducting studies among PWID

HIV, HBV, and HCV are among the most influential infections globally and the problem among PWID is especially serious, among whom co-infections are common. In most regions of the world, hetero- and homo-sexual routes of infection are the most frequent, and studies among these groups have been abundant. However, in Eastern-Europe, IVDU is one of the leading causes of new HIV infections and PWID represent an important study group (DeHovitz et al., 2014; Kroner et al., 1994; Soodla et al., 2015). Most fascinating are PWID who are exposed to the viruses yet remain seronegative. Knowing the factors distinguishing ESN from the general population makes it possible to reveal new insights regarding the factors that protect some individuals from viruses and thereby develop vaccines or drugs against these infections.

Sexual and parenteral exposure are somewhat different, for example because of the different first cell populations affected during HIV exposure (Levy, 2007). During sexual exposure, Langerhans and dendritic cells are the first to encounter HIV, whereas during parenteral exposure, CD4+ blood cells are the first (Hu et al., 2000; Koppensteiner et al., 2012). As mentioned, sexually exposed individuals have been studied more than PWID, because (1) it is the most frequent exposure route and (2) these individuals are relatively easy to recruit. In contrast, numbers of PWID are relatively smaller and various legal and socio-economic issues make their enrollment into studies – especially into long-term or cohort studies – much more challenging. This thesis is important owing to the analysis of PWID, which has provided new insights into the mechanisms that affect susceptibility to HIV during parenteral exposure.

One important characteristic of PWID populations are the HIV/HCV/HBV triple or HIV/hepatitis dual infection individuals that are rare among sexually exposed populations (Uuskula et al., 2006). Co-infections increase immune activation, cause cell exhaustion, and increase viral susceptibility and afterwards quicken disease progression (Gudo et al., 2009; Markowitz et al., 2016). Therefore, it was possible to investigate the effects of various infections on each other's susceptibility and progression.

The second important characteristic of infections among PWID is the homogenous nature of the virus transmitted, which enables the evaluation of human genetic factors and T cell distribution, without taking into account additional

viral factors (Adojaan et al., 2005; Avi et al., 2014). Among sexually exposed populations, the viruses are much more diverse, making it hard to differentiate between genetic and immunological reactions of the host and viral strain differences.

6.2. Study design

The most important factor of this study was the study population. As mentioned, PWID subjects are hard to recruit and therefore studies among PWID are limited. Especially important were the ESN and HESN study groups, which revealed new factors that protect against HIV. The study was cross-sectional and the T cell distribution and HIV, HBV, and HCV seropositivity in PWID blood samples was evaluated only at one time-point, thus giving an overview of the study subjects' immune status at just one time-point, as past/future infections are not possible to assess. This was important in both the IL-10 polymorphism and T cell analyses, because the study subjects could seroconvert and as the HESN and ESN study groups were small, the seroconversion of a few individuals could significantly have changed the study results.

In the IL-10 study, the frequency of two polymorphisms (–592 and –1082) were detected, giving the opportunity to analyze IL-10 genotype pairs. The detection of two main polymorphisms influencing IL-10 protein production, provided a good overview of how IL-10 effects susceptibility to HIV and HBV. As the number of HCV highly exposed seronegative subjects was very low (only four individuals), larger studies involving highly exposed individuals should be conducted to evaluate the effects of IL-10 polymorphisms on HCV seropositivity. In addition, during this study the stage of hepatitis infections was not assessed, and the role of IL-10 polymorphisms on HBV and HCV disease progression among PWID was not clarified.

One of the main strengths of the study is the 8-colour flow cytometry analyses of samples. It enabled the simultaneous evaluation of multiple immunological markers, without having to color the samples of the same patients multiple times, which might have created bias amongst the analyses. In addition, it was possible to analyze multiple CCR5+ and HLA-DR+ memory cell subpopulations. Studies involving so many markers in the same flow cytometry analyses have not been carried out among similar PWID populations. Such flow cytometry panels are complicated to design due to spectral overlap. Although some study groups have investigated T cell differences among PWID, they have not used as many markers or have pooled the PWID with sexually exposed/infected subjects (Tran et al., 2006; Truong et al., 2003).

In this study, two classifications of memory T cell subsets were used: an older CD45RA/CD45RO expression based division; and a newer CD45RA/CCR7 based classification (Mahnke et al., 2013). The simplified CD45RA/RO classification enabled us to confirm the results of HIV exposure studies conducted among sexually exposed populations and among PWID, to study the percentages of CD45A+RO+ double positive cells (Biasin et al., 2000; Killian et al., 2004; Suy

et al., 2007), and to analyze the CCR5 expression during simplified memory cell division. The analyses of both the number and surface density of CCR5 cells provided a comprehensive overview of this marker's expression, whereas in most studies only the percentage of cells or MFI has been used (Suy et al., 2007; Tran et al., 2006; Yang et al., 2012). In the co-infection study, the use of the newer memory cells classification gave a more comprehensive overview of the topic

6.3. Study limitations

The main limitation was the cross-sectional nature of the sample collection, whereby T cell distribution could be influenced by extreme values at the specific time-point. It was not possible to analyze T cell distribution over a longer time period to evaluate median values, thus changes in immunological markers (such as percentages of CD4+ or CD8+ cell proportions) remained unknown. In addition, the risk-behavior (syringe sharing) of the study subjects was based only on self-reporting. This may have resulted in an underestimation of the study subjects' risk behavior and should not have produced false positive results when analyzing the effect of drug exposure or HIV exposure to T cell distribution.

As mentioned, differentiation between hepatitis disease stages was not possible during this study. Disease stage (acute, chronic, and self-limiting) is especially important in HCV studies, where re-infection is common among PWID (Osburn et al., 2010). Both HBV and HCV cause acute and chronic infections. As during these infections different types of memory cells are increased (T_{CM} or T_{EM}), it makes the stage of hepatitis important to assess (Appay et al., 2002; Carotenuto et al., 2011; Urbani et al., 2005; Urbani et al., 2002). Unfortunately, a lack of clinical data did not allow us to evaluate the course of infection or distinguish between chronic and acute hepatitis infections, or HIV disease stage. Nonetheless, this cross-sectional study enabled the evaluation of the factors that affect the susceptibility to infections of PWID with high cost-effectiveness.

The detection of differences between genetic polymorphisms and the studied infections, may be limited owing to the small number of exposed seronegative subjects. The recruitment of PWID was based on calculations of previous similar cross-sectional studies conducted among PWID in Tallinn; unfortunately is was not possible to recruit more triple negative subjects as they are very rare among this population (Uusküla et al., 2015a). Therefore, the power of IL-10 study was low. This lack of exposed seronegative subjects would mainly cause false negative results, together with an underestimation of the effect on polymorphisms on the studied infections. The study could be used in future meta-analyses combining different studies, and advanced by analyzing similar polymorphisms in larger sample sizes, especially with regard to HCV.

In flow cytometry analyses, the levels of cell surface markers could be influenced by the freezing of PBMC samples, for example CCR5 expression decreases during storage (Shalekoff and Tiemessen, 2001). Analysis of whole blood was not available, therefore the absolute numbers of T cells and T cell

subsets were not measured. The data are presented in the current thesis as subpopulation percentages of the parent population. The multicolor flow cytometry panel might have caused false positive results due to spectral overlap, however, FMO controls and a strict gating strategy were used to reduce this problem. Regardless of these limitations, the results are thought to accurately describe the studied parameters among PWID.

6.4. The influence of IL-10 polymorphisms on susceptibility to HIV, HBV, and HCV infections

One of the main genes of interest in HIV studies is the virus's co-receptor CCR5 Δ32 mutation, which is protective against HIV (Samson et al., 1996). A lot of effort is being put into finding new genes that could have a similar protective effect. One of the possibilities for finding candidate gene variants are genome wide association studies (GWAS). These have revealed multiple genetic variants that might be protective against HIV (Joubert et al., 2010; Limou et al., 2012; Lingappa et al., 2011). However, GWAS studies overlook genes with smaller effects on susceptibility to HIV, but these can be found during monogenetic studies. During these monogenetic studies, multiple immune response gene variants, such as IL-10 single nucleotide polymorphisms, have been associated with protection against HIV. IL-10 is responsible for the balance between immunosuppression and inflammation, making it an interesting study subject during persistent viral infections. As 50–70% of IL-10 production levels are genetically determined, single nucleotide polymorphisms can affect susceptibility to viral infections via the regulation of interleukin production (Reuss et al., 2002; Westendorp et al., 1997). Part of this regulation is caused by the transcription factor Sp1 binding to only IL-10 –1082G alleles, increasing IL-10 production. In addition, IL-10 increases CCR5 expression on T cells during HIV infection and inhibits virus specific T cell responses (Alfano and Poli, 2005; Brockman et al., 2009; de Waal Malefyt et al., 1991; Juffermans et al., 2000; Larsson et al., 2010; Sozzani et al., 1998; Wang et al., 2002). IL-10 levels increase during persistent viral infections and high IL-10 producing genotypes might increase susceptibility to other infections seen among PWID (Barcellini et al., 1995; Ejrnaes et al., 2006; Talaat et al., 2014). The level of IL-10 is also dependent on other cytokines and immune activation, which links IL-10 genetic polymorphisms to T cell distributions and immune activation among HIV positive and HIV exposed individuals (Groux et al., 1998; Santin et al., 2000).

In addition to HIV infection, IL-10 levels increase during multiple other persistent viral infections, such as chronic HBV and HCV infection (Cheong et al., 2006; Ejrnaes et al., 2006; Paladino et al., 2006; Wilson and Brooks, 2011). Although the results are still controversial, depending on the study groups and hepatitis stage analyzed (self-limiting, chronic, and occult), more data confirms the beneficial effect of low IL-10 protein production in fighting HBV and HCV infections (Cheong et al., 2006; Edwards-Smith et al., 1999; Ramezani et al., 2012;

Shin et al., 2003). For example, a meta-analyses of multiple ethnical groups showed that –592A is associated with spontaneous HCV recovery and –1082G with increased risk of chronic HCV infection (Sun et al., 2013). However, these results were highly dependent on the ethnicity of the specific study group. As the influence of IL-10 polymorphisms in HBV and HCV progression clears, the effects of these polymorphisms to hepatitis susceptibility remain poorly studied. The current study showed a beneficial effect of –592A to remaining HBV sero-negative, but no differences were observed in relation to HCV infections. Similar studies could be conducted among populations of other ethnicities and risk groups, to further evaluate the effect of IL-10 polymorphisms on hepatitis susceptibility.

6.5. T cell distribution and HIV, HBV, and HCV infections

Similar to genetic studies, data regarding T cell responses among PWID subjects is limited, especially among ESN. Only a few studies have focused on T cell subsets, CCR5 expression, and immune activation among PWID (Markowitz et al., 2016; Tran et al., 2006). The study showed that PWID exhibit increased immune activation, increased numbers of CD45RA+RO+ cells, and lower densities of CCR5 on multiple T cell populations, compared to controls. Although PWID are – additionally to HIV – exposed to other infections and illicit drugs, most of the influences on T cell distribution are similar between sexual and parenteral HIV exposure (Biasin et al., 2000; Killian et al., 2004; Lo Caputo et al., 2003; Messele et al., 2001; Restrepo et al., 2010; Suy et al., 2007; Yang et al., 2002). The one exception to these similarities, was the number of CCR5+ cells and or CCR5 expression of T cells. The current study showed that both ESN and HIV positive individuals had lower CCR5 expression on T cells than controls. In contrast, numbers of CCR5+ cells increase during sexual HIV exposure (Suy et al., 2007). However, Tran et. al (2006) found decreased numbers of CCR5+ cells among Vietnamese PWID, which suggests that in addition to HIV exposure, CCR5 expression is affected by drug use.

There has been much debate about the differences in T cell percentages and immune activation seen among ESN, and two possible hypotheses have been proposed. First, that the alterations in T cell distribution are the result of HIV exposure. Second, these alterations – compared to blood donors – represent an individual's normal state of protection against HIV infection (Suy et al., 2007). As shown by Suy et al (2007), the increases in immune activation and memory cell subsets depends on the HIV positive partner's viral load, i.e. seems to be a result of viral exposure, which also supports the first hypothesis. These are inde-pendent of the route of exposure and correlate with the level of exposure (Suy et al., 2007). CCR5 expression might follow a different trend, as low CCR5 expression of T cells may give an advantage in remaining HIV negative, and during HIV infection slow the progression of the disease (Reynes et al., 2001).

Multiple other studies have shown T cell subset changes during HBV and HCV infections, however, these studies have focused on acute or chronic infections, not

seropositivity (Appay et al., 2002; Appay and Rowland-Jones, 2004; He et al., 1999; Urbani et al., 2005). The current study found only one difference in CD4+ percentages and analyzing the stages of hepatitis infections in larger groups might reveal additional differences in memory cell subset distributions or immune activation. The effect of this small CD4+ decline is not yet known, but it might influence an individual's overall immune response. In addition to T cell distribution, multiple other factors apparent in co-infected individuals must be taken into account. These factors include cytokine profile and virus-specific T cell responses.

6.6. T cell distribution and intravenous drug use

In addition to HIV, HBV, and HCV infections, the effect of IVDU on T cell distribution was evaluated. The current study showed a decrease in CD4+ T_{EM} cells, and an increase in percentages of CD8+ T_{CM} cells, immune activation, and the number of CCR5+ cells among triple negative PWID compared to controls. There are not many studies that have analyzed the effect of illicit drugs on the immune system from PWID blood samples.

Opioids, such as fentanyl, are commonly used by Estonian PWID (Uusküla et al., 2010). Opioids are usually regarded as immunosuppressive, thus the increase in immune activation and memory T cells found during the current study can be regarded as somewhat contradictory. However, these suppressive mechanisms are very complex, acting via T cell response, opioid receptors, and cytokines (Barcellini et al., 1995; Börner et al., 2009; Mahajan et al., 2005). For example, opioids can inhibit IL-6 production and even IL-2 production in activated T cells (Börner et al., 2009; Roy et al., 2004). Increased numbers of immune activated cells therefore do not reflect the overall immune response or levels of anti-inflammatory cytokines.

Our results showing an increase in CCR5+ cells complement earlier studies of cell culture and animal models that indicated an increase due to opioid exposure (Li et al., 2002; Platt et al., 2016; Steele et al., 2003; Wang et al., 2003; Wang et al., 2011). As CCR5 and immune activation markers are expressed on the same cells, further research is needed to determine if the increase in CCR5+ expression is caused by opioids or by an increase in HLA-DR. However, it is not possible to rule out that these changes were owing to viral exposure (e.g. HIV, HBV, and or HCV) and further studies are needed to clarify CCR5 expression among PWID.

6.7. Future research

The current study investigated relationships between IL-10 polymorphisms and T cell distribution, HIV, HBV, and HCV infections, and IVDU. The results showed that IL-10 genetic polymorphisms may influence susceptibility to HIV and HBV. No effect was not seen among HCV exposed seronegative subjects.

One reason for this could be the small size of the HCV exposed seronegative group. An additional analysis with more HCV exposed individuals could reveal an effect of IL-10 genetic polymorphisms on susceptibility to HCV. It would be interesting to analyze the effect of additional interleukin polymorphisms (such as IL-2, IL-6, and IL-12) on susceptibility to the infections, because as in the case of IL-10, data regarding polymorphisms among PWID populations are almost entirely absent. Among sexually exposed populations, these polymorphisms have an impact on susceptibility to HIV susceptibility and or its progression.

Of T cell subpopulations, data regarding the influence of the stage of HIV, HBV, and HCV infections on T cell distributions remains insufficient. Not much research has been carried out among PWID, whose immune system is influenced by repeated exposure to multiple viruses and opioids. A new prospective study analyzing the effects of acute, self-limiting, and chronic hepatitis types and HIV disease stage could better describe the role of T cell changes and immune activation during the progression of each disease. Much more research is needed to further reveal the effects of opioid use on susceptibility to HIV, HBV, and HCV.

7. CONCLUSIONS

1. The presence of at least one low IL-10 producing allele (–1082A or –592A) might protect highly exposed seronegative subjects (HESN) against HIV infection. Furthermore, the –592AC genotype and –592AC containing genotype pairs seem to protect against both HIV and HBV infections among PWID.

2. HIV negative PWID who share needles and thus have likely been repeatedly exposed to blood-borne infections, including HIV, have altered T cell distributions. More specifically, in comparison to people without this risk behavior, ESN PWID have increased immune activation and lower CCR5 surface density on CCR5+ T cells. This increase in immune activation seems to be a result of viral exposure, however, the differences in CCR5 expression density could be caused by intravenous drug use itself.

3. HIV/HBV/HCV co-infected individuals demonstrate similar T cell changes to HIV mono-infected individuals, such as a decrease in percentages of CD4+ T cells, an increase in memory cell subsets, immune activation, and CCR5+ cell percentages. These changes are likely triggered by HIV infection; neither hepatitis viruses added to the immunosuppression caused by HIV. Among HBV/HCV seropositive individuals, only a limited drop in CD4+ cell percentages was observed, suggesting once again that seropositivity to HBV and or HCV has minimal effects on T cell distribution and also likely to immune disturbances in HIV positive subjects co-infected with hepatitis viruses.

4. Triple negative PWID had lower percentages of CD4+ T_{EM}, higher percentages of CD8+ T_{CM} cells, and higher percentages of activated and CCR5+ cells among both CD4+ and CD8+ T cells compared to controls. This indicates that even while remaining seronegative for all the studied viruses, the immune systems of PWID are disturbed, likely as a consequence of injecting drugs and or exposure to various pathogens.

8. SUMMARY IN ESTONIAN

Immunoloogiliste faktorite mõju HIV-i, B-hepatiidi ja C-hepatiidi viirustesse nakatumisele süstivate narkomaanide hulgas

Inimese immuunpuudulikkuse viirus 1-ga (HIV) on maailmas nakatunud üle 35 miljoni inimese ning aastal 2014 suri HIV tõttu 1,2 miljonit inimest. Lisaks HIV-le põhjustavad terviseprobleeme ka B hepatiidi (HBV) ja C hepatiidi viirused (HCV). Kõigil kolmel viirusel on sarnased nakatumisteed ja selle tõttu on ka nende ko-infektsioonid tavalised. Eriti on ohustatud süstivad narkomaanid (SN).

Eelnevad uuringud on leidnud, et mõned inimesed ei nakatu HIV viirusega hoolimata korduvast kokkupuutest. Selliseid inimesi nimetakse sageli kõrgelt eksponeeritud seronegatiivseteks isikuteks (KESN) või eksponeeritud seronega-tiivseteks isikuteks (ESN). Põhiliselt on uuritud seksuaalsel teel viirusele ekspo-neeritud isikuid ja uuringuid SN-ide seas on olnud vähe. Kuigi mitte nakatuvaid inimesi on uuritud epideemia algusaegadest saati, ei ole siiani teada nende resis-tentsuse põhjuseid. Üheks põhjuseks võib olla HIV ko-retseptori CCR5 puudu-mine rakkude pinnal, mida põhjustab 32-aluspaarine deletsioon CCR5 geenis. Samas kõik inimesed, kes ei nakatu HIV-ga, ei ole vastava mutatsiooni kandjad ja resistentsust võivad põhjustada mutatsioonid mitmetes teistes immuun-süsteemi geenides (näiteks interleukiinide, HLA regiooni või CCR5 ligandide geenides).

Lisaks geneetilistele polümorfismidele on ESN-ide seas uuritud ka mitmete teiste immunoloogiliste faktorite mõju. Näiteks on vaadeldud erinevate T raku populatsioonide muutumist viirusele eksponeeritud indiviididel ning on näi-datud, et neil suurendab kokkupuude viirusega mälurakkude hulka veres. Samuti esineb eksponeeritutel rohkem immuunaktiveeritud rakke. Sarnaselt geneetika uuringutele on vähe teavet süstivate narkomaanide kohta ja eriti mitmetele viirus-tele eksponeerituse või ka HIV, HBV ja HCV ko-seropositiivsuse mõjude kohta T raku populatsioonidele. Lisaks on vähe teavet süstiva narkomaania mõjude kohta T raku jaotusele ja immuunsüsteemile ilma kaasuvate viirusnakkusteta.

Uurimistöö eesmärgid
Töö üldine eesmärk oli hinnata immunoloogiliste faktorite mõju HIV-i nakatu-misele ja uurida, kuidas seropositiivsus kolme viiruse (HIV, HBV ja HCV) suhtes mõjutab T raku jaotust europiidsesse rassi kuuluvatel SN-idel.

Uuringu alameesmärgid
1. Kirjeldada kuidas ja kas IL-10 polümorfismid −1082 ja −592 mõjutavad HIV, HBV ja HCV-ga nakatumist.
2. Kirjeldada seoseid HIV ekspositsiooni, CCR5 ekspressiooni, immuunaktivat-siooni ja T rakkude jaotuse vahel SN-ide populatsioonis.
3. Kirjeldada seoseid HIV, HBV ja HCV ko-infektsioonide, CCR5 ekspressiooni, immuunaktivatsiooni ja T rakkude jaotuse vahel SN-ide populatsioonis.

4. Kirjeldada seoseid süstiva narkomaania, CCR5 ekspressiooni, immuunaktivatsiooni ja T rakkude jaotuse vahel SN-ide populatsioonis.

Uuritavad ja metoodika

Uuring viidi läbi kahes osas.

1. Esimesse uuringusse kaasati 345 SN-i ja 496 tervet vabatahtlikku veredoonorit. Veredoonorite demograafilised andmed ei olnud teada. SN-idest 79% olid mehed ja uuritavate mediaanvanus oli 30 aastat. SN-idest 20 olid viimase kuu aja jooksul kasutanud süstalt, mida oli eelnevalt kasutanud keegi teine. Need 20 uuritavat klassifitseeriti KESN-ideks. SN-id värvati 2011. aastal Tallinna süstlavahetuspunktidest.

2. Teise uuringusse kaasati 88 SN-i (eelmise uuringu 345-st), kellest 41 olid HIV positiivsed SN-id ja 47 HIV negatiivsed (viimase kuue kuu jooksul jaganud süstalt) SN-id. Kontrollgrupina kaasati 47 tervet vabatahtlikku, kes olid soo ja vanuse alusel sarnased ESN-idele.

Kõikidelt uuritavatelt koguti veri EDTA katsutitesse ja täisverest eraldati perifeerse vere mononukleaarsed rakud. IL-10 polümorfismide määramiseks eraldati rakkudest inimese genoomne DNA ja IL-10 polümorfismid määrati Real-Time PCR-iga, kasutades *Allelic Dicrimination Assay*'d.

T rakkude jaotuse määramiseks kasutati läbivoolutsütomeetriat. Perifeerse vere mononukleaarsed rakud värviti fluorokroomidega konjugeeritud antikehadega (8 pinnamarkerit: CD3, CD4, CD8, CD45RA, CD45RO, CCR7, HLA-DR ja CCR5 jaoks) ning seejärel fikseeriti. Fikseeritud rakke analüüsiti LSR Fortessa voolutsütomeetril.

Statistilises analüüsis kasutati geneetiliste polümorfismide analüüsil Fischeri täpset testi ja T rakkude populatsioonide analüüsimisel Wilcoxoni testi.

Peamised tulemused ja arutelu

Kõikides gruppides olid IL-10 alleelid –592C ja –1082A kõige sagedasemad ja 1082AG/–592CC levinuim genotüübi paar. Kõikidel KESN-idel oli vähemalt üks –1082A alleel, samas kui HIV positiivsetel SN-idel oli 81,4% ja doonoritest 79% vastav alleel (vastavalt p=0,029 ja p=0,019). KESN-idel ei olnud GG/CC genotüübi paari, kuid see esines 18,6% HIV positiivsetel SN-idel ja 21% doonoritel (vastavalt p=0,029 ja p=0,019). HBV nakkuse puhul vähendasid –592AC alleel ja AG/AC genotüübi paar šansse HBV-ga nakatuda (vastavalt OR=0,28; 95% CI 0,09–0,87; p=0,028 ja OR=0,19; 95% CI 0,06–0,61; p=0,052). Kokkuvõttes võib öelda, et alleelid, millelt ekspresseeritakse vähe IL-10 (–1082A and –592A) kaitsevad sageli eksponeeritud isikuid HIV ja HBV infektsioonidega nakatumise eest.

Voolutsütomeetriline analüüs näitas, et ESN-idel on suurenenud immuunaktivatsiooni tase (rohkem HLA-DR+ rakke), rohkem CD4+ CD45RA+RO+ ja CD8+ CD45RA+RO+ rakke võrreldes tervete vabatahtlikega. Kuigi kõikide uuringugruppide CCR5+ rakkude protsendid olid sarnased, oli CCR5 tihedus

raku pinnal ESNidel madalam kui tervetel vabatahtlikel. Siit järeldub, et süstiv narkomaania võib mõjutada CCR5 ekspressiooni.

Analüüsides HIV+HBV+HCV+ kolmikpositiivseid SN-e (n = 35) oli näha, et neil on vähem CD4+ rakke ja rohkem immuunaktiveeritud rakke kui HIV negatiivsetel SN-idel (p < 0,001 kõikidel juhtudel). HBV+HCV+ SN-del oli väiksem CD4+ rakkude protsent kui kolmiknegatiivsetel SN-del (52,1% ja 58,6%, p = 0,021). Võrreldes omavahel kolmiknegatiivseid SN-e ja terveid vabatahtlikke selgus, et esimestel on kõrgem immuunaktivatsioon ja rohkem CCR5+ rakke. Kokkuvõttes võib öelda, et HIV positiivsetel on põhiliseks immuunsüsteemi mõjutajaks HIV ja lisanduvad ko-infektsioonid (HBV ja HCV) T rakkudele suurt lisamõju ei avalda. Samas HBV+HCV+ kaksikinfektsiooni korral vähendavad infektsioonid CD4+ rakkude hulka.

Analüüsimaks süstiva narkomaania mõju T rakkudele, võrreldi kolmiknegatiivseid SN-e (HIV–HBV–HCV–) tervete vabatahtlikega. Uuringus selgus, et kolmiknegatiivsetel SN-idel on vähem CD4+ T_{EM} rakke, rohkem CD8+ T_{CM} rakke, rohkem immuunaktiveeritud rakke ja rohkem CCR5+ rakke võrreldes tervete vabatahtlikega.

Järeldused:
1. SN-e kaitsevad HIV nakkuse eest IL-10–1082A ja –592A alleelid ning HIV ning HBV nakkuste eest –592AC genotüüp koos –592AC-d sisaldavate genotüübi paaridega.
2. Sarnaselt viirusele seksuaalsel teel eksponeeritud isikutele, on SN ESN-idel kõrgenenud immuunaktivatsioon ja kõrgenenud CD45RA+RO+ rakkudeprotsent võrreldes tervete vabatahtlikega. Lisaks on SN ESN-idel madalam CCR5 pinnatihedus kui tervetel vabatahtlikel.
3. HIV+HBV+HCV+ SN-idel on sarnased T raku muutused (kõrgenenud immuunaktivatsioon, mälurakkude hulk ja CCR5 ekspressioon) kui HIV monoinfektsiooniga indiviididel. HBV+HCV+ kaksiknakkusega SN-idel on ainult väiksem CD4+ rakkude arvukus, mis näitab, et HBV ja/või HCV seropositiivsusel on vähene mõju T raku jaotusele.
4. HIV–HBV–HCV– SN-idel on muutunud mälurakkude jaotus, kõrgem immuunaktivatsioon ja rohkem CCR5+ rakke võrreldes tervete vabatahtlikega. Seega mõjutab ka süstiv narkomaania HIV, HBV ja HCV nakkusest hoolimata T rakkude jaotust.

9. ACKNOWLEDGEMENTS

These studies were performed as a teamwork and I would like to express my gratitude to following persons:

- My supervisors Irja Lutsar and Radko Avi for their great support and objective criticism, that always helped me forward in my work.
- My supervisor in the laboratory and colleague Kristi Huik for all her help during my studies.
- My colleagues Merit Pauskar, Ene-Ly Jõgeda, Tõnis Karki, Pilleriin Soodla and Silver Türk in my research group for their help, support and friendship.
- All my colleagues in the Department of Microbiology for their help during my studies; especially Merle, who was the first to welcome me to the Deparment of Microbiology.
- Our collaborators from other institutions professor Anneli Uusküla, professor Don Des Jarlais, Marina Šunina, Kristina Marsh, and Karolin Toompere who assisted me in conducting the studies.
- All persons who participated in the study for their cooperation.
- Kai Kisand, Reet Kurg, Kai Truusalu, and Kristi Huik for the critical reading of the manuscript and for their valuable comments.
- My parents, family, and friends, for all their support during the studies and for keeping my spirits up.

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PUBLICATIONS

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List of publications:
Avi, Radko; Pauskar, Merit; Karki, Tõnis; **Kallas, Eveli**; Jõgeda, Ene-Ly; Margus, Tõnu; Huik, Kristi; Lutsar, Irja (2016). Prevalence of drug resistance mutations in HAART patients infected with HIV-1 CRF06_cpx in Estonia. Journal of Medical Virology, 88 (3), 448−454, 10.1002/jmv.24361.

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Avi, Radko; Pauskar, Merit; Karki, Tõnis; **Kallas, Eveli**; Jõgeda, Ene-Ly; Margus, Tõnu; Huik, Kristi; Lutsar, Irja (2016). Prevalence of drug resistance mutations in HAART patients infected with HIV-1 CRF06_cpx in Estonia. Journal of Medical Virology, 88 (3), 448−454, 10.1002/jmv.24361.

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