Aspects of immune activation in HIV-1 infection

Piotr Nowak

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To my family
Abstract

Abnormal immune system activation is central in HIV-1 pathogenesis leading to CD4+ T-cell depletion and inappropriate immune responses. Disease stage and HIV load strongly correlate with the immune activation level which decreases under antiretroviral therapy (ART).

The general aim was to investigate the role exerted by immune activation in HIV-1 infection and to characterise the contribution of high mobility group box protein 1 (HMGB1). This was fulfilled by: (a) studying two unique cohorts consisting of four orthotopic liver transplant (OLT) patients receiving ART and immunosuppressive therapy, and 22 patients with early primary HIV infection (PHI) who underwent both ART and analytical treatment interruption (ATI); (b) characterising the interaction between HMGB1 and HIV-1 in vitro and in vivo.

HIV-1 was controlled by ART, without any major contribution of the adaptive immunity, as shown by low or undetectable viral load (VL) and restricted viral diversity-divergence, in the four OLT patients. Also, the kinectics of VL during PHI in an OLT subject suggested that the initial rapid viral decline is caused by the innate immunity or reduced immune activation. Furthermore, the lower degree of immune activation in 22 PHI-patients was associated with lower viral replication and improved long-term virological outcome, as implied by the kinetics of 13 cytokines and chemokines during PHI and post ATI. We showed that PHI is indeed associated with generalized immune activation not restricted to a specific cytokine/chemokine pattern.

HMGB1 is an abundant protein with intracellular localisation and function exerting DNA flexibilisation and regulation of transcription. Additionally, this protein can be released actively after activation of macrophages, and dendritic cells, and passively by necrotic cells. Exogenous HMGB1 is a proinflammatory mediator that has been attributed a plethora of functions. Our data indicate that HMGB1 has an important role during HIV-1 pathogenesis. Thus, intracellular HMGB1 could inhibit the LTR mediated HIV-1 transcription in epithelial and monocytic cells. The physiological overexpression of HMGB1 in myeloid cells might be responsible for the restricted HIV-1 replication in these cells. Moreover, extracellular HMGB1 had a regulatory function on HIV-1 replication, tested in two models, latent and acute. We found upregulation of viral replication after HMGB1 stimulation of U1 cells and downregulation in acutely infected primary macrophages. The latter effect could be explained by β-chemokines (MIP-1α, MIP-1β, RANTES) release from these cells after HMGB1 induction. ELISA analysis revealed that the plasma levels of HMGB1 are significantly elevated in HIV-1 infected individuals (n=43) as compared to HIV-1 negative individuals (n=14). Additionally, the HIV-1 infected patients with clinical complications (n=16) had higher levels of HMGB1 than asymptomatic patients with preserved (n=14) or deteriorated immune system (n=13).

In conclusion, the data included in this thesis stress the essential role of immune activation in HIV-1 pathogenesis and place HMGB1 as an important factor that can modify the HIV-1 replication and contribute to the dysregulation of the immune system.

Keywords: immune activation, HMGB1, PHI, HIV-1 LTR-transcription, cytokines, chemokines
List of Publications

Follow-up of antiretroviral treatment in liver transplant recipients with primary and chronic HIV type 1 infection. 

Intracellular high mobility group B1 protein (HMGB1) represses HIV-1 LTR-directed transcription in a promoter – and cell –specific manner. 
*Virology* 2003, Sep 15; 314 (1): 179-189

HMGB1 activates replication of latent HIV-1 in a monocytic cell-line, but inhibits HIV-1 replication in primary macrophages. 
*Cytokine* 2006 Apr;34(1-2):17-23.


V. Nowak P, Barqasho B, Sönnerborg A. 
Elevated plasma levels of high mobility group box protein 1 in patients with HIV-1 infection. *AIDS* 2007, Apr 23, 21(7): 869-71
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List of abbreviations

AIDS Acquired Immune Deficiency Syndrome
APOBEC3G Apolipoprotein B mRNA–Editing Enzymes
ART Anti Retroviral Therapy
CCR CC chemokine receptor
CMV Cytomegalovirus
CTL Cytotoxic T Lymphocyte
CXCR CXC chemokine receptor
EBV Epstein Barr Virus
HAART Highly Active Anti-Retroviral Therapy
HIV-1 Human Immunodeficiency Virus 1
HIV-2 Human Immunodeficiency Virus 2
HMGB1 High Mobility Group Box protein 1
IL Interleukin
LTNP Long Term Non Progressor
LTR Long Terminal Repeat
MCP Monocyte Chemotactic Protein
MDM Monocytes Derived Macrophages
MIP Macrophage Inflammatory Protein
mRNA Messenger RNA
NFAT Nuclear Factor of Activated T cells
NF-κβ Nuclear Factor Kappa B
NK Natural Killer Cell
PBMC Peripheral Blood Mononuclear Cells
PCR Polymerase Chain Reaction
PHI Primary HIV-1 Infection
RAGE Receptor for Advanced Glycation Endproducts
RANTES Regulated on T cell Activation, Normal T cell Expressed and Secreted
RT Reverse Transcriptase
SIV Simian Immunodeficiency Virus
TAR Tat-Responsive Element
TLR Toll-like Receptor
TNF Tumor Necrosis Factor
UNAIDS Joint United Nations Program on HIV/AIDS
WHO World Health Organization
Introduction

Despite the increased knowledge and available chemotherapy HIV remains a global health threat. The epidemic is still successfully spreading with 15,000 people being infected every day, particularly in low to middle-income countries. In the end of 2006 40 millions people have been infected with HIV [1]. The virus can not be eradicated even with successful treatment and the preventive vaccine is far from the pipe-lines of pharmaceutical companies. Even if previous sentences are not encouraging, substantial progress has been made both in the field virology and immunology since the discovery of HIV. This advancement in the understanding of the viral pathogenesis and the interactions with the immune system gives us the hope for the future combat against the HIV.

The work included in this thesis has focused on the role of immune system activation in HIV-1 infection. Particular emphasis has been placed on the characteristics of the high mobility group box protein 1 (HMGB1) that we believe is important for this process.

The epidemic

In 1981 a new clinical syndrome, the acquired immunodeficiency syndrome (AIDS) was first described, after the identification of an increased incidence of opportunistic diseases in previously healthy young homosexual men [2-4]. The first indication that AIDS could be caused by a retrovirus came in 1983, when the agent was isolated in France from a patient with a pre-AIDS syndrome [5]. The virus was given the name lymphadenopathy virus (LAV). Researchers in USA isolated an identical virus the year after and named it AIDS associated virus [6] or human T-cell lymphotropic virus (HTLV III) [7].

The virus was later given the name human immunodeficiency virus (HIV). In 1986 a second immunodeficiency virus was discovered [8], named HIV-2, and the former strains of HIV were referred to as HIV-1. HIV-1 and HIV-2 are lentiviruses belonging to the Retroviridae family [9]. These viruses were found to be spread by sexual contacts, parenteral contact including blood and by vertical transmission from mother to child. HIV-2 also causes AIDS but appears to be less infectious and pathogenic than HIV-1 [10].

HIV infection has spread to all continents and has been responsible for the death of 25 million people. Approximately 4.3 million people became infected and 2.9 million people died due to HIV/AIDS only during year 2006 (www.unaids.org).
Virological aspects of HIV-1 infection

Virion structure

The HIV-1 virion is about 110 nm in diameter and has a spherical shape [9]. The envelope is a host derived lipid bi-layer which contains anchored viral trimeric structures consisting of surface (gp120) and transmembrane (gp41) subunits bound non-covalently [11]. The glycoprotein gp41 is attached covalently to the matrix (MA) p17 protein that lines the inner surface of the virus envelope, surrounding the nucleocapsid complex [12]. As an immature virus, the HIV-1 core has a doughnut-like structure which is then transformed into cone-shaped structure upon maturation as a result of Gag processing [13]. The capsid core contains the viral genome which are two copies of single stranded RNAs tightly bound to the nucleocapsid (NC; p7) protein. Other viral proteins contained here are the reverse transcriptase (RT; p66/p55), the protease (PR; p15) and the integrase (IN; p32). A schematic picture of HIV-1 can be seen in figure 1.

Figure 1. Schematic overview of HIV-1 structure

Genomic structure and replication

The HIV-1 genome is approximately 9200 bases in length [9]. In common with all replication competent retroviruses, the HIV-1 genome has three major structural genes (gag, pol, env), which are flanked on both sides by repetitive sequences called long-terminal repeats (LTR) [14]. The gag gene encodes the structural proteins: capsid (p24), matrix (p17) and nucleocapsid (p9/p7). The pol gene encodes reverse transcriptase, integrase and protease. These proteins are essential enzymes for transcription of viral RNA into DNA, integration of viral DNA into the cell genome and cleavage of HIV-1 proteins, respectively. The env gene codes for the envelope
glycoproteins (gp120 and gp41) that are critical for viral attachment and subsequent infection of the target cell [15]. Beside the three structural genes, the HIV-1 provirus contains six additional open reading frames, coding for the regulatory proteins (Tat, Rev) and the accessory proteins (Vif, Vpr, Vpu and Nef) which functions are of importance for viral replication and infection (Table 1) [16, 17].

Table 1. Additional proteins of HIV-1 and their functions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>vif</td>
<td>p23</td>
<td>enhances infectivity of virions/ counter-acts APOBEC3G</td>
</tr>
<tr>
<td>vpr</td>
<td>p15</td>
<td>involved in targeting the nuclear localization of preintegration complexes; arrest cell cycle at G2 phase</td>
</tr>
<tr>
<td>vpu</td>
<td>p16</td>
<td>degradates CD4 in the endoplasmic reticulum; assists budding</td>
</tr>
<tr>
<td>tat</td>
<td>p14</td>
<td>potent transactivator</td>
</tr>
<tr>
<td>rev</td>
<td>p19</td>
<td>RNA transport and stability; regulation of viral gene expression</td>
</tr>
<tr>
<td>nef</td>
<td>p27</td>
<td>interferes with endosome trafficking and downregulates expression of CD4 and MHC-1; effect on cell activation / apoptosis induction in bystander cells</td>
</tr>
</tbody>
</table>

The HIV-1 infection of a cell is initiated by interactions between the viral envelope glycoprotein (gp120) and the cellular receptor, the CD4 molecule (Figure 2) [15]. Thus, the major targets for HIV-1 are cells expressing the CD4 antigen on their surface. These cells include T-lymphocytes, monocytes, macrophages, and dendritic cells [18-20]. After binding to the CD4 molecule, gp120 subsequently interacts with one of the coreceptors of the chemokine receptor family (CCR5 and/or CXCR4) [21]. Also other chemokine receptors have been shown to be functional as coreceptors in the viral entry, although their relative importance seems to be limited [22]. This interaction results in a conformational change of gp41, which in turn leads to a pH-independent fusion between the virus envelope and the cell membrane [23]. After that the fusion is accomplished the virus is unpackaged and the nucleocapsid is dismantled. The reverse transcriptase then transcribes viral RNA into DNA, which subsequently enters the cell nucleus in the form of a preintegration complex [24]. The proviral DNA is then incorporated into the host cell DNA by the viral integrase [25]. Frequently transcription of the integrated proviral DNA is initiated immediately, but in some cells the proviral DNA remains latent for a variable length of time until host cell activation [26]. The cellular activation initiates transcription of the structural genes into mRNA, which are then transported out of the nucleus into the cytoplasm [27]. Following translation, the viral proteins begin to assemble at the host cell surface which is modified by insertion of gp41 and associated gp120 [28]. The viral RNA and the core proteins assemble beneath the modified membrane, acquiring the modified host plasma membrane as its envelope during the budding process [29]. The viral Gag and Gag-Pol polyproteins are cleaved by the viral protease during or shortly after budding, generating mature infectious virions [30].
The long terminal repeats (LTRs) sequences contain promoters, enhancers and other sequences that interact with host-cell transcription factors as well as virus encoded proteins and modulate expression of viral RNA and proteins [31]. LTR function has a direct impact on the productive infection of the host cell, viral expression and assembly, production of cytotoxic viral proteins and the transition from latency level of viral expression to highly productive infection [32]. Although, both the 5’ and 3’ LTRs facilitate integration of the viral genome into the host chromosome these two LTRs are distinct in function. The 5’ LTR functions as a promoter/enhancer and therefore directs HIV-1 transcription, whereas the 3’ LTR is required for cleavage and polyadenylation of the viral mRNAs [33].

The LTR extends from -454 to +184 (related to the site of transcription) and is structurally divided into three regions: the 454 bp U3 region, the 97 bp R region and the 85 bp U5 region [31]. Two nucleosomes have been positioned in the 5’LTR regions: the first one located in the 5’ of the U3 region (nuc-0) and the second one immediately after the transcription initiation site in the R region (nuc-1) [34]. These sites define two large nucleosome-free regions encompassing upstream and downstream of the site of transcription initiation. After initiation of viral transcription, nuc-1 is disrupted and an additional hypersensitive site appears in the R region of the 5’ LTR. Thus the U3-R part of the HIV-1 LTR is associated with an open chromatin configuration which point to the regulatory role of this region in the viral gene expression. The U3-R part contains cis-acting sequences and may be subdivided into several elements due to function during the transcription: the modulatory (-454 to -104), the core enhancer (-105 to -79), the core promoter (-78 to -1) and the Tat Responsive Element termed TAR (+1 to +60) (Figure 3). U5 region...
also contains several constitutive and inducible regulatory elements that are important for efficient HIV-1 transcription and replication [32, 35].

HIV-1 LTR-directed transcription is RNA polymerase II (Pol II) regulated by the complex interactions of host transcription factors and viral proteins, with specific DNA as well as RNA sequences on the HIV-1 5'-LTR [31]. The initiation of viral transcription occurs in the core promoter which contains the key regulatory elements required for transcription. These sites include: three SP-1 binding sites, a conserved TATA box and an initiator element (Inr) [36-38]. The presence of these three elements is crucial for minimal promoter activity in vitro and in vivo [39, 40]. The LTR contains two initiator elements. Several different cellular factors have been identified to interact with initiator elements and positively or negatively regulate HIV-1 basal transcription [35]. The enhancer region consists of two tandem binding sites for the transcription factor NF-κβ. Several proteins members of the Rel family can bind to these sites, associating as homo or hetero-dimers [41]. This binding to NF-κβ motifs in HIV-1 enhancer increase expression of HIV-1 proteins in T-cells and macrophages [42]. However the NF-κβ sites are not absolutely required for viral replication and appear to serve primarily to enhance viral replication in response to cellular activation and differentiation [43, 44]. It has been shown that NF-κβ can functionally compensate for Sp1 in activating HIV replication [45]. Several cellular, constitutively expressed or inducible host-cell transcription factors, which bind to the modulatory element within the LTR have been identified [32]. This element contains a negative regulatory region (NRE) of about 260 bp sequences. NRE despite its name confers also positive regulation and contains binding sites for many cellular transcription factors including: USF, hLEF and NFAT [46, 47].

![Figure 3: Schematic diagram of the HIV-1 5'-LTR](image)

**Figure 3.** Schematic diagram of the HIV-1 5'-LTR. The U3 and R regions are divided into the TAR, core promoter, enhancer and modulatory elements. Binding sites for the identified cellular transcription factors are indicated. Modified from [31].
The early phase of HIV-1 transcription is regulated by cellular transcription factors and results in the production of early viral products (Tat, Rev and Nef) [48, 49]. The late phase of transcription is under control of Tat which potently enhances gene expression by a direct binding to TAR-RNA and association with cyclin T1 (Cyc T1), which recruits the cyclin dependent kinase 9 [50]. This leads to phosphorylation of the C-terminal domain of RNA polymerase II and efficient elongation. Tat can also stimulate transcriptional initiation and it has been shown that Tat can synergize NF-kβ activity [51, 52]. These two steps of provirial transcription occur in various cell types, which indicate that distinct combination of factors mediate transcription in a cell type-specific manner [31, 49].

**Variability and heterogeneity**

HIV-1 is characterized by a remarkable genomic heterogeneity [53]. HIV-1 genetic variants appear readily in individuals, which result in a swarm of closely related genomes termed ‘quasiespecies’ [54]. The rapid evolution of HIV-1 and the high genetic diversity are results of the low fidelity of the reverse transcriptase (RT), recombination events, selective host immune responses and the high turnover rate of virus production. The low fidelity of the RT is caused by the lack of the 3’exonuclease proof-reading activity, which makes it very error prone, and thus allows for nucleotide substitution, deletions, insertions, duplications and recombinations [55, 56]. The rate of nucleotide substitution caused by the RT is about one substitution per virus genome per cycle of replication [53, 57]. Also, host RNA-polymerase II, responsible for viral genomic RNA synthesis, is lacking 3’exonuclease proof-reading activity. This high error rate in combination with the fact that in HIV-1 infected individuals approximately 10^{10} new virus particles are produced and cleared per day with a life span in cells about two days results in the high viral diversity [58]. The sequence variation is due to nucleotide substitutions that are either synonymous (ds), which do not change the amino acid, or nonsynonymous (dn) that cause changes. The ds/dn ratio varies between different genomic regions and is in part influenced by the selective pressure of the immune system [59, 60]. Products of the pol gene are essential for viral replication, which is the reason why this region shows limited variability. The largest variation occurs in the env gene. This is illustrated by the fact that the gp120 part of envelope polyprotein has evolved to contain hypervariable domains that can accept extensive variation, thereby escaping from the host immune system. This region can thus be used as an indicator for viral evolution [61, 62]. The gp120 is divided into five variable (V1-V5) and five constant (C1-C5) regions [63]. The V3 region of env has attracted most attention. It is known that this region is a major target for humoral and cellular immune responses [64-66]. Changes of certain aminoacids within the V3 loop result in changes of the phenotype of the virus [67, 68], e.g in the use of co-receptors [21]. The variability in the p17 region of the gag is higher than in pol, but lower than in env. Because of these properties and the higher rate of ds the p17 region has been proposed as being a useful marker for epidemiological studies [69]. The phylogenetical studies have revealed the dynamics of the viral population in the infected host. Thus, early after infection, the virus population is usually relatively homogenous [70-72]. During the disease progression, in the asymptomatic period the population of viral variants becomes more
heterogeneous probably as a result of continuous immunological selection [73-75]. A comprehensive study with frequent sampling from PHI up to twelve years defined three distinct phases within this interval: an early phase of variable duration with a linear increase of both viral divergence and diversity (~ 1% per year); an intermediate phase of mean 1.8 year with a continuous increase in divergence but stabilizing or declining diversity; and finally a slowdown or stabilization of the viral divergence and declining diversity [76]. In support of these data several reports show that, at the AIDS stage, the virus population returns to a relative homogeneity, due to lack of the pressure from damaged immune system [53, 77].

**Natural course of HIV-1 infection**

The acute infection with HIV-1 may be associated with clinical symptoms of variable severity and duration [78, 79]. Those symptoms usually occur within two weeks after viral entry [80]. The viral load (that can be detected around a week after infection) rapidly increases to reach the peak about one week after the onset of symptoms. The increase of viral load inversely correlates with CD4+ T-cell counts, depletion of which has been claimed to be caused mainly by direct virus effect [81]. The viremia peak is followed by a phase of rapid decay and subsequent phase of slow decay that starts after about 3 weeks after onset of symptoms [82]. The viral load reaches the steady-state 3 to 4 months after infection. It is believed that events occurring during the acute/primary phase of HIV-1 infection (PHI) determine the disease prognosis [83-85]. A correlation between the peak of viremia and viral set point as well as disease progression has been shown [86]. The chronic phase of infection, usually asymptomatic, can last for many years without a need for therapeutic intervention [87]. However, a continuous viral replication and gradual loss of CD4+ T cells lead to dysfunction of the immune system and the appearance of opportunistic conditions associated with AIDS, the final stage of HIV-1 infection [88, 89].

Opportunistic conditions include:

- Candidiasis of esophagus, bronchi, trachea, or lungs
- *Mycobacterium tuberculosis* (TB),
  - pulmonary
  - extrapulmonary
- *Mycobacterium*, other species, disseminated or extrapulmonary
- *Pneumocystis jirovecii* pneumonia (PCP)
- Pneumonia (other than PCP), recurrent
- Cryptosporidiosis, chronic intestinal
- Cytomegalovirus retinitis
- Toxoplasmosis of the brain
- Encephalopathy, HIV-related (AIDS dementia complex)
- Histoplasmosis, disseminated or extrapulmonary
• Kaposi's sarcoma (KS)
• Lymphoma: non-Hodgkin's (NHL), primary brain/CNS
• Progressive multifocal leukoencephalopathy (PML)
• Invasive cervical cancer

Treatment of HIV-1 infection

Drugs that are approved as antiretroviral agents target either the viral entry (introduced recently), reverse transcriptase, protease or integrase enzymes (currently introduced) [90-92]. A combination of these drugs is called highly active antiretroviral treatment (HAART) and is very efficient in reducing the HIV-1 viral load as well as immune activation [93, 94]. Morbidity and mortality have been dramatically decreased after HAART introduction [95]. However, HAART is unable to eradicate HIV-1 because replication competent viruses persist under therapy in an integrated latent form and/or in a slowly replicative manner in several cell types and anatomical sites [96, 97]. Moreover suboptimal ART, which allows the viral replication to continue, is known to select for virus variants with reduced sensitivity to the antiretroviral agents and drug-resistance is therefore a common problem [98]. From the patient perspective HAART although effective may have side effects such as neuropathy/myopathy (due to mitochondrial damage), gastrointestinal intolerance, a metabolic syndrome including lipid abnormalities and diabetes mellitus, a body change syndrome (lipodystrophy) and a slightly increased risk for myocardial infarctions [99, 100]. The appearance of long-term side effects is why alternative strategies such as structured treatment interruptions (STI) came into focus in the beginning of 2000s [101, 102]. These strategies have given the opportunity for studying the interactions of HIV-1 with immune system under monitoring conditions. The recently published results of SMART trial may change the approach to wider usage of intermittent therapies [103]. This open-label study assigned more than 5400 HIV-1 infected patients who were divided into the group receiving continuous or intermittent (CD4+ T-cell guided) ART. The episodic ART significantly increased the risk of opportunistic disease or death from any cause as compared with continuous ART. These findings have lead to early termination of SMART trial [104].

Immune activation

A complex combination of viral and host factors contribute to the pathogenesis of AIDS [15, 105]. The direct effects of the virus on T-cell death cannot explain the AIDS pathogenesis because of the low frequency of infected CD4+ T cells [106]. Additionally, virus preferentially infects activated CD4+ T cells, most of which are destined to die regardless of their infection, because of activation process itself [107]. An activation of the immune system is an important part of a proper immune response to pathogens. In general, after that the immune response adequately has dealt and cleared the foreign antigen, the system returns to the state of relative quiescence until the next stimulus is introduced. This does not happen in the case of
HIV-1 infection. The persistence of the virus leads to a constant exposure of the immune system to viral antigens and is likely to contribute to the chronic activation of the immune system observed in the HIV-1 infected patients [108]. The immune activation is the result of both the continuous attempt of the immune system to clear the infection and the HIV-1 associated dysregulation of its homeostasis. Thus, HIV-1 both directly (cell damage/induction) and indirectly (immune compensatory mechanisms) can contribute to immune activation [81]. This contributes the state of chronic inflammation, which may also be responsible for non-HIV-1 related complications, such as the increased rate of atherosclerosis in HIV-1 disease [109].

This process starts very early after PHI and persists throughout the whole disease course [110, 111]. The theory that HIV-1 causes T-cell dysfunction and T-cell loss by chronic immune activation could feasible explain AIDS pathogenesis [81, 112].

The study on chronic activation in a murine model [113] has elegantly showed that also in the absence of active infection the animals with hyperactive immune system could present signs of immunodeficiency syndrome with T-cell loss and opportunistic infections. Contrary, the lack of generalized immune activation in the natural hosts for SIV infection, sooty mangabeys, is associated with lack of pathogenicity despite high viral production and poor host immune response [114].

HIV-1 can infect both activated and quiescent cells, however the virus requires cell activation signals for genome integration as well initiation of its replication [27]. The in vivo connection between immune activation and HIV-1 replication is demonstrated by the increases in viremia in HIV-1 infected individuals transiently or persistently exposed to exogenous immune stimuli [115, 116]. Individuals living in Africa, chronically exposed to parasites and other pathogens, usually express higher levels of activation markers [117, 118] and harbour high viral loads [119] that are associated with faster disease progression [120]. In addition, co-infection with opportunistic pathogens results in significant increases of viral load, which decreases when the opportunistic infection is treated [121, 122]. Moreover, immunisation of HIV-1 infected patients with influenza or tetanus toxoid antigens, results in transient increase of plasma viremia [123]. In conclusion, immune activation is a requisite of HIV-1 infection, closely connected to the viral replication.

The immune activation can be characterized by many immunological signs including:

- increased T-cell turnover
- increased frequencies of T- and B-cells with activated phenotypes
- increased CD8+ T-cell counts
- increase in T-cell death rate apoptosis (AICD activation induced cell death)
- impairment of T-cell responses to antigens
- decrease of regulatory T-cells
- polyclonal B-cells activation-hypergammaglobulinemia, presence of autoantibodies
- activation of monocytes
- increased serum concentrations of proinflammatory cytokines and chemokines
Several studies have implicated that the levels of these markers represent independent predictors of disease outcome in antiretroviral-untreated and –treated individuals [124, 125].

Of these, the best characterized marker of immune activation is the CD38 expression on T-cells. CD38 is a multifunctional transmembrane glycoprotein that is up-regulated during the earliest stages of T-cell activation [126]. Giorgi et al [127] reported that CD38 expression on CD8+ T-cells had stronger prognostic value concerning HIV-1 progression than other commonly used markers of activation. However, even serial quantification of CD8+ T-cells gives significant predictive information of the natural progression of HIV-1 infection in patients with moderate to severe immunodeficiency [128]. An immune activation set point is established during PHI and predicts subsequent CD4+ T-cell loss, independent of VL [110]. Furthermore, chronic HIV-1 infection is associated with increased T-cells turnover [129]. Elevated markers of CD4+ T-cell apoptosis correlate with CD4+ cell depletion and virus production [130, 131]. Activation induced cell death (AICD) appears to be the major mechanism of uninfected “bystander” T-cell loss due to direct action of viral proteins and inflammatory milieu [132]. Dysregulation of the cytokine network is characteristic of all phases of HIV-1 infection [133-135]. A number of cytokines have been shown to direct modulate HIV-1 replication in T-cells and macrophages in vitro and influence disease progression [134, 136]. Proinflammatory cytokines like TNF-α, IL-1β, and IL-6 are reported to be elevated during the primary and late phases of HIV-1 infection, contributing to immune activation and inducing apoptosis signals [136, 137]. β-chemokines, although exerting antiviral effects, can be also responsible for attraction of cells and contribute to viral spreading [138, 139]. Thus, diverging results about the levels of β-chemokines in relation to viral replication and disease progression are found in the literature [94, 140, 141]. Additionally, Brenchly et al have recently suggested [142] that microbial products originating from intestinal flora could contribute to immune activation. The authors reported that bioactive LPS is found in plasma of HIV-1 infected individuals with levels correlating to immune activation markers. Interestingly, the levels of LPS decrease with effective HAART and long-term non-progressors (LTNP) contain lower levels of this potent immune activator in plasma. The hypothesis that pathogenic events during PHI that lead to breach in the integrity of gastrointestinal mucosal barrier and cause microbial translocation is an attractive explanation of HIV-1 systemic immune dysregulation [81, 143].

Deactivation of immune system

The introduction of HAART has altered the course of HIV-1 infection. A rapid decrease in the viral load (VL) and a significant decrease of cellular and soluble markers of immune activation are followed by increase in CD4+ T-cell counts [129, 144]. However, successful suppression of the VL is not immediately followed by normalisation of T-cell levels that takes many years and is not always achieved [145, 146]. This observation implies that activation of the immune system, not direct viral effect followed by homeostatic compensatory mechanism is a major cause of T-cell
death. In any case, the effect of HAART strongly supports the role played by the virus in the cause of abnormal activation of immune system. Suppressing the virus and hampering of the immune activation not only restore number and function of the T-cells but also has the important impact on the other immune cells, such as CD8+ T-cells, B-cells and polymorphonuclear cells [112]. However, the deactivation of immune system may not be the ‘golden approach’ in the fight with HIV-1, as the proper level of immune activation is essential for its function. This could also be illustrated by the rapid decline in the HIV-specific CD8+ T-cells counts during the first month of an efficient antiretroviral therapy [144, 147]. Furthermore, clinical trials with the use of anti-inflammatory and/or immunosuppressive drugs have not shown a clear picture of advantage in delaying disease progression [148, 149]. Hydroxyurea, prednisone and mycophenolic acid have been tested for these purposes, showing limited benefit (or even harm) that dampened the enthusiasm for this approach [150]. Interesting results have been obtained with another immunosuppressive agent, Cyclosporin A (CsA). This drug acts on lymphocytes and prevents T-cell activation, reduces cytokine production and adhesion molecule expression [151]. Additionally interacting with cyclophilin, CsA has been shown to exert direct anti-HIV effect [152]. A study involving treatment of PHI patients with HAART and 8-weeks CsA regime showed positive effect of this combination on CD4+ T-cell counts and function [153]. This effect was pronounced and sustained for >1 year as the follow up study reported [154]. However this beneficial effect of CsA was not confirmed in a clinical trial involving chronically infected individuals [155].

There are still gaps in the basic knowledge of the concomitant regulation of viral replication and immune activation. A better understanding of how HIV-1 induced immune activation leads to CD4+ T-cell depletion may provide new targets for immune-based interventions that could be used in addition to HAART to slow disease progression [156].
High mobility group box protein 1 (HMGB1)

HMGB1 is a member of the family of high mobility group (HMG) proteins (A, B, N) (http://www.informatics.jax.org/mgihome/nomen/genefamilies/hmgfamily.shtml). They were first described in the 1970s, as a group of non-histone DNA binding proteins and named because of their rapid mobility on polyacrylamide gels under electrophoresis [157]. HMGB1 is the most ubiquitous of the high mobility box (HMGB) sub-family, being constitutively expressed in all nucleated cells, though in variable levels [158, 159]. HMGB2 and HMGB3 are primarily expressed during embryogenesis, however expression of HMGB2 in the testis and lymphoid tissue in adult mice has also been reported [160-162].

HMGB1- the intracellular protein

HMGB1 consists of 215 amino acids with the gene localisation on chromosome 13q12 [163]. It is highly conserved through evolution with 99% homology between rodents and humans. Structurally, HMGB1 is organized into two DNA-binding domains (named A-box and B-box) and a negative charged C-terminus (Figure 4) [164]. The two DNA-binding domains have an α-helix conformation and attach to minor grooves of linear DNA, which results in DNA bending. This is considered as an allosteric transition of DNA, which can promote the binding of other proteins. HMGB1 implies to regulate the transcription of several genes through two potential mechanisms [159]. The first mechanism is the ability of HMGB1 to interact directly with nucleosomes (by loosening the wrapped DNA) and enhance accessibility to chromatin-remodelling complexes and possibly to transcription factors. The second mechanism is exerted by establishing protein-protein interactions. Thus, HMGB1 binds various transcription factors, including p53, recombination activating gene 1 / 2 (RAG 1 / 2) proteins, several NF-κβ subunits and all steroid (class I) receptors [165, 166]. These interactions enhance/regulate transcriptional activation. HMGB1 overexpression, observed in many tumour cells, accelerates cell-cycle progression probably through its ability to promote access to some transcriptional complexes [167]. The nuclear functions of HMGB1 are critical for survival but not for foetal development, as HMGB1-knockout mice are born alive although they die within 24h due to hypoglycaemia [168]. Cell lines obtained from these animals grow normally, but the activation of the gene expression by different factors, including the glucocorticoid receptors, is impaired.

![Figure 4. Schematic structure of HMGB1 protein. The two nuclear localisation sequences (NLS) are positioned according to the primary amino acid sequences of human HMGB1. Modified from [164]](image-url)
**HMGB1 - the extracellular protein**

The year 1999 publication of Wang et al [169] has attracted intensive interest in the properties of HMGB1. The authors have described that this intracellular protein could be actively released after stimulation of macrophages/monocytes and functions as a late mediator of lethal endotoxemia and sepsis. However, prior to Wangs article other groups have proposed that HMGB1 could function as an extracellular factor, in particular with regard to cell differentiation and motility [170, 171].

**Release of HMGB1**

Active secretion of HMGB1 has been described in macrophages/monocytes, dendritic cells, pituicytes, NK and endothelial cells, following the stimulation with exogenous bacterial products such as endotoxines or proinflammatory cytokines (IL-1β, INF-γ, TNF-α) [164]. The mechanism of active HMGB1 secretion is not yet thoroughly understood. It is established that HMGB1, like IL-1β and fibroblast growth factors, is lacking a classical leader sequence and can not be released by classical endoplasmic reticulum-Golgi mediated pathway. Instead, activated macrophages/monocytes acetylate HMGB1 at potential nuclear localization sequences, leading to its cytoplasmic translocation and subsequent release into the extracellular milieu through secretory vesicles [172]. The acetylation step seems to control active HMGB1 release, at least in myeloid cells [173]. Scaffidi et al [174] reported an alternative mechanism for HMGB1 release, demonstrating that necrotic or damaged cells release bioactive HMGB1. The underacetylation of chromatin that precedes formation of apoptotic bodies causes tightly binding of HMGB1 in apoptotic cells that prevents HMGB1 release. Even secondary necrosis or partial autolysis was not affecting the HMGB1 release. Moreover, necrotic cells lacking the HMGB1 were not able to induce inflammation as compared to normal cells. This distinction in HMGB1 liberation between the models of cell death could explain the presence of inflammation associated with necrosis and its absence during apoptosis [166, 175].

**Alarmin**

Recently a new term ‘’alarmin’’ was proposed to describe endogenous molecules that could signal tissue and cell damage to immune cells [176, 177].

They have been applied characteristics such as:

1) Rapid release following the non-programmed cell death, but lack of release during apoptosis
2) Alternative pathway of active secretion by cells of immune system
3) Stimulation of innate and adaptive immunity
4) Role in the tissue repair and regeneration
All above criteria can be met by HMGB1. This molecule activates immune cells and promotes the release of proinflammatory cytokines as well as chemotactic activity [164]. Andersson et al [178] have reported secretion of TNF-α, IL-1α, IL-1β, IL-1RA, MIP-1α, MIP-1β, IL-6 and IL-8 by macrophages stimulated with HMGB1. A similar pattern of cytokine release has been described in other cells [179]. The chemotactic properties of HMGB1 have been described on many cells including monocytes, macrophages, dendritic cells, enterocytes, endothelial and smooth muscle cells [166, 180]. Moreover, HMGB1 has been attributed proangiogenic activity [181]. Neurons respond to the protein by extending neurites, the process that also involves cytoskeleton remodelling and resembles chemotaxis [182]. HMGB1 stimulates maturation of dendritic cells which implicates a role in switch from innate to adaptive immune system [183, 184]. In addition, HMGB1 can recruit stem cells and its involvement in tissue regeneration after myocardial infarction has been reported [185].

Extracellular HMGB1 binds to a cellular receptor for advanced glycation endproducts (RAGE) in a concentration-dependent manner [186]. RAGE is a transmembrane protein that belongs to the immunoglobulin superfamily, and acts as a receptor for the diverse ligands including the amyloid peptide and members of S100/calgranulin family of inflammatory mediating peptides [187]. It is expressed at low levels in normal tissues and in the vasculature. Increased levels of RAGE expression has been found in diabetes, Alzheimer’s disease and atherosclerosis [188]. Stimulation of RAGE results in the activation of multiple intracellular signalling pathways including the mitogen activated protein kinases, Rho GTPases Rac and CDC42, as well as NF-κB translocation [179]. These signalling pathways allow the transcription of pro-inflammatory genes, cytokine production finally inducing inflammatory response. Recent structural/functional studies have localized the cytokine functional motif (amino acids 106-123) in the “B-box”, but the RAGE-binding motif in C-terminus (Figure 4), suggesting a potential involvement of other receptors in HMGB1 induced activation [164]. Accumulating evidence indicate that in addition to RAGE, toll like receptors (TLR-2 and TLR-4) and syndecan-1, also mediate the cytokine activity of HMGB1 [182, 189].

**Role of HMGB1 in inflammation**

Several experimental models have been used to demonstrate that HMGB1 plays a central role in acute and chronic inflammation, including endotoxemia, septicemia, rheumatitis and acute respiratory distress syndrome (ARDS) [164]. Thus, high systemic levels of HMGB1 in animals and correlation to symptoms as well survival have been shown [190]. Injection of recombinant HMGB1 into mice causes the development of clinical signs of sepsis and multiple organ failure [191]. In addition, passive immunization of anti-HMGB1 neutralizing antibodies increased the survival rate in the mice sepsis model [192]. In humans, increased systemic HMGB1 levels have been reported in different organ disorders and diseases characterized by inflammatory responses, including sepsis, pancreatitis, disseminated intravascular coagulopathy, rheumatoid arthritis (RA) and ARDS [169, 193-196]. However, the correlation between survival rate, clinical symptoms, parameters of immune
activation and levels of HMGB1 vary significantly between studies most probably due to differences in the methodology and treatment-related interventions [197]. Neither animal, nor human studies have answered the questions about the origin and biological importance of circulating HMGB1 [179]. In the recent publication Urbonavicute et al. [198] reported the presence of anti-HMGB1 antibodies in plasma/sera not only in individuals with autoimmune diseases but also in healthy subjects. The authors have also described the occurrence of elevated HMGB1 levels among the latter group. These findings are not theoretically astonishing, but presume complex regulation of HMGB1 function in vivo. Their importance, both in the relation to methodology of previous reports and clinics, remains to be elucidated.

The plethora of functions governed by HMGB1, especially the potent proinflammatory activity, have attracted a lot of interest and resulted in a magnitude of publications after the re-discovery of HMGB1. During the last 6 years the view on the HMGB1 protein has fluctuated from being a danger signal and a potent proinflammatory cytokine per se to rather being an immunomodulatory factor [176, 199]. The role of HMGB1 as an important mediator in cell death and modulation of immune responses is not questioned, however future research would probably place this interesting molecule in a more correct position [177]. Even though, HMGB1 is a potential target for immunotherapy [192].

**Role of HMGB1 in viral infections**

The function of HMGB1 depends on its localisation [200], which also attributes to viral infections. The nuclear localization and function of HMGB1 imply its possible influence on the viral transcription in infected cells. HMGB1 has been shown to stimulate transcription from viral promoters, such as the adenovirus major late promoter (MLTF) and the SV40 late promoter [201]. Gamma-2 herpesviruses, including Kaposi sarcoma-associated virus (KSHV) and murine gamma herpesvirus 68 (MHV-68), exploit HMGB1 to facilitate binding of their RTA (replication and transcription activator) protein to different promoters [202]. Moreover, MHV-68 replication, in addition to its gene expression was reduced in HMGB1 -/- cells. Phosphoprotein P of Borna disease virus (BDV) binds A-box subunit of HMGB1 and represses the transcriptional activity of p53 in infected cells [203]. This strategy of P protein may be involved in noncytolytic replication and persistent infection of BDV in CNS cells. As different cell types express diverse amounts of HMGB1 its intracellular interaction might be cell specific [159].

Viral infections trigger inflammatory responses that involve both innate and adaptive immunity [204]. This is reflected by increased levels of local and systemic immune activation. If the inflammatory response is appropriate the infection will be cleared, however the overproduction of cytokines and chemokines may become harmful to the host and cause organ injury [205]. Many viruses are directly cytopathic causing cell necrosis, release of HMGB1 and inflammation. This has been proven for West-Nile (WN) virus belonging to the Flaviviridae family, which at a high infectious dose induces necrotic cell death caused by profuse budding of WN progeny virus particles [206]. Similar observations have been made for in vitro infection with Salmon
anaemia virus [207]. Death of CD4+ T-cells is a hallmark of HIV-1 infection [15], even if the relative contribution of apoptosis/necrosis to this process is still a matter of discussion [208]. The direct cytopathic effect HIV-1 seems to be essential in the primary and late phases of the infection [81]. Unpublished data from our laboratory (P. Nowak et al, Manuscript in preparation) reveal that HIV-1 infection of T-cell lines and PBMCs results in cytopathic effects and subsequent HMGB1 release (Figure 5).

Figure 5. Kinetics of HMGB1 release from MT4 cells infected with HIV-1 virus. Western-blot technique was applied to detect the HMGB1 protein in supernatants of HIV-1 infected (+) and uninfected (-) cells at 48, 54, 60, 72 hours after infection. Cytopathic effect in the HIV-1 infected wells was present at 60h. Supernatants from necrotic MT4 cells (obtained by three rapid freezing and thawing cycles) served as a control.

Besides the contribution to immune activation of HMGB1 caused by the passive release, an active secretion could be assumed based on several observations [205]. In viral infections increased local and systemic levels of several pro-inflammatory cytokines have been documented [209, 210]. An upregulation of the HMGB1 expression in a mice model of experimental hepatitis has been described [211]. Moreover, treatment of CTL-induced liver disease in HBV transgenic mice with inhibitors of extracellular HMGB1 significantly decreased the intrahepatic recruitment of inflammatory cells and diminished the severity of liver disease [212]. These findings implicate that HMGB1 released both in active and passive manner could initiate an inflammatory response and contribute to viral pathogenesis (Figure 6).
Figure 6. Hypothetical role of HMGB1 in the pathogenesis of viral infectious diseases. Adapted from [205].
Aims of the study:

General aim

- To investigate the role exerted by immune activation in HIV-1 infection, as well as to characterise the contribution of HMGB1.

Specific aims

- To analyse the impact of a heavy immunosuppressive regime on the HIV-1 kinetics and sequence variability after liver transplantation in patients with anti-HIV-1 treatment (Paper I)

- To determine the effect of intracellular HMGB1 overexpression on HIV-1 transcription (Paper II)

- To investigate the effect of extracellular HMGB1 on HIV-1 replication in acute and chronic models of HIV-1 infection (Paper III)

- To determine systemic levels of HMGB1 in HIV-1 infected patients (Paper V)

- To investigate the pattern of immune activation by studying cytokines and chemokines during primary HIV-1 infection and after analytical treatment interruption during chronic HIV-1 infection (Paper IV)
**Material and Methods**

For details about the Material and Methods used in this thesis, see respective paper.

**Ethical clearance**

The studies included in thesis were performed in the spirit of Helsinki agreement, after approval from the Institutional Review Boards and Ethical Committees at each participating site. All subjects included in the study gave their signed informed consent prior to study enrolment.

**Statistical analysis**

For details about the statistical analysis used in this thesis, see respective paper.
Results and Discussion

Background- role of immune activation in HIV-1 infection

The central question that I have tried to address in my thesis is; what is the role of immune activation in HIV-1 pathogenesis? Being a physician taking care of patients and with the knowledge that the activation of the immune system in the end cause severe harm to the infected individual, I chose to perform both in vivo and in vitro studies. In order to analyse whether an advanced immunodeficiency induced by chemotherapy could affect the course of the HIV-1 infection in patients, my studies started with the characterisation of the HIV-1 disease in an extraordinary group of patients - those who underwent orthotopic liver transplantation (OLT) and were treated with both immunosuppressive and antiretroviral regimes. In addition, I also studied another unique group of patients with primary HIV-1 infection (PHI), the largest in the world ever studied, in whom ART where initiated very early and who also participated in the first study ever performed with therapeutic vaccination in such patients – the Quest study. Studying these patients I managed to characterise the very early immune activation events and compare the events occurring after analytical treatment interruption (ATI) in the same patients. This was done by analysing the kinetics of 13 soluble markers of immune activation, both cytokines and chemokines, in samples obtained both during PHI and after ATI. In the latter part of my work, I characterized the involvement of the newly re-discovered HMGB1 protein in the interaction with HIV-1 both in in vitro and in vivo settings.

Paper I: Follow up of antiretroviral treatment in liver transplant recipients with primary and chronic HIV-1 infection

Background for the study

Introduction of ART has dramatically improved the prognosis of HIV-1 infected patients and has changed the picture of the disease course [95]. Progression to end-stage liver failure now frequently occurs in subjects co-infected with hepatitis C virus (HCV) [213]. This has raised again the issue of organ transplantation in such patients, however due to experience from pre-ART era, HIV-1 infection was considered a contraindication to organ transplantation in many transplant centres [214]. There have been several concerns:

1) Immunosuppressive treatment would lead to an increased viral replication and disease progression [215].
2) Drug interactions between the components of ART: protease inhibitors, nonnucleoside analogues and immunosuppressive compounds [216]. These drugs utilize similar cellular transport systems and the same metabolizing enzymes of the cytochrome P450. Concomitant usage of these agents would require careful monitoring.
3) The limited access to liver transplants made transplantation ethically questionable as long as the prognosis of the HIV-1 infection itself was bad [217].
Nevertheless, the increased need for transplants, accumulating information about the negative role of immune activation in HIV-1 pathogenesis as well as increased possibilities to perform therapeutic drug monitoring of the immunosuppressive and antiretroviral drugs resulted in the re-evaluation of transplant lists criteria. At Karolinska University Hospital, one of the first liver transplantations of an HIV-1 infected patient ever done was performed in 1998 and a further four patients were transplanted during the following years. In view of aspects discussed above, we decided to study how intensive immunosuppression in combination with potent anti-HIV therapy could influence the viral and immunological events in four patients after OLT.

Absence of increased HIV-1 replication and of increased viral evolution in patients under immunosuppressive treatment

Three HIV-1 infected patients (patients 1, 2, 3) with liver cirrhosis due to chronic HCV infection and one patient (patient P) with primary biliary cirrhosis (PBC) were followed up 3, 9, 14 and 36 months after OLT. All subjects were treated with both antiretroviral and immunosuppressive drugs. The PBC patient was ‘‘exceptional’’ as he developed PHI at the time of transplantation. We investigated the effect of the combined therapy on HIV-1 viral load (VL), viral diversity and divergence as well CD4+ and CD8+ T-cell counts.

Our data strongly support the pivotal role of immune activation in the HIV-1 pathogenesis. A detailed analysis of the viral events in our four patients showed that HIV-1 replication was efficiently inhibited by the potent ART without any significant support provided by the HIV-1 specific adaptive immunity. Thus, in the chronically infected patients, VL was undetectable (patients 1 and 3) or declined (patient 2) during post OLT follow up. The kinetics of VL in patient P (Figure 3, paper I) followed initially the pattern generally observed in PHI patients [82]. The peak of VL occurred 1 week after onset of symptoms and decreased thereafter, before the initiation of ART but under the immunosuppressive regime. It is thus unlikely that this initial viral decrease was due to effects of the adaptive immunity. We suggest that this decline was more likely due to an innate immune response or rather, as implied from the data of Paper IV, a decrease of immune activation and in consequence a lack of target cells for the virus. In opposite to the third phase of slower viral decline usually seen in PHI, the viremia increased in patient P. As similar VL kinetics were observed in CD8+ T-cell depleted SIV infected monkeys [215], the role of CTLs in controlling these early viral events could be considered [218]. In patient P, a decreased immune activation was not only restricting the access to target cells but also dysregulating appropriate immune response to the pathogen. The efficacy of ART was further illustrated by the gradual plasma HIV-1 RNA and cellular HIV-1 DNA decrease after treatment initiation.

Moreover, the restriction of the viral evolution in PHI and chronic HIV-1 infected patients, measured as viral diversity and divergence (Table 1, paper I), was similar to data reported from corresponding patient groups who did not received immunosuppression due to transplants [76, 219]. We did not observe a deterioration
of CD4+ T-cell counts in our patient group. On the contrary a stable increase was seen in 3 out of 4 individuals.
In conclusion, our data show that suppression of immune system in HIV-1 infected patients under effective HAART does not eventually lead to increase of viral replication and to rapid disease progression.

Papers II, III and V- The role of HMGB1 in HIV-1 infection.

Background for the study

We intended to characterise the function of the HMGB1 in HIV-1 infection by studying different aspects of the virus and protein interaction in vitro and by analysing plasma HMGB1 levels in HIV-1 infected patients.

HMGB1 is a remarkable molecule. Although, very abundant in all nucleated cells, the clear-cut intracellular function is still the matter of debate [159]. Based on sequence homologies, the DSP-1 protein in Drosophila has been suggested to be the equivalent of vertebrate HMGB1 [220]. DSP-1 can convert members of Rel family of transcription factors, NF-κβ and the Drosophila protein Dorsal, from being transcriptional activators to repressors [221]. Similar to DSP1, the HMGB1 can interact with Dorsal and Rel proteins: p50 and p65 [165]. Thus, cells express different amounts of HMGB1, which could be attributed to a cell specific modulation of transcription [159]. The data presenting HMGB1 interactions with viral transcription factors with stimulatory/inhibitory consequences have implicated its potential role during viral infections [201-203]. Moreover, the presence of NF-κβ binding sites, as well as cell specific differences in HIV-1 pathogenesis, has encouraged us to test the possibility of HMGB1 involvement in the intracellular modulation of HIV-1 transcription.

Reports showing that HMGB1 could be released to the extracellular environment and there act as a proinflammatory cytokine [190], brought to us another important facet of HMGB1 and HIV-1 interactions. An obvious question was if exogenous HMGB1 could act on HIV-1 infected cells and stimulate viral replication. Several proinflammatory cytokines can stimulate viral production from infected cells and contribute in vivo both to an increase of VL as well as immune activation and viral spreading [133]. The biology of HMGB1 could add an additional mechanism to immune activation in HIV-1 infection. Necrotic cell death present in HIV-1 infection [222] could liberate HMGB1 [174] and add an endogenous factor that will endeavour a vicious cycle of immune activation and viral replication. Elevated levels of HMGB1 have been shown in several conditions to be associated with acute and chronic inflammation/immune activation [190]. Activation of the immune system is a hallmark of HIV-1 infection correlating to disease progression [108]. The potential in vivo link between systemic levels of HMGB1, virological and immunological parameters of HIV-1 disease was therefore worth testing.
To test the effects of intracellular HMGB1 on HIV-1 replication, the full length HIV-1 infectious clone (pNL4-3) was cotransfected in the absence or presence of various concentrations of HMGB1 sense, HMGB1 antisense, or the empty pcDNA vector, into HeLa, Jurkat and Thp-1 cells. In HeLa cells, we observed inhibition of HIV p24 gag production from pNL4-3 in the presence of HMGB1 sense plasmid, at 24 hours post transfection. This inhibitory effect of HMGB1 was dose dependent with a maximum of five fold inhibition at ≥ 5µg of HMGB1 sense plasmid (Figure 1, Paper II). Moreover, this repressive effect was seen independently of hGH normalization. No inhibition of viral replication was observed in the presence of empty pcDNA or antisense HMGB1 plasmid indicating that the inhibitory effect was induced by ectopic production of HMGB1. In Jurkat cells, we could not observe any modulatory effects on viral replication with any of the transfected HMGB1 plasmids. This may suggest an inhibitory effect of HMGB1 through NF-κβ since there is no activated NF-κβ in the nucleus of Jurkat cells, in contrast to HeLa cells. Analysis of cell lysates, revealed that Thp1 cells carried more HMGB1 than the epithelial and T-cells. Therefore, transfection with HMGB1 sense plasmid did not have any effect on pNL4-3 replication. On the opposite, a two fold upregulation of viral replication was observed in the presence of 5µg antisense HMGB1 plasmid at ≥ 24 hours post transfection (Figure 6, Paper II).

**Intracellular HMGB1 represses HIV-1 LTR mediated transcription in HeLa cells (Paper II).**

To investigate whether the HMGB1 repressive effect was at the level of LTR-directed transcription, a CAT reporter gene construct containing HIV-1 LTR derived from subtype B virus (pNLCATB) was cotransfected in the presence or absence of sense and antisense HMGB1 plasmids. Ectopic overexpression of HMGB1 inhibited transcription directed from PNLCATB HIV-1 LTR (Figure 2, Paper II). Analogous effect was present when HeLa Tat cells were used as target for transfections. This inhibitory effect of HMGB1 was LTR specific in the sense that HMGB1 in contrast stimulated the transcription from SV40 promoter/enhancer. Additionally, HMGB1 had no substantial effect on CMV promoter. In accordance with these findings, HMGB1 has been shown to stimulate transcription from adenovirus major late promoter and the SV40 late promoter [201].

We further investigated a possible functional interaction between HMGB1 and NF-κβ in context of HIV-1 LTR. By deletion of the extra NF-κβ site (NF-κβI) present in the LTR of subtype C viruses, we could show a higher inhibitory activity of HMGB1 on the NF-κβI mutant subtype C LTR compared to the WT subtype C LTR. The interaction of HMGB1 with NF-κβ alone cannot be the only explanation for HMGB1 repression of HIV-1 gene expression because several numbers of NF-κβ sites are also present in the SV40 promoter/enhancer in which, in contrast to HIV-1 LTR is stimulated by HMGB1. It is generally acknowledged that transcription factors bind to each other in a three-dimensional jigsaw puzzle creating a multiprotein/DNA complex [159]. Thus, it can be speculated that a ternary complex forms between HMGB1, NF-κβ and another, yet unidentified repressor(s) which directly or
indirectly interact with specific element within LTR. Similar regulation of HIV-1 LTR transcription for other proteins interacting with NF-κβ site has been reported [31, 223-225].

**Extracellular HMGB1 upregulates HIV-1 replication in latently infected monocytic cells (Paper III)**

To investigate the role of extracellular HMGB1 on HIV-1 replication, we added recombinant HMGB1 to the U1 cell line, which is accepted as a model of latent HIV-1 infection in monocytic cells [226]. We could observe three fold increase of viral replication (measured as RT upregulation in supernatants) in HMGB1 stimulated cells as compared to controls (Figure 1, paper III). This effect was dose dependent and specifically linked to the protein as Polymyxin B was used to inhibit endotoxin contamination. Additionally, presumed amounts of LPS tested in 100 fold higher concentrations did not have stimulatory effect on HIV-1 replication in U1 cells. Experiments with dexamethasone inhibition of the stimulatory effect suggested that the downstream pathway of HMGB1 stimulation in U1 cells involves utilization of NF-κβ complex. HMGB1 did not have any effect on stimulation of viral replication in chronically infected T cells (ACH-2). This observation is in line with the initially suggested absence of stimulatory effect of HMGB1 on lymphoid cells [178]. Our findings implicate that HMGB1 could act as a proinflammatory cytokine on latently infected cells of monocytic origin. Recently published work by Thierry et al [227] has confirmed our findings in the U1 cell model. Moreover, their experiments with protein synthesis inhibitor cyclohexamide, revealed that HMGB1 acts directly on viral transcription, without additional stimulation of proinflammatoric mediators. The authors report also divergent finding concerning the effect of HMGB1 on HIV-1 replication in ACH-2 cells. One possible explanation of this apparent discrepancy might be different basal levels of viral expression as well different read-outs used in our experiments. Thus, this finding is surprising, as there is no information on direct effect of exogenous HMGB1 on function of T-cells. However, T-cells express RAGE receptor and the possibility of such interaction can not be dismissed.

**Extracellular HMGB1 represses HIV-1 replication in acutely infected macrophages (Paper III)**

Addition of HMGB1 to acutely infected (7 days) monocytes derived macrophages (MDM) resulted in inhibition of viral replication present already at 24 hours post HMGB1 stimulation (Table 1, paper III). This effect was similar in MDM cultured in adherent and non-adherent conditions. Analysis of the culture supernatants revealed HMGB1 induced release of RANTES, MIP-1α, and MIP-1β from MDM cells. These cytokines have been attributed the inhibitory effect, not only during HIV-1 entry, but also on viral replication in macrophages [228]. Thus, it is very likely that their anti-HIV-1 effect was exerted in the HMGB1 stimulated HIV-1 infected MDMs. Induction of MIP-1α and MIP-1β from monocytes by HMGB1 stimulation has been previously described [178]. Our data indicate that RANTES should also be included in this pattern. This double mode of action; suppressive in the acute model and stimulatory in the chronic model of HIV-1 infection is not unusual for the effects of
cytokines on HIV-1 replication. Similar properties have been documented for e.g. TNF-α, INF-γ and endotoxin [139, 228-231].

**Increased plasma levels of HMGB1 in HIV-1 infected patients (Paper V)**

In Paper V, we determined the levels of HMGB1 in plasma from 14 healthy HIV-1 negative volunteers and 43 treatment-naïve HIV-1 infected individuals using an ELISA system. Three patient-categories were defined based on immunological and virological parameters, as well as presence of opportunistic conditions at the time of blood sampling (Table 2).

**Table 2.** Characteristics of HIV-1 infected patients studied in Paper V.

<table>
<thead>
<tr>
<th></th>
<th>Group A (n= 14) Patients with CD4+ T-cells &gt;600/ul</th>
<th>Group B (n= 13) Patients with CD4+ T-cells &lt;400/ul</th>
<th>Group C (n=16) Patients with CD4+ T-cells ≤300/ul and opportunistic conditions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/Male, n</td>
<td>6/8</td>
<td>4/9</td>
<td>8/8</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>39 (27-66)</td>
<td>36 (24-53)</td>
<td>39 (23-62)</td>
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<tr>
<td>Transmission</td>
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<td>7</td>
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<td>2</td>
</tr>
<tr>
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<td>CD4+ T-cell count</td>
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</tr>
<tr>
<td>(cells/μl)a</td>
<td>(655-1 519)</td>
<td>(58-374)</td>
<td>(8-300)</td>
</tr>
<tr>
<td>CD8+ T-cell count</td>
<td>1165</td>
<td>815</td>
<td>667</td>
</tr>
<tr>
<td>(cells/μl)a</td>
<td>(429-3050)</td>
<td>(153-1498)</td>
<td>(88-1864)</td>
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<tr>
<td>HIV-1 RNA load</td>
<td>350</td>
<td>347 000</td>
<td>541 000</td>
</tr>
<tr>
<td>(copies/ml)b</td>
<td>(19-1 900)</td>
<td>(20 000-10⁶)</td>
<td>(8 900-10⁶)</td>
</tr>
</tbody>
</table>

*a*median (range) bIVDU, Intravenous drug users

*Opportunistic conditions in 16 HIV-1 infected patients (group C): Patient (P) 1: Pneumonia (bacterial); sepsicaemia; P2: Tuberculosis (TB) (disseminated); P3: B-cells lymphoma; P4: Oropharyngeal candidiasis; TB (lymph node); P5: Aspergillosis; TB (lymph node); P6: *Pneumocystis jiroveci* pneumonia (PCP); P7: TB (pulmonary); Oesophageal candidiasis; Toxoplasmosis; P8: Wasting syndrome; Oropharyngeal candidiasis; P9: Pneumonia (bacterial); P10: Oropharyngeal candidiasis; P11: TB (lymph node); P12: Oesophageal candidiasis; Kaposi’s sarcoma; Wasting syndrome; Neurosyphilis; P12: *Mycobacterium avium* complex infection (disseminated); Oesophageal candidiasis; Toxoplasmosis; PCP; P13-16: TB (pulmonary).
We found higher plasma levels of HMGB1 in the HIV-1 infected patients, as compared to HIV-negative healthy controls (p<0.001). Additionally, HIV-1 infected patients with opportunistic conditions had higher HMGB1 levels as compared to asymptomatic patients with preserved (p<0.05) or deteriorated immune system (p<0.05) (Figure 1, Paper V). There was no significant difference in HMGB1 levels between the two latter groups. No difference was observed in HMGB1 levels within the symptomatic patients with regard to specific opportunistic conditions. Additionally, no statistically significant correlation was found between the plasma HMGB1 levels and sex, age, viral load, CD4+ or CD8+ T-cell counts, respectively. Our results thus show that HMGB1 can be detected in the circulation of chronically infected HIV-1 individuals with the highest concentrations in patients with clinical complications. The data could support the view that a profound activation of immune system is related to the high HMGB1 levels. In addition, cytopathic effects of HIV-1 may contribute to the enhanced HMGB1 levels as suggest our unpublished data derived from in vitro system (Figure 5).

The levels of HMGB1 found in HIV-1 infected patients were comparable to those in patients with disseminated intravascular coagulation or with acute pancreatitis [194, 196]. The clinical relevance of the high systemic HMGB1 levels remains to be elucidated. The work showing existence of anti-HMGB1 antibodies not only in patients with autoimmune diseases but also in healthy individuals [198], implicate that the regulation of HMGB1 function is very complex [177].

**Paper IV. Kinetics of plasma cytokines and chemokines during PHI and after analytical treatment interruption.**

_Rationale for the study_

Although systemic immune activation is an important pathogenic factor in chronic HIV-1 disease, its role in primary HIV-1 infection (PHI) is poorly defined [108]. However, the events occurring during this early phase of HIV-1 infection are critical for disease prognosis [81, 110]. Thus, a functional impairment of the cellular response early in the course of infection has provided a rationale for the initiation of ART during acute infection to preserve HIV-1 specific T cell responses and decrease immune activation [232]. Cytokine imbalances play a key role in HIV-1 pathogenesis through the upregulation of viral replication and immune activation [136]. There is a lack of comprehensive data about the kinetics of these soluble factors during PHI. A better understanding of their contribution to the immune activation during PHI could help develop novel treatment strategies.

In this study, we analysed the pattern of immune activation by measuring the kinetics of 13 cytokines and chemokines in 22 patients during PHI and after analytical treatment interruption (ATI). Patients enrolled in this sub-study were participants of the QUEST study, the first larger controlled treatment trial in acute HIV-1 infection [233]. It was also the first clinical trial of therapeutic vaccination [234]. The Quest cohort was very unique as the patients were enrolled very early after exposure to HIV-1. The first sample was drawn before a complete antibody pattern was
developed (as identified on the Western blot), corresponding to two-three weeks after HIV-1 infection. The selection of the patients, in our substudy, was based on the virological outcome at the end of the study as well as availability of plasma samples. The individuals were divided into two groups: further referred as responders (R) who had VL levels <1000 HIV-1 RNA copies/ml and non-responders (NR) who had VL >9000 copies/ml at 6 months after treatment interruption [235]. The ART was initiated very early during the PHI and lasted at least for 96 weeks for all subjects.

Intensive immune activation during PHI is associated with worse virological outcome after analytical treatment interruption

During PHI, the NR had significantly higher levels of HIV-1 RNA, IFN-γ, TNF-α, IL-1β, IL-10 and Eotaxin than the R (p≤0.05). Furthermore, a positive correlation was found between VL and IFN-α, TNF-α, IL-1β, MIP-1α and MIP-1β, respectively. On the contrary, R had significantly higher levels of IFN-γ, MIP-1β and MCP-1 than NR after ATI, while NR had higher levels of HIV-1 RNA, IL-15 and Eotaxin (Figure 7). Comparison between the cytokine/chemokine concentrations during the highest viraemia point of both phases showed significantly higher levels of IFN-γ, TNF-α, IL-10 and chemokines (with exception of MCP-1) during PHI than post-ATI.

The data describe different cytokine profiles during PHI and post ATI. All subjects showed an intense state of immune activation during PHI, the degree of which correlated with the VL, and was higher in the non-responders. Thus, subjects who had lower levels of VL and of all soluble factors at PHI (with the exception of MCP-1) also had lower VL after ART cessation. It implicates that the problem during PHI is not a lack of certain immune mediators as seen in other infections [236-238]. The profound activation of immune system is likely to be caused primarily by the extensive viral replication during PHI [81]. The question remains to be answered if and which genetic factors [239] determine the lower degree of immune activation in R.

This lower degree of immune activation might cause better preservation of the innate and adaptive immunity in those individuals. Betts et al has recently [240] suggested that presence of polyfunctional CD8+ T-cells characterized by strong cytokine (INF-γ, TNF-α, IL-2) and chemokine (MIP-1β) production is negatively correlated with disease progression. Our data from the plasma compartment are in line with this hypothesis.

In conclusion, our work adds some important information in terms of the pattern of cytokine and chemokine production during early HIV-1 infection and after cessation of ART. We have shown that, the degree of immune activation was highest during PHI and associated with VL levels. We also showed that lower levels of viral replication and immune activation during PHI are associated with improved long-term virological outcome. Further investigations are required to clarify the role of ART in the change of cytokine pattern observed after treatment interruption and its potential impact in the long-term virological outcome.
Figure 7. The graph show the comparison between the area under the a) INF-γ curve and b) IL-15 curve during the post ART phase in responder and non-responder group. * P<0.05
Concluding remarks

1) Potent antiretroviral therapy without any support from concomitant HIV-1 specific adaptive immunity was sufficient to efficiently control HIV-1 replication in patients after OLT. Chemotherapy induced suppression of the immune system under successful ART did not lead to an increase of viral sequence evolution or a more rapid disease progression.

2) Endogenous HMGB1 expression represses HIV-1 LTR-directed transcription in a promoter and cell specific manner. Constitutive higher expression of HMGB1 in monocytes/macrophages could be responsible for slow mode of HIV-1 replication in these cells.

3) Extracellular HMGB1 induces HIV-1 replication in latently infected monocyctic cells but not T-cells. The opposite effect of exogenous HMGB1 stimulation is seen in acute infected primary macrophages. The inhibition of HIV-1 replication in these cells could be explained by induction of β-chemokines production by HMGB1.

4) Plasma levels of HMGB1 are elevated in HIV-1 infected patients, with the highest concentration in patients with clinical complications. HMGB1 is likely to contribute to the activation of the immune system in HIV-1 infection.

5) High levels of immune activation during PHI are associated with high HIV-1 RNA levels rather than better control of the viral replication. Additionally, the degree of immune activation during PHI may determine the functionality of immune system as well as the virological outcome post-analytical treatment interruption. This interaction does not seem to be affected by early antiretroviral therapy.
**Populärvetenskaplig sammanfattning**

En abnorm aktivering av immunsystemet är central för sjukdomsutvecklingen vid HIV-infektion och AIDS. Denna leder till ett oproportionellt kraftigt och missriktat immunsvar och till att specifika celler i immunförsvaret dör, vilka också angrips av HIV. Hypotesen är att HIV leder till en kronisk inflammation med ett inadekvat immunsvar som förklarar det avvikande immunologiska och virologiska förloppet. Cellulära immunaktivering markörer samt cytokin nivåer ökar under sjukdomen, och sjunker under effektiv HIV behandling. Frånvaro av immunaktivering samt sjukdomsutveckling hos apor som är naturliga värdar av aviruset SIV och som inte blir sjuka förstärker ytterligare denna teori.

Huvudsyftet med denna avhandling var att karakterisera immunaktiveringen hos HIV-infekterade patienter och kartlägga betydelsen av proteinet ”high mobility group box protein 1” (HMGB1) i denna process. Därför har jag studerat: a) två unika patient kohorter. Den ena bestod av fyra patienter som genomgått levertransplantation och som behandlades med immundämpande läkemedel samt anti-HIV läkemedel. Den andra bestod av 22 patienter med HIV-infektion i tidig fas som fick anti-retroviral behandling och genomgick så kallad ”analytical treatment interruption”; b) interaktioner mellan HIV-1 och HMGB1 in vitro och in vivo.

Den antiretrovirala terapin som de HIV-infekterade levertransplanterade individerna erhöll var effektivt och ledde till odetekterbara virusnivåer samt få virusmutationer. Dessutom tydde förändringarna av virusnivåerna under den tidiga fasen av HIV-infektionen hos en av dessa patienter på att den initiala virus minskningen i plasma sannolikt var orsakad av det medfödda immunförsvaret eller reducerad immunoaktivering. I gruppen med 22 patienter med HIV-infektion i tidig fas var låg immunoaktivering korrelerat till lägre virusförökning samt bättre virus kontroll 2 år senare. Vi kunde också bekräfta att primär HIV-infektion karakteriseras av generaliserad immunoaktivering.

HMGB1 är ett protein som finns i cellkärnan i alla kroppens celler. Detta protein verkar både intra- och extracellulärt. Extracellulärt HMGB1, fungerar som en proinflammatorisk cytokin och kan stimulera ett stort antal celler. Proteinet har sannolikt en central roll i inflammatoriska reaktioner vid ett stort antal sjukdomstillstånd. Våra data visar att HMGB1 har stor betydelse vid HIV-infektion. Intracellulärt HMGB1 hämmade HIV’s förökning i vissa cellslag, bland annat monocyter. Vår tolkning är att en hög produktion av HMGB1 i denna celltyp leder till en låggradig virusförökning i cellerna och därmed utgör de en viktig reservoar för HIV-1. Vi kunde dessutom visa att HMGB1 tillsatt extracellulärt kunde modulera HIV’1s förökning i två cellsystemsmodeller för kronisk och akut HIV infektion. Sålunda ökade virusföröknings i celler (U1 celler) som är en modell för latent, sovande, HIV-1 infektion och hämmade HIV-1 i akut infekterade makrofager. Den senare effekten kunde förklaras av att vissa signalmolekyler (B-kemokin; RANTES, MIP-1a, MIP-1b) utsändes efter HMGB1 stimulering. Vi har också visat att HIV infekterade patienter har förhöjda plasma nivåer av HMGB1 med högsta koncentrationerna hos personer med kliniska komplikationer.
Sammanfattningsvis, denna avhandling betonar vikten av immunoaktivering i sjukdomsutvecklingen vid HIV-infektion. HMGB1 är en viktig faktor i patogenesen. Proteinet påverkar HIV-förökningen samt leder till att immunsystemet inte fungerar adekvat. Fortsatta studier bör utvärdera om anti-HMGB1 läkemedel kan bidra till en normalisering av det hyperaktiva immunsystemet vid HIV infektion.
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