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HMGB1 AND OTHER SOLUBLE FACTORS IN HIV-1 PATHOGENESIS

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Knowledge is the breath of the soul
The Culture is the life of the human beings
He, who does not love them,
Is like the dead without a soul.

Wisdom is stronger than weapon
Knowledge is better than mammon
A wise little child
Is better than an old foolish king.

Saint Aphrem,
A great teacher of the Syrian Orthodox Church
303-373 AD

To my dear Family

ABSTRACT

The innate immune system is the first defense mechanism invading pathogens encounter. Macrophages, dendritic cells and cytokines/chemokines are important factors for the functionality of the innate immunity, and provide the adaptive immune system with sufficient signals for the proper action. Immune activation occurring during HIV-1 infection is essential to understand and investigate.

The aims of this thesis were to evaluate the role of immune activation factors of the innate immune system, such as cytokines and chemokines, in different sets of patient categories, cellular systems and phases during the HIV-1 infection. We were most interested in a particular cytokine, the high mobility group box protein 1 (HMGB1). In addition to our in vitro experiments, we had access to an unique cohort, the Quest study, which is the first placebo controlled treatment trial in acute HIV-1 infection. For our sub-study, we selected 22 patients, categorized into responders and non-responders regarding the outcome of their viral load after analytical treatment interruption (ATI). We found that high levels of immune activation as determined by the pattern of cytokines/chemokines during PHI, did not favor a better virological outcome after ATI. The early initiation of ART did not seem to affect the preservation of the immune system.

HMGB1 is a proinflammatory cytokine, ubiquitously expressed in all nucleated cells, with a functional importance as a regulator of transcription and stabilization of the nucleosomal structure. HMGB1 is actively secreted from LPS- or TNF-activated macrophages/monocytes, pituicytes and other cells. HMGB1 can also be passively released by damaged necrotic or apoptotic cells. We studied the role of HGMB1 in different systems and modes during HIV-1 infection. Extracellular HMGB1 upregulated HIV-1 infection in latently infected U1 monocytic cells, but did not have impact on viral replication in ACH-2 T-lymphocytic cells. In acute HIV-1 infected monocyte-derived macrophages (MDMs), HIV-1 production was downregulated, most likely due to the increased production of the chemokines MIP-1α, MIP-1β and Rantes. Furthermore, higher levels of HMGB1 were found in HIV-1 infected patients with deteriorated immune status and opportunistic conditions, compared to uninfected individuals and HIV-1 infected patients with less preserved immune status. Additionally, HMGB1 was released by HIV-1 infected MT4 cells and CD4+ T-cells, in connection with virus induced cell death. This release could be interfered by addition of a pan-caspase inhibitor Z-vad to MT4 cells cultures. We suggest that HMGB1 was released passively from MT4 cells not only during necrosis but also during apoptosis.

In conclusion, the presented data cast a light on the importance of the immune activation process during HIV-1 pathogenesis. HMGB1 is released during the viral cell infection and may be a molecule connecting the cell death processes and the immune activation during HIV-1 infection.

Keywords: HIV-1, PHI, cytokines, chemokines, immune activation, HMGB1, cell death

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LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to by their roman numbers [I-IV].

I. Nowak P, Barqasho B, Treutiger CJ, Harris HE, Tracey KJ, Andersson J, Sönnerborg A
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

II. 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

III. Barqasho B, Nowak P, Tjernlund A, Kinloch S, Goh L-E, Lampe F, Fisher M, Andersson J, Sönnerborg A
Kinetics of plasma cytokines and chemokines during primary HIV-1 infection and after analytical treatment interruption.
HIV Medicine. Accepted for publication 2008

IV. Barqasho B, Nowak P, Walther-Jallow L, Sönnerborg A
Release of HMGB1 during HIV-1 infection in-vitro; implications on the cell death mode.
In manuscript 2008

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen-presenting cells
ART	Anti Retroviral Therapy
ATI	Analytical Treatment Interruption
CD	Cluster of determination
CCR	CC chemokine receptor
CTL	Cytotoxic T Lymphocyte
CXCR	CXC chemokines receptor
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
HAART	Highly Active Anti-Retroviral Therapy
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HMGB-1	High Mobility Group Box protein 1
HTLV	Human T-lymphotropic virus
IL	Interleukin
IFN	Interferon
IVDU	Intravenous drug user
LDH	Lactate Dehydrogenase
LTNP	Long Term Non Progressor
LTR	Long Terminal Repeat
MCP	Monocyte Chemotactic Protein
MDM	Monocyte Derived Macrophages
MIP	Macrophage Inflammatory Protein
mRNA	Messenger RNA
M-tropic	Macrophage tropic
NF-κβ	Nuclear Factor Kappa B
NK	Natural Killer Cell
NSI	Non syncytium inducing
PBMC	Peripheral Blood Mononuclear Cell
PHI	Primary HIV-1 Infection
RAGE	Receptor for Advanced Glycation Endproducts
RANTES	Receptor on T cell Activation, Normal T cell Expressed and Secreted
RNA	Ribonucleic acid
RT	Reverse Transcriptase
SI	Syncytium inducing
SIV	Simian Immunodeficiency Virus
Th1/Th2	T helper cell type 1/2
TLR	Toll-like receptor
TNF	Tumour necrosis factor
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organization
WB	Western Blot
Zvad-fmk	Val-Ala-Asp-fluoromethyl-ketone

1 General Introduction Human Immunodeficiency Virus

1.1 Background

The HIV pandemic remains as one of the most serious challenges to public health. The estimated number of HIV-1 infected individuals and deaths due to AIDS since the beginning of the pandemic exceeds 60 and 25 million people, respectively [1]. Every day, over 6800 persons become infected with HIV and over 5700 persons die from AIDS, mostly because of inadequate access to HIV prevention and treatment services. Recent global data confirms the disproportionate impact of HIV/AIDS on Sub-Saharan Africa [1]. Although HIV-1 has caused many deaths, we have gained remarkable knowledge when studying the HIV-1 disease, both for the HIV pathogenesis and immune system interactions. Despite these advances, no effective HIV-1 vaccine has been developed yet, and new treatment strategies are needed in order to induce long-term remission or even cure of the HIV-1 infection.

1.2 The HIV pandemic

In early 1980's, the first cases of opportunistic infections as Pneumocystis carinii pneumonia (PCP) and Kaposi's sarcoma (KS) among previously healthy young homosexual men in the United States [2-4] were reported. Later on, heterosexual Haitian immigrants [5], intravenous drug users (IVDU) [6] and haemophiliacs [7] began to present similar symptoms. Laboratory analysis identified low ratios of CD4+ T-cells to effector cells (CD8+) in the blood, which was believed to account for the deficiency of the immune system. In 1982, the phenomenon had become known as the acquired immune deficiency syndrome (AIDS) and the number of reported cases had increased worldwide.

1.3 The discovery of HIV

In 1983, Luc Montagnier and co-workers at the Pasteur Institute in Paris, reported the isolation of a retrovirus from a patient with persistent multiple lymphadenopathy, and the virus was given the name Lymphadenopathy-associated virus (LAV) [8]. Another group, under the guidance of Robert Gallo

at the National Cancer Institute in Bethesda, Maryland, also made the discovery of a retrovirus from a patient with PCP suffering from advanced AIDS. Gallo went on and named the virus human T-cell lymphotropic virus type III (HTLV III) [9]. Consequently, in 1985 the term human immunodeficiency virus (HIV) had been coined by the International Committee on Taxonomy of Viruses (ICTV) [10] to denote the similarity with other members of the Retroviridae family. The French researchers, Luc Montagnier and Françoise Barre-Sinoussi, were rewarded with the Nobel Prize in physiology and medicine in year 2008 for the discovery of HIV-1.

Two related viruses were also discovered in 1986: The HIV-2 in Western Africa and the simian immunodeficiency virus (SIV-1), which cause AIDS-like symptoms in rhesus macaque monkeys. In the natural hosts of SIV (e.g. sooty mangabeys, African green monkeys and chimpanzees), the virus does not cause AIDS even in the presence of high viral loads. The former strain of HIV was referred to as HIV-1. Epidemiological studies show that HIV-2 is less contagious, the time to develop AIDS is longer and the morbidity is lower as compared to HIV-1 [11].

The earliest documented case of HIV-1 infection in humans was identified in a frozen plasma sample from 1959, collected in the Democratic Republic of Congo, Kinshasa [12]. It is suggested that the virus likely entered the human population through zoonotic transmission from chimpanzees in Central Africa [13].

1.4 HIV-1 structure

1.4.1 Morphologic structure

HIV-1 is a member of the Lentivirinae genus in the Retroviridae family. Each free virus particle (virion) is of a spherical shape with a diameter of approximately 110 nm [14, 15] and consists of a capsid protein core (CA; p24) surrounded by a host-derived lipid bilayer membrane (envelope). The envelope is equipped with trimers of the surface glycoprotein called gp120 and the transmembrane glycoprotein gp41 that protrude from the membrane. The matrix protein (MA, p17) lines the inner surface of the viral envelope,

surrounding the nucleocapsid complex [16]. The cone shaped capsid core contains two copies of single stranded RNA of approximately 9.2 kbp which is flanked by long terminal repeats (LTR) on both sides and tightly bound to the nucleocapsid (NC; p7). The virus capsid also contains several proteins that are required for infecting the host cell and for early stages of viral replication; reverse transcriptase (RT; p51/66), enzyme integrase (IN; p32), and protease (PR; p15). The morphologic structure is illustrated in Figure 1.

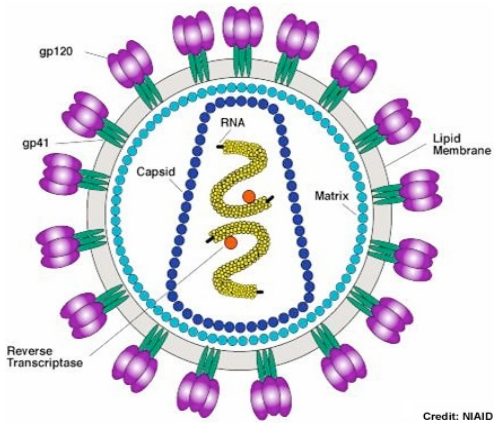

Figure 1: The morphologic structure of HIV-1.

1.4.2 Genetic structure

The HIV-1 genome is composed of three structural genes that are common to all retroviruses; *gag*, *pol* and *env* (Figure 2). HIV-1 *gag* codes for the major structural proteins; nucleocapsid (p9 and p7), capsid (p24) and matrix proteins (p17). The HIV-1 *pol* gene encodes for reverse transcriptase (RT), integrase and protease, which are essential enzymes for transcription of viral RNA to DNA, integration of viral DNA into the human genome and cleavage of HIV-1 proteins, respectively. HIV-1 *env* codes for the envelope glycoproteins (gp120 and gp41) that are critical for viral attachment and hence infection of the target cell [17].

The HIV-1 provirus contains six additional open reading frames encoding the regulatory proteins Tat and Rev, and the accessory proteins Nef, Vif, Vpr and Vpu [18]. The accessory proteins are thought of as supplementary proteins since they are not needed for viral production although they facilitate it. The

regulatory proteins Tat and Rev are involved during transcription and regulation of the viral mRNA expression, respectively.

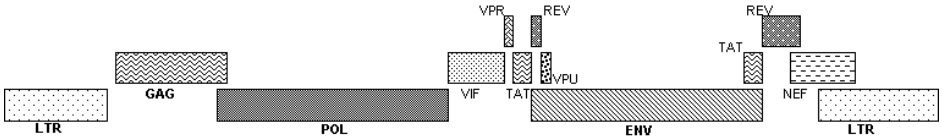

Figure 2: The genomic structure of HIV-1.

1.5 HIV-1 viral life cycle and replication

The HIV-1 life cycle begins when the viral envelope glycoprotein gp120 attaches to host CD4-expressing cells [17], including T-lymphocytes, monocytes, macrophages, dendritic cells and microglia cells [19-22] that circulate in the bloodstream (peripheral blood lymphocytes) or lymphatic system (Figure 3). To complete the binding, gp120 interacts with a co-receptor, usually the chemokine receptor CCR5 and/or CXCR4 [23]. HIV-1 isolates that utilize CCR5 to enter the target cell are referred to as non syncytium inducing (NSI) and R5-using isolates, while CXCR4 using isolates are called X4 isolates and syncytium inducing (SI) [24-26]. The interaction between gp120 and the co-receptor results in a conformational change of the gp41, which leads to the fusion between the virus envelope and the cell membrane [27]. After the fusion, the virus enters the cell cytoplasm and is unpackaged and the nucleocapsid is dismantled. The viral RNA genome is reverse transcribed by RT to obtain a double stranded DNA copy. The proviral DNA is integrated in the target cell genome in the form of a preintegration complex [28] by the viral enzyme integrase [29]. The transcription of the integrated proviral DNA is usually initiated immediately, but in some cells it can remain dormant for a long period of time, until the cell is activated [30]. The cellular activation starts transcription, and the integrated provirus is copied into mRNA, which are then transported out of the nucleus into the cytoplasm [31]. After translation, the viral mRNA and proteins assemble at the cell membrane, which in turn is modified by insertion of gp41 and associated gp120 [32]. The budding process starts, where the

modified host plasma membrane is acquired as the viral envelope [33]. During this process the viral gag and gag-pol polyproteins are cleaved by the viral protease enzyme, generating mature infectious particles [34]. Due to the high replication rate [35, 36], lack of proof reading activity of the RT enzyme [37, 38] and recombination events [39] the replication process is very error prone. The result is the occurrence of a swarm of viral variants called quasispecies, which allows the virus to evade neutralizing antibodies and also develop resistance to pharmacological therapies.

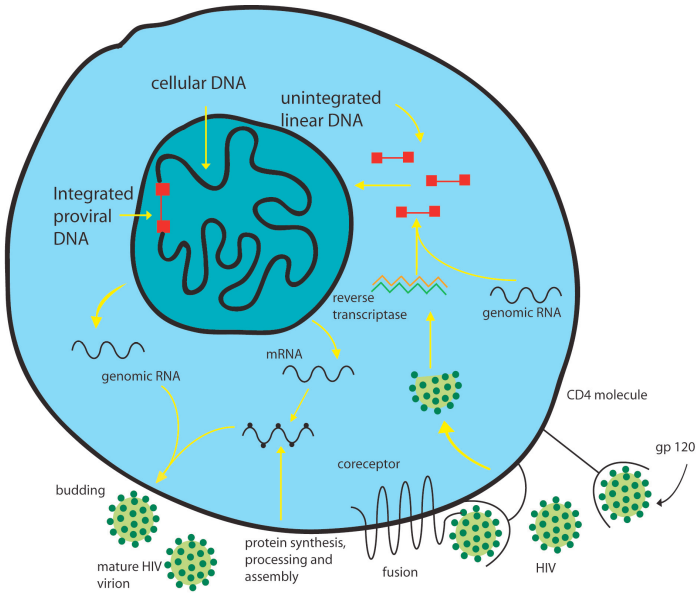

Figure 3: A schematic illustration of the HIV-1 replication cycle during infection of a human cell. Entry, reverse transcription, nuclear entry, integration, transcription, translation, virus assembly and budding are shown. Adapted from [40].

1.6 Transmission and pathogenesis of HIV-1 infection

The probability of HIV-1 transmission depends on the infectiousness of the source individual and the susceptibility of the exposed person [41]. HIV-1 can be transmitted horizontally via sexual contact and via any infected body substance that comes into contact with mucous membranes, non-intact skin or the bloodstream. It can also be transmitted vertically from mother to child during pregnancy, delivery and breastfeeding [42-44].

Infection with HIV-1 initiates series of host-virus interactions, leading to a profound impairment of the immune system. The natural history of HIV-1 infection can be divided into three phases; primary (PHI) or acute phase, the chronic phase and the final stage of AIDS (Figure 4). Infected individuals can either develop symptoms the first weeks after exposure to HIV-1 or have an asymptomatic infection. Symptomatic PHI infection is characterized by fever, sore throat, enlarged lymph nodes, rash and lethargy, which can persist for one to two weeks**.** The plasma levels of viral RNA are very high, while antibodies against HIV-1 are not detected initially. Seroconversion usually occurs within a few weeks after the onset of the acute illness. As the immune response against HIV-1 develops, the viral load dramatically decreases, reaching the viral steady state three-four months after HIV-1 infection [45]. This plateau is individual and determines the rate of disease progression [46-48].

The chronic phase of HIV-1 infection is generally a symptom-free period that usually last several years [49-51]. During this phase a gradual loss of CD4+ T-cells is observed, and the virus is replicating continuously, although the levels are lower than during the acute phase. Eventually the dysfunction of the immune system (CD4+ T-cells <200/μl) and the appearance of various opportunistic infections, e.g. Kaposi's sarcoma, lymphoma, tuberculosis, PCP, and neurological disorders, characterize the final stage of the HIV-1 infection, AIDS [52, 53].

A subset of HIV-positive individuals termed as long-term non-progressors (LTNPs) [54, 55] maintain high CD4+ T-cell counts and low viral load, and remain therapy naive and persistently infected with HIV-1 for more than 15 to 20 years. It is believed that combination of host genetic factors [56] influencing the innate and adaptive immunity results in the control of the infection in these subjects.

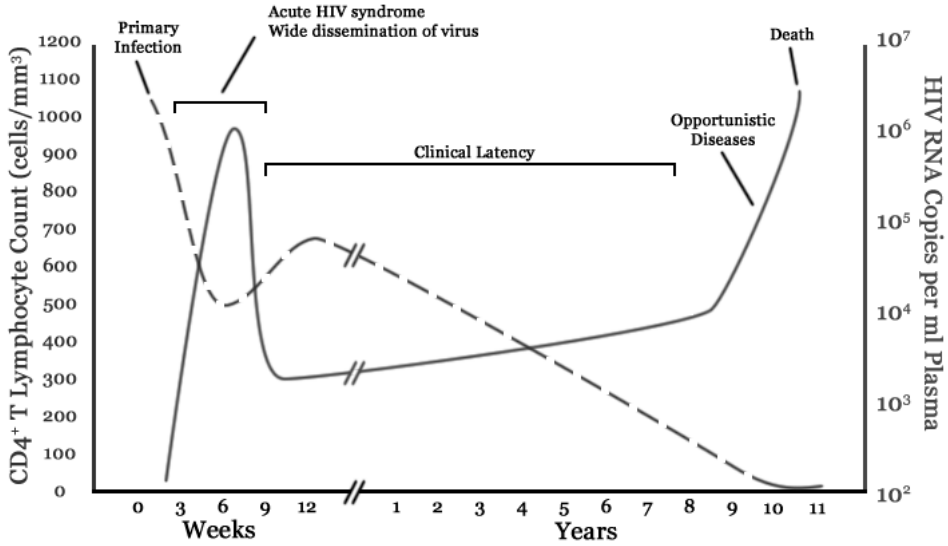

Figure 4: Illustration of the natural course of HIV-1 infection showing viral load (solid line) and CD4+ T-cell counts (dashed line).

1.7 Treatment of HIV-1 infection

1.7.1 Treatment drugs and strategies

Despite the continuing advances in the treatment of HIV-1 infection, there is still no cure. Efficient antiretroviral therapies (ART) are costly, and are still out of reach for the majority of the infected population living in developing countries.

The first developed agent against HIV-1 was zidovudine (ZDV), a nucleoside analogue reverse transcriptase inhibitor (NRTI) that interferes with the RT enzyme activity during the HIV-1 replication in the host cell. A typical treatment combination consists of two NRTIs and one protease inhibitor (PI) or an NNRTI (non- nucleoside analogue reverse transcriptase inhibitor). Drugs that hamper the viral entry or the integrase activity, have recently been introduced [57, 58]. HAART has significantly delayed the HIV-1 disease progression [59-61], morbidity and mortality [62], even though replication-competent viruses persist under therapy in an integrated latent form in several cell types and anatomical sites [63, 64]. Although HAART is beneficial to most HIV-1 infected patients, individual differences affect the outcome of the treatment, such as selection of multi-resistant HIV-1 strains and side effects. HIV-1 is able to quickly create drug resistant mutants due to its high error rate, combined with its enormous virion turnover rate. This highlights the importance of careful monitoring of antiretroviral combination therapy. Side effects vary from different drugs and

can give nausea, headaches, neuropathy/myopathy, gastrointestinal intolerance, diabetes mellitus, and lipodystrophy [65, 66] but are less frequent with the newer generation of drugs.

Although HIV treatment has improved since the introduction of ART, the only way to stop the epidemic is a protective vaccine. The properties of HIV-1infection with remarkable viral diversity, early establishment of latent reservoirs and lack of effective immunity in infected host makes this effort difficult. In the past few years, several different vaccine candidates (recombinant proteins, synthetic peptides, HIV-1 peptides or lipopeptides, viral or bacterial vectors expressing HIV-1 antigen(s), DNA vaccines and virus-like particles) were tested. The latest vaccine project included an adenovirus type 5 (Ad5)-based vaccine regimen, developed by Merck. It failed to either prevent the HIV-1 infection or suppress viral load in the STEP human phase 2b trial [67, 68]. An optimal vaccine should stimulate the innate immunity, generate neutralizing antibodies and induce HIV specific polyfunctional (IL-2 and IFN-γ) CD4+ and CD8+ T-cell responses [69]**.** Most vaccines were designed to stimulate strong cell-mediated immune responses to HIV-1 [70-72].

1.7.2 Discontinuation and interruption of HAART

In the vast majority of individuals, discontinuation of HAART results in rapid virological relapse or rebound of viral replication [36, 73-75]. This strategy, also called structured treatment interruption (STI) was introduced in the beginning of the 2000 [76, 77] due to long-term side effects. STI was thought to stimulate the immune response (compared to “booster inoculation”) which then would be capable to control the viral replication. However, studies as the SMART-trail demonstrated increased risk of opportunistic diseases and death with cessation of therapy as compared to continuous ART [78].

2 The influence of the immune system

2.1 Background Immune response

The host immune system consists of two major defence pathways: the innate and adaptive immunity, each with a different function and role. The innate immune system, the initial response of defence, is rapid with absence of memory and recognizes the pathogens by pattern recognition receptors (PRRs) by unique surface components on the microbial (called pathogen-associated molecular patterns [PAMPs]), rather than by a specific sequence. PAMPs can include lipopolysaccharide (LPS), peptidoglycan and lipotechoic acids, the sugar mannose, bacterial and viral unmethylated CpG DNA, bacterial flagellin, the amino acid *N*-formylmethionine found in bacterial proteins, double-stranded and single-stranded RNA from viruses, and glucans from fungal cell walls.

The components included in the innate immune system consist of a number of different cell types; dendritic cells (DCs), natural killer cells (NKs), γδ T-cells, macrophages, monocytes, neutrophils, and of soluble components; mannose-binding lectin (MBL), members of the complement system, cytokines, chemokines and defensins. These factors create a network with a capacity of limiting or even eradicating the spread of pathogenic activity, and also to provide the stimuli and the time for the adaptive immune system to react [79]. From the innate immune system, the more sophisticated adaptive immune system has evolved [80]. The adaptive (acquired) immunity refers to antigen-specific defence mechanisms that take several days to become protective and are designed to react with and remove a specific antigen. There are two major types of cells that are responsible for the specificity exhibited by the adaptive immune system: the immunoglobulin (Ig) secreting B lymphocytes, which synthesize and secrete antibodies with specificity against foreign substances, i.e. humoral immunity. The other subset is the T lymphocytes, which conduct the cellular immunity, involving the production of cytotoxic T-lymphocytes (CTLs), antigen-presenting cells (APCs) such as macrophages and dendritic cells, activated macrophages and NK cells, and cytokines in response to an antigen. The T-cells interact with the B cells. Adaptive immunity usually improves upon repeated exposure to a given infection.

2.2 Immune response against HIV-1

2.2.1 Innate immune response against HIV-1

The host defence systems must be able to recognize both free infectious virus and virus-infected cells to prevent and control infection. The response must be rapid, and have the ability to inactivate and prevent the spread of HIV-1 into other cells. Both the innate and adaptive immune responses have a central importance in the virus control with humoral and cellular immune activities. Here I will focus on the innate immune response against HIV-1. The advantage with innate immunity is the lack of being virus specific, and therefore can respond to a wide variety of HIV isolates. Resistance to this kind of immunity has not been described.

Recently, HIV-1 infection was suggested to trigger immune mechanisms that are normally associated with innate immune responses, not only during early phases of infection but also through the course of the chronic phase [81]. DCs, NK cells and a special subset of CD8+ T-cells play an important role in innate immunity. There are two subsets of DCs; myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs produce IL-12, which is important to induce cell mediated Th1 like immune response [82]. The pDCs are found in CD4-rich regions of lymphoid tissues and in low numbers in the blood. They produce IFN-α and IFN-β [83-85], which among other effects can inhibit HIV-1 replication and activate NK cells [86, 87]. pDC levels are reduced during HIV-1 infection, particularly in patients who have progressed to AIDS. In LTNPs the levels of pDCs are higher than in healthy controls [88], suggesting that pDCs might play a role in restricting disease progression.

NK cells have an important part in destroying HIV-1 infected cells, and their function depends on efficient production of cytokines and chemokines such as IFN-γ, IL-12, TNF-α, MIP-1α, MIP-1β and Rantes. NK cells can kill virus infected cells directly or by antibody dependent cellular cytotoxicity (ADCC). Other cells that play a role in the innate response toward HIV-1 are $\gamma\delta$T-cells. They are able to lyse virus infected cells and produce β-chemokines [89].

Immunity to HIV-1 has been reported in African sex workers who have remained HIV-1 negative despite frequent high-risk behavior [90, 91]. In some

high-risk uninfected subjects, nontoxic HIV-1-specific CD8+ T cell immune response has been correlated with resistance to infection [92].

2.2.2 Soluble components

Several circulating components of the innate immune system can have anti-HIV activity. The mannose-binding lectin (MBL) attaches to HIV and can enhance its engulfment and destruction by macrophages or, in the presence of complement, directly lyse the virus [93-96]. As HIV-1 uses chemokine receptors on cells to enter and infect, soluble factors as chemokines and cytokines play an important role in controlling the homoeostasis during HIV-1 infection. Infection with HIV-1 results in a dysfunctional network of the cytokine profile in vivo and in vitro (reviewed in [97]). The cytokines and chemokines can have inhibitory, stimulatory or bifunctional effect on the HIV-1 replication. The HIV-suppressive cytokines are IFN-α, IFN-β, IL-16, IL-10 and IL-13 and inhibit HIV-1 replication in e.g. T-cells and Macrophage Derived Monocytes (MDMs). The proinflammatory cytokines, such as TNF-α, IL-1 and IL-6 have been shown to enhance HIV-1 replication through NF-κB activation and upregulation of CCR5 expression. IFN-γ is a byfunctional cytokines, meaning it has both stimulatory and inhibitory effects on HIV-1. The α-chemokine SDF-1 exerts its inhibitory effect via binding to the co-receptor CXCR4 and thus does not affect infection by M-tropic strains of HIV-1. The other subset of chemokines is referred to as the β-chemokines and includes MIP-1α, MIP-1β, Rantes and Eotaxin. They are natural ligands for the CCR5 co-receptor and have been suggested to compete with or block HIV-1 entry, respectively [23, 98, 99].

2.3 Immune activation

In the danger model, immune activation is the result of recognition and clearance of an intruding pathogen in the host. Also, HIV-1 infection results in remarkable activation of immune system that eventually leads to the loss of immune control. The question is what is driving the chronic immune activation in HIV infection; does the virus drive the immune activation, is the immune activation driving viral replication, or both. How do the host genetics interfere?

Chronic immune activation include polyclonal B-cell activation [100], increased T-cell turnover [101], increased frequencies of T cells with an activated phenotype [102], and increased serum levels of proinflammatory cytokines and chemokines [103]. Thus, through immune activation, HIV-1 generates its own substrate for replication. The inability of the immune system to clear the HIV-1 infection, leads to a constant exposure of viral antigens to the immune system and contribute to the chronic activation observed in HIV-1 infected patients. The simian immunodeficiency virus (SIV) is nonpathogenic in its natural host, the sooty mangabeys. SIV-infection is associated with low immune activation even in the presence of high viral loads [104].

Many investigators had previously assumed that direct infection of CD4+ T-cells accounts for the selective CD4+ T-cell losses observed in AIDS patients. However, when it was clear that only a small portion of T-cells are actually infected by HIV-1 [105], it became obvious that mechanisms other than direct loss of infected CD4+ T-cells must play a role in the CD4+ T-cell depletion [106]. Indeed, HIV-1 infection induces widespread activation of uninfected bystander T-cells, leading to apoptotic cell death [107-109]. HIV caused non-antigen-specific T-cell activation [110, 111], drains the naïve and resting memory CD4+ T-cell pools, leading to failure of the T-cell homeostasis and subsequent T-cell depletion [112].

Furthermore, Schacker et al [113] showed that continued immune activation causes destruction of lymph node architecture, which is critical for supporting the normal T-cell homeostasis through interactions with APCs. The loss of normal architecture may shorten the half life of resting naïve and memory T-cell, limiting CD4+ T-cell recovery during suppressive ART [114].
In untreated HIV-1 infected patients, higher percentages of CD8+ T-cells expressing the activation marker CD38 predict rapid clinical progression to AIDS and death more strongly than CD4+ T-cell counts and plasma HIV-1 RNA levels [115, 116].

How does HIV cause T-cell activation? It has been suggested that the binding of gp120 to CD4 or coreceptor may induce cellular activation in the absence of direct infection [117, 118]. Also, continued activation of innate immune

responses may lead to elaboration of inflammatory cytokines, causing T-cell activation [119]. Chronic co-infections may promote further T-cell activation in the inflammatory milieu of HIV-1 infection [120-122]. Furthermore, microbial translocation is observed in HIV-1 infected patients with progressive disease, due to the defects in the gastrointestinal (GI) tract during the acute phase of infection [123]. Elevated levels of LPS are found in plasma during the chronic phase of infection, which suggest that bacterial derived products may contribute to the systemic immune activation.

2.4 Deactivation of immune response

HAART due to the effective suppression of viral replication decreases the level of immune activation and allows immune reconstitution in the majority of patients. By suppressing the virus and obstructing the immune activation, T-cells are restored and the immune system gets a chance to recuperate. However, the beneficial inhibition of immune activation with HAART, have consequences, such as leading to a rapid decline of HIV-1 specific CD8+ T-cell titers during the first months of efficient ART [124]. Therefore, a balance between activation and deactivation of the immune system must exist.

Immune-based therapies should help restore normal T-cell function in HIV-1 infected individuals, who have failed to do so with ART alone. The modulation of immune activation is one of the exciting approaches. Rizzardi et al [125] showed that the treatment with immunosuppressive agent cyclosporine A in combination with ART during early HIV infection, resulted in significantly higher CD4+ T-cells. However the larger study could not confirm these results in the chronically infected patients [126]. Similar attempts to decrease T-cell activation with immunosuppressive therapy (prednisone, hydroxylurea, mycophenolate mofetil and cyclosporine) have failed to show clinical benefit [127-130]. The reasons for this may be the suppressive effect by the agent on protective, antigen-specific immune responses.

The ideal immune-based therapy would block widespread non-antigen-specific T-cell activation during HIV-1 infection while preserving functional antigen-specific T-cell responses to foreign antigens.

3 High Mobility Group box protein 1 (HMGB1)

3.1 The protein

High Mobility Group (HMG) proteins consist of three families: HMGA, HMGB and HMGN [131]. The HMG proteins bind to DNA and mediate various biochemical functions, such as cell cycle regulation and gene expression, through condensing or unfolding of chromatin [132]. Currently there are four known members of the HMGB family in mammals: HMGB1-4 [133]. In addition to mammals, HMGB proteins are widely expressed in eukaryotic organisms [134].

HMGB1 was isolated in 1973 in an effort to better describe the specific regulators of gene expression, and identified as an abundant chromosomal protein with important structural functions in chromatin organization [135]. HMGB1 (also known as amphoterin) is a nuclear protein that binds to nucleosomes and promotes bending of DNA [132]. This structural change facilitates interactions between DNA and various proteins including p53, nuclear factor-κB (NF-κB), homeobox-containing proteins, recombination activating gene 1/2 (RAG1/2) proteins, and steroid hormone receptors. HGMB1 is an extremely mobile protein, resting on a specific DNA site for only fractions of a second. HMGB1 is ubiquitously present at variable levels in most nucleated cells [136], whereas the expression of HMGB2 and HMGB3 is more restricted. The HMGB2 proteins are widely present during embryonic development, and expression is limited to only testis and lymphoid tissue in adult mice [137], while HMGB3 seem to appear only during embryogenesis [138]. HMGB4 is expressed in late and round spermatids [139, 140].

HMGB1 is localized on the human chromosome 13q12 [141] and encodes a 215 amino acid (aa) polypeptide with a uniquely conserved sequence among species with 99% identity among rodent, bovine and human proteins [142]. Structurally, HMGB1 consists of two separate “boxes”, the A- and B-boxes, each containing ~80 amino acids in a L-shaped fold, followed by a cluster of 30 acidic residues in the Carboxy-terminus (Figure 5), [143, 144]. Several studies have identified the A-box as the non-pro-inflammatory domain, which competes

with HMGB1 for binding sites leading to attenuation of the inflammation cascade [145]. The B-box is important for many of the pro-inflammatory properties of HMGB1 including cytokine release [146, 147].

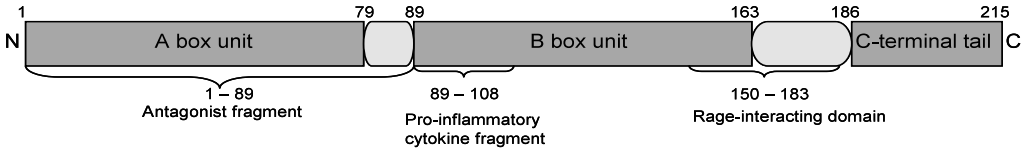

Figure 5: Structure of HMGB1.

HMGB1 was first implicated as an important endogenous signaling molecule in 1999 when Wang et al described the cytokine activity by the identification of HMGB1 as the late mediator of endotoxin-related lethality in mice [148]. This led to the increased interest of HMGB1 as an important component of the immune response. Also, the nuclear functions of HMGB1 are critical for the survival, as $HMGB1^{-/-}$ mice are born alive, but die within 24 hours (h) due to hypoglycemia [149].

The major cell surface receptor of HMGB1 is the receptor for advanced glycation end-products (RAGE). RAGE is a transmembrane protein expressed at low levels in normal tissues that is upregulated at sites where its ligands accumulate [150]. The receptor was first identified to bind advanced glycation end products (AGE) in diabetes, but has since been identified to bind other ligands and is involved in multiple inflammatory states [150, 151]. The RAGE-HMGB1 interaction promotes chemotaxis and production of cytokines in a process involving the activation of the transcription factor NF-κB, [152, 153]. Recently, members of the toll-like receptor (TLR) family have been implicated in the HMGB1 signaling. Generally, TLRs can recognize both damage associated molecular pattern molecules (DAMPs) and PAMPs, and hence are involved in immune response to both infection and injury. TLR2, TLR4 and TLR9 have all been concerned as HMGB1 receptors. TLR4 is most well known for its role in LPS recognition complex [154]. HMGB1-binding to TLR2 and TLR4 results in NF-κB upregulation [155-157] leading to cytokine release. There are speculations that HMGB1 does not act alone in the triggering of receptor activation. Tian et al [158] demonstrated that HMGB1-DNA complexes

activate TLR9 signaling. Other interactions with other cytokines such as lL-1β, IFN-γ and TNF-α also lead to an increased pro-inflammatory response compared to HMGB1 stimulation alone [159].

3.2 The liberation of HMGB-1

3.2.1 Passive and active release

There are two mechanisms for cells to liberate HMGB1 into the extracellular milieu. The first system describes the passive release of HMGB1 from damaged cells or during cellular necrosis. Here, HMGB1 represents an intracellular marker selected by the innate immune system to signal tissue damage and initiate reparative responses [160, 161]. Once in the extracellular environment, HMGB1 acts as an immune-stimulatory cytokine that indicates the extent of tissue injury [160], recruits mononuclear cells to clear cellular debris and protects against possible infection that often follows trauma [162]. Recently, studies have shown that late apoptotic cells can undergo secondary necrosis and hence release HMGB1 [163, 164].

The second mechanism, illustrate the active secretion of HMGB1 by immune cells such as monocytes, macrophages and dendritic cells [148, 165, 166]. Active secretion of HMGB1 is in general through non-traditional, leaderless pathways which are not routed through the classical endoplasmic reticulum (ER) or Golgi apparatus [167]. Upon stimulation with IL-1β, IFN-γ and TNF-α, macrophages, monocytes and pituicytes actively release HMGB1 in a time- and dose dependent manner [148, 168-170]. Activated macrophages/monocytes acetylate HMGB1 at potential nuclear localization sequences, leading to its cytoplasmic translocation and subsequent release into the extracellular environment [167, 168, 171]. When HMGB1 is not acetylated, it remains localized to the nucleus and is not secreted or released, such as claimed during apoptosis [160], subsequently confirming the necessity of translocation of HGMB1 from nucleus to cytosol for its active release.

Once HMGB1 is released, the production of pro-inflammatory cytokines is stimulated from macrophages, dendritic and endothelial cells and promotes inflammatory responses, reviewed in [165].

3.3 The role of HMGB-1 in inflammation

The two mechanisms described earlier allow HMGB1 to notify responding cells of immune activation or the presence of pathogens or cellular damage. The interest in HMGB1 extends far beyond sepsis and systemic inflammatory response. As a pleiotropic cytokine, HMGB1 is involved in inflammation, wound healing and tissue damage in various conditions from autoimmune, cardiovascular diseases, cancer and infections. HMGB1 can mediate a range of biological responses in diverse cell types and tissues. Low levels of HMGB1 produced under appropriately controlled circumstances can mediate "beneficial" responses that range from antibacterial activity to the activation of locally protective immune defense mechanisms and wound repair. High levels of HMGB1 cause acute damage because they mediate epithelial-barrier failure in several organ systems.

In late 1990s, Wang et al identified HMGB1 as a late mediator of endotoxin lethality in murine models [148]. They reported that HMGB1 was actively secreted by monocytes and macrophages after stimulation with LPS. In addition, passive immunization with anti-HMGB1 neutralizing antibodies increased the survival rate in mice sepsis model [172].

Enhanced HMGB1 mRNA concentrations are found in rats exposed to thermal injury [173], which is consistent with the proposal that HMGB1 is a prototypical DAMP, driving recruitment of inflammatory cells and tissue repair in the absence of other pathogenic signals.

Suda et al reported correlation between serum levels of HMGB1 and pre- and postoperative complications [174], and Yasuda et al [175] detected enhanced HMGB1 serum levels in severe acute pancreatitis. Increased levels of HMGB1 were found in cerebrospinal fluid in patients with bacterial meningitis [176], and in serum of cerebral malaria nonsurvivors [177], respectively. Data from our group showed increased levels in HIV-1 infected patients [178].

Still, either animal or human studies, have answered the questions about the origin and biological importance of circulating HMGB1 [179].

Depending on the activation status of the cells during the passive release of HMGB1, different isoforms may occur extracellularly. There is a great interest in the characterization of HMGB1 isoforms and their relative contribution to

infectious and inflammatory disorders [145]. Studies have also shown that extracellular HMGB1 can make complexes with IgG [180, 181], LPS, CpG [158, 182, 183], and IL-1b [159]. All these modified forms of HMGB1 influence the mode of receptor binding, the choice of receptor and the subsequent signaling cascade.

In the context of therapeutic targets, inhibition of HMGB1 secretion by e.g. ethyl pyruvate [184] or with antibodies against HMGB1 [148], have been reported. Blockade of the HMGB1-RAGE interaction is also a target of therapy [185]. Further characterization of the intracellular signaling pathways activated by HMGB1 and the isoforms contributing to a specific immune response may provide additional pharmacological targets to control inflammation.

3.4 The role of HMGB-1 in viral infection

HMGB1 has become more and more interesting in the field of pathogenesis of acute or chronic infectious diseases. West Nile encephalitis and acute hepatitis are viral-induced pathologies associated with necrotic cell death either via direct cytotoxic effects of the virus itself or the inflammatory response to viral infection [186]. Chu et al showed that in West Nile virus in vitro infection, HMGB1 release was associated with high but not low levels of infection, which mainly resulted in apoptotic cell death [187]. A number of viral illnesses including SARS and influenza result in the elevation of proinflammatory cytokines, that could be responsible for inducing the release of HMGB1 from inflammatory cells, and leading to the proliferation of the immune response [186]. Gaillard et al [188] have also shown that HMGB1 can be detected in HSV-2 infected HEC-1 cells by using a complex formation method of hemicatenated DNA (hcDNA) and HMGB1. Moreover HMGB1 is involved in the cross-talk between NK-DC cells during HIV-1 infection in vitro and may have a pivotal role in promoting viral dissemination in vivo [189].

4 Aims of the thesis

The general aim of this thesis was to study the soluble components of immune system during HIV-1 infection, with the focus on the HMGB1 protein.

Specific aims were:

To study the effects of extracellular HMGB1 on viral replication in acute and chronically HIV-1 infected cells (**Paper I**).

To analyze the systemic levels of HMGB1 in HIV-1 infected patients during different stages of the HIV-1 infection (**Paper II**).

To describe the kinetics of cytokines and chemokines in immune activation during primary HIV-1 infection and after antiretroviral treatment interruption (**Paper III**).

To assess the kinetics of HMGB1 release in relation to the cell death mode during HIV-1 infection in MT4 and CD4+ T cells (**Paper IV**).

5 Material and Methods

For detailed information about material and methods used in this thesis, see respective paper.

Ethical clearance

The studies included in thesis were performed after approval from the Institutional Review Boards and Ethical Committees at each participating site. All subjects included in respective paper gave their signed informed consent prior to study enrollment.

Statistical analysis

For details about statistical analysis used in this thesis, see respective study.

6 Results and Discussion

My focus in this thesis was to study soluble components of the immune system contributing to immune activation during the course of HIV-1 infection. To be able to gather as much understanding about HIV-1 pathogenesis and the immune activation as possible, I conducted studies both *in vitro* and *in vivo*. We had access to a very unique patient group – the Quest cohort, for the investigation of cytokine and chemokine pattern during early acute HIV-1 infection and after cessation of several years of successful HAART. Three of the papers presented in this work involve HMGB1 in different settings of the HIV-1 infection.

Paper I, II and IV: The properties of HMGB1 during different settings of HIV-1 infection

HMGB1 has been proposed as a molecule that could have an impact on the pathogenesis of viral infections, both related to viral cytopathic effects as well as activation of the immune cells [186, 187, 190-192]. This re-discovery of HMGB1 as a proinflammatory protein opened the doors to a growing new field of HMGB1 research. Hence, in vitro data implicated that HMGB1 could act as a cytokine with pleiotropic effect on monocytes and endothelial cells.

The initial data from mice sepsis model showed that interference with HMGB1 resulted in increased animal survival which brought attention to anti-HMGB1 agents as potential drug targets [148]. Further, reports that necrosis but not apoptosis lead to HMGB1 extracellular release [160] provided additional interesting mechanism linking the protein to innate and adaptive immune responses.

Our interest in studying the role of HMGB1 protein in HIV-1 infection was quite obvious. Thus, HIV-1 infection is a proinflammatory condition with excessive immune activation where cell death ratios are markedly elevated.

We performed both *in vitro* and *in vivo* studies, investigating if HMGB1 could play an important role in HIV-1 infection. At first, we studied if HMGB1 acting as an extracellular cytokine could affect the HIV-1 replication during the acute and chronic in vitro infection (Paper I). Additionally, we wanted to describe the mode of T-cell death during the acute HIV-1 infection and describe the potential link between the virus induced cell death and HMGB1 release (Paper IV).

To obtain a clinical relevance, *in vivo* experiments were conducted, whereas we analyzed plasma HMGB1 levels in samples from HIV-1 infected patients categorized into three different subsets, due to their immune status (Paper II).

Paper I: HMGB1 activates replication of latent HIV-1 in a monocytic cell-line, but inhibits HIV-1 replication in primary macrophages

In this paper we used U1 cell line, an accepted model of latent HIV-1 infection of monocytic cells [193], ACH-2 cells which are of T-lymphocytic origin and HIV-1_{BAL} infected MDMs, to evaluate the effect of HMGB1 on HIV-1 replication *in vitro*. Recombinant HMGB1 in concentration of 1 μg/ml was added to cultures. Cell viability was assessed and also Reverse Transcriptase (RT) activity was measured. The chemokines Rantes, MIP-1a and MIP-1b were quantified by in-house ELISA and a multiplex assay, respectively. Statistical analysis was performed using GraphPad Prism 4.0.

HMGB1 upregulates HIV-1 replication in U1 cells, but not in ACH-2 cells

The viral replication was measured in the supernatants of U1 cell cultures 72h after HMGB1 exposure. HMGB1 was found to upregulate the RT production three fold in U1 cells as compared to unstimulated controls. Also, the stimulatory effect of HMGB1 on HIV-1 replication in U1 cells was dose dependent. We also showed that the stimulatory effect by HMGB1 on the viral replication was negatively influenced by supplementing the cultures with dexamethasone. This suggests that the downstream pathway of HMGB1 stimulation of U1 cells involves utilization of the NF-κβ complex. On the contrary, the viral RT production in the other chronically infected cell line, ACH-2, was not affected by the addition of extracellular HMGB1 as compared to unstimulated controls. This observation is in line with earlier stated findings of lymphocytic cells [194]. In U1 cells, HMGB1 acts as a proinflammatory cytokine and induces viral replication. This is further supported by Thierry et al [195], where divergent findings concerning the effect of HMGB1 in HIV-1 replication in ACH-2 cell cultures were stated. One possible explanation of this apparent discrepancy might be different basal levels of viral expression as well as different read-outs used in our experiments. As T-cells do express RAGE receptor, the possibility of such interaction can not be neglected.

Viral replication in MDMs was suppressed by chemokines

The stimulation of acutely HIV-1 infected MDMs (isolated from PBMCs) with HMGB1 resulted in a decrease of the HIV-1 production as compared to unstimulated cells. To assess if this downregulation was associated with an increased production of chemokines, we measured the concentration of Rantes, MIP-1α and MIP-1β. Indeed, we detected significantly increased levels of the three chemokines present already at 24h after HMGB1 stimulation. These chemokines have been attributed inhibitory effect on HIV-1 replication, acting not only during the HIV-1 entry but also during later stages of the viral cycle. It is rational to conclude that HMGB1 induced increased chemokines levels resulted in downregulation in HIV-1 replication in MDMs. The presented double mode of action on HIV-1 replication is not unique for the HMGB1 protein as similar findings have been observed for TNF-α [196], LPS [197] and other cytokines and chemokines depending on the cell system and surrounding environment [79, 198].

Paper II: Elevated plasma levels of high mobility group box protein 1 in patients with HIV-1 infection

HIV-1 infected patients had elevated HMGB1 levels as compared to controls

In paper II, we measured the plasma levels of HMGB1 in 14 healthy individuals and 43 treatment naïve HIV-1 infected patients using a commercial ELISA. The HIV-1 infected patients were divided into three categories due to the preservation of their immune system. Group A (n=14) was defined as patients with preserved immune status (>600 CD4 T-cell/μl; low VL, median 350 copies/ml); group B (n=13) with deteriorated immune status (<400 CD4 T-cells/μl; high VL, median 347 000 copies/ml); group C (n=16) with deteriorated immune status ($\leq$300 CD4 T-cells/μl; high VL, median 541 000 copies/ml) and opportunistic conditions [199]. Plasma HIV-1 RNA levels and peripheral blood CD4 and CD8 cell count were quantified by routine methods.

We found higher HMGB1 plasma levels in HIV-1 infected patients as compared to uninfected ($p<0.001$). Interestingly, patients with opportunistic conditions had higher HMGB1 levels as compared to group A and B, separately. No significant difference was found between group A and B. In addition, no significant difference was seen between plasma HMGB1 levels and sex, age, viral load, CD4 or CD8 T-cell counts, respectively.

The presence of HMGB1 in plasma of HIV-1 infected patients with the highest concentrations in those with clinical complications and highest VL could be explained by the excessive immune activation. Thus, both active (due to increased proinflammatory milieu) and passive (increased cell death) HMGB1 release from cells could contribute to systemically elevated levels. Although, we could not see correlation between the CD8+ T-cells counts and HMGB1, analysis of LPS plasma levels in this patient group, have shown similar distribution as HMGB1 in the three patient categories (data not included). That confirms differences in immune activation levels in this patient setting. However, in our study there was no direct correlation between LPS and HMGB1 levels, which implicates that the picture is more complex. The clinical relevance of elevated systemic HMGB1 levels in HIV-1 infection should be further investigated in the context of ART.

Paper IV: Release of HMGB1 during HIV-1 infection in vitro; implications on the cell death mode

The aim of the study was to characterize the mode of cell death and events occurring during the liberation of HMGB1 in HIV-1 infection in vitro. We infected MT4 cell line and CD4+ T-cells with HIV-1 isolates and monitored the release of HMGB1 in relation to cytopathic effect and apoptosis during the course of infection. Moreover, we evaluated the effect of a broad caspase inhibitor on HMGB1 release in both cell systems. HMGB1 release from cells was analyzed by Western Blot, ELISA and ELISPOT assay which was adapted to detect the protein release in MT4 cell system. The disintegration of outer membrane was confirmed by increasing Lactate dehydrogenase (LDH) activity in cell supernatants. Flow cytometry was used to measure the degree of infection, apoptosis events and caspase 3 (C3) activation.

High grade of HIV-1 infection and apoptosis in MT4 cells, but not in CD4+ T-cells

At 48h post infection (p.i.) with HIV-1_{HXB2}, >90% of the MT4 cells were positive for HIV p24 antigen. CPE were visualized at 60h p.i. and at 72h two distinct cell populations were observed at the forward scatter-side scatter (FSC-SSC), indicating increased death of the infected cells. LDH activity in cell supernatants showed 0.2-fold increase between 24-48h, and 2-fold increase from 24h to 72h.

During the course of infection, the caspase 3 activity was increasing indicating increased apoptosis process, corresponding to the cell morphological changes. In the primary cell system, we were not able to induce as high grade of HIV-1 infection as in the MT4 cell system, and the caspase 3 activity was lower in HIV-1 infected CD4+ T-cells.

Subsequent release of HMGB1

The kinetics of the HMGB1 release were characterized by Western blot, whereas the HMGB1 release started at 54h, was substantially higher at 60h, and increased thereafter in a time-dependent manner. The uninfected controls had only a weak signal throughout the infection. The same observations concerning HMGB1 and LDH release were seen in acutely infected CD4+ T-cells.

The ELISPOT assay showed higher levels of secreted HMGB1 spots in the HIV-1 infected wells at 72h p.i. (40±14 spots) as compared to 48h p.i. (5-10 spots). Necrosis due to osmotic cell damage resulted in 234±14 spots. The spots detected during the passive HMGB1 release were larger and more diffuse as compared to spots due to LPS stimulated RAW cells.

Z-vad inhibits the caspase 3 activity and the release of HMGB1

In order to evaluate if the cell death in our model can be modulated, we treated MT4 cells with broad caspase inhibitor Z-vad. With 50 μM of Z-vad we managed to inhibit the disintegration of cells into two populations and the C3 activity as compared to non-Zvad treated infected cells at the different timepoints. Furthermore, Z-vad treatment resulted in reduction of LDH and RT

activity in corresponding supernatants, and in western blot analysis HMGB1 release was substantially inhibited after adding Z-vad at 24h or 40h p.i. as compared to controls. In CD4+ T-cell system, treatment with Z-vad had effect on the C3 activation but not on the HMGB1 release.

Data from the present study show clearly that infected T-cells can passively release HMGB1 during membrane disintegration and cell death in connection with HIV-1 replication. As described by Wahamaa [200], the ELISPOT method detects HMGB1 forms released by active and passive processes or necrosis as in our system.
Due to the inhibitory effect by Z-vad on the cell death and HMGB1 release, the relationship to HIV-1 induced apoptosis can be established. The differences between the HMGB1 release and Z-vad effect in MT4 cell system and primary CD4+ T-cells can be explained by the differences in cell infectivity and cytopathicity of the HIV-1 strains used in these experiments.

In earlier studies, the release of HMGB1 was reported to only occur during necrotic cell death [160, 187, 190]. However, other groups have recently reported that apoptosis also lead to HMGB1 release [163, 201]. Furthermore, in conditions of systemic lupus erythematosus (SLE), non-ingested apoptotic cells may undergo secondary necrosis leading to the release of HMGB1 in complexes with nucleosomes [202].

One of the strategies developed by HIV-1 to escape the host immune system involves both necrosis and apoptosis that destroys the immune effectors [203-205]. HIV-1 induced, bystander CD4+ T-cell killing by apoptosis is recognized as central to immunodeficiency [206] driven by excessive inflammation. As necrosis is a frequent condition in organs targeted by opportunistic diseases during AIDS, this could also be an additional source of HMGB1 release during late stage of HIV-1 disease.

With the presented data, we suggest that passively released HMGB1 should be considered as a molecule connecting cell death with immune activation not only at the systemic levels but also at local compartments where HIV-1 replication occurs.

Paper III: Kinetics of plasma cytokines and chemokines during primary HIV-1 infection and after analytical treatment interruption.

The early events occurring during the primary phase of HIV-1 infection, predict the outcome of disease [207, 208]. The treatment strategies which aimed to hit ‘’strong and early’’ were designed to decrease the initial damage to the immune system and to prevent the saturation of viral reservoirs. However, the underlying scientific evidence was not consistent concerning the time frame of the therapy initiation due to the lack of clinical studies during PHI.

Several studies have earlier addressed the question about the role of cytokines during HIV-1 infection [209-211]. However, the knowledge about kinetics of cytokines and chemokines involved during the PHI phase and after analytical treatment interruption (ATI) was incomplete. We had access to sequentially taken samples from the first placebo controlled vaccination trial during PHI, the Quest cohort [212]. The Quest study is unique, as the participants were enrolled at a very early stage of infection, and started HAART immediately after diagnosis.

In this study we measured 13 different cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-10, IL-12 and IL-15) and chemokines (MIP-1α, MIP-1β, Rantes, MCP-1 and Eotaxin) in plasma of 22 HIV-1 infected individuals during PHI and after analytical treatment interruption (ATI). The selection of patients in the present study was based on the virological outcome and available samples at the end of the study, i.e. 24 weeks after stopping ART. The subjects were categorized into two groups; responders (R, n=10) with viral load (VL) levels <1000 HIV-1 RNA copies/ml and non-responders (NR, n=12) who had VL >9 000 copies/ml [213].

The increased immune activation during PHI resulted in worse outcome after ATI

Throughout PHI, the NR had significantly increased levels of HIV-1 RNA and IFN-γ, TNF-α, IL-1β, IL-10 and Eotaxin, as compared to R. This was also confirmed when concentrations were compared at different time-points during PHI. During the ATI phase the pattern changed; R had significantly higher

levels of MIP-1β, MCP-1 and IFN-γ levels as compared to NR, while the R had higher concentrations of IL-15 and Eotaxin.
Furthermore, we could not detect any significant differences in terms of cytokine/chemokine profiles between vaccinated and non-vaccinated patients after ATI.
When comparing the cytokine and chemokine levels at the time of the highest VL between the two phases we found significantly increased levels of IFN-γ, TNF-α, IL-10 and all chemokines, except of MCP-1, during PHI as compared with ATI.

The cytokine and chemokine profiles presented during PHI and ATI show an intense immune activation in NR during the acute phase as compared to R. This immune activation is probably not a lack of a certain immune mediator as seen in other infections [214, 215], rather a result of the excessive viral replication during PHI. An important aspect to assess is whether genetic reasons [216] can determine the degree of immune activation among R. Betts et al suggested that polyfunctional CD8+ T cells characterized by strong production of IFN-γ, TNF-α, IL-12, MIP-1β is negatively correlated with disease progression [217]. Our data are in line with this theory.

In conclusion, the data presented in this study add new insight concerning the kinetics of cytokines and chemokines during PHI and after cessation of ART. We showed that the enhanced levels of immune activation during the PHI in non-responders were associated with poorer control of VL despite two years of successive ART.

7 Concluding remarks

1) Extracellular HMGB1 stimulates dose dependently HIV-1 replication in the latently infected U1 monocytic cell line. The effect was not present during stimulation of lymphocytic ACH-2 cells. In acutely HIV-1 infected monocyte-derived macrophages, HMGB1 downregulated the viral production. The increased β-chemokine production due to HMGB1 stimulation could feasible explain the inhibitory effect.

2) HIV-1 infected patients with deteriorated immune status and opportunistic conditions had significantly elevated plasma HMGB1 levels, as compared to uninfected individuals and HIV-1 infected patients with less deteriorated immune status. The finding suggests that HMGB1 could contribute to immune activation in vivo during HIV-1 infection.

3) The primary HIV-1 infection is associated with excessive production of cytokines and chemokines. The immune activation levels at PHI affects the functionality of the immune system after the ATI phase, and does not seem to be modulated by early introduction of ART.

4) HMGB1 is released during the HIV-1 infection of T-cells in connection with cell death processes, both during apoptosis and necrosis.

Populärvetenskaplig sammanfattning

Humant immunbrist virus typ 1 (HIV-1) orskar en kronisk infektion som obehandlat leder till grav immunbrist och död. Detta tillstånd kallas AIDS. Viruset smittar via blod, sexuellt umgänge och från mor till barn framför allt vid förlossning. HIV-viruset upptäcktes 1983, och över 60 miljoner människor beräknas ha infekterats och 25 miljoner har dött, enligt WHO. HIV-1 har från början överförts till människa från chimpanser i Afrika, vilken är den kontinent som är mest drabbad.

Den viktigaste målcellen för HIV-1 är en typ av vita blodceller, T-hjälparceller (=CD4+ T-celler), som är centrala för immunförsvarets funktion. I en HIV-1 infekterad cell bildas flera miljoner nya viruspartiklar, vilka i sin tur kan infektera nya celler. Idag finns mycket effektiva bromsmediciner som tas flera tillsammans i en kombination. Infektionen botas inte, men patienten får i det närmaste ett normallångt liv. De vaccinstudier som prövats hittills, har misslyckats och något vaccin är inte i sikte.

Vårt immunsystem delas upp i två delar; det medfödda (innate) som är ospecifikt, och det förvärvade (adaptive) som reagerar mer specifikt. HIV aktiverar båda dessa system och ett stort antal signalmolekyler utsöndras därmed från cellerna på ett till slut okontrollerat sätt vilket är en viktig orsak till att immunbrist uppstår.

I denna avhandling ingår fyra delarbeten. Ett antal lösliga signalmolekyler (cytokiner/chemokiner) studeras då de kan bidra till den intensiva immunaktiveringen under HIV-infektionen. Störst fokus har legat på proteinet high mobility group box 1 protein (HMGB1). HMGB1 har många funktioner beroende på om den är lokaliserad i cellkärnan eller om den utsöndrats från cellen. Proteinet finns i alla celler med kärna, mest i en viss typ av immunförsvarsceller som kallas för makrofager.
Vi har undersökt HIV-1 patienter som fått behandling väldogt tidigt som några veckor efter att de smittats, s.k PHI-fasen. Våra resultat visar att de HIV-infekterade patienterna som hade lägst virus nivåer i början av infektionen hade svagare

immunaktivering bestämt utifrån cytokin/kemokin mönstret. Detta visar att en hög immunaktivering under PHI, inte bidrog till ett bättre försvar mot infektionen.
I de andra delarbetena har vi undersökt hur HMGB1 beter sig under olika omständigheter. Effekten av HMGB1 studerades på tre HIV-1 infekterade cell linjer som representerar en sovande (”latent”) infektion i två typer av vita blodkroppar (”monocyter” och "lymfocyter”), respektive en aktiv infektion i en annan monocyt-celltyp, ”makrofager”. Vi fann att HMGB1 satte igång den sovande infektionen i monocyterna men inte i lymfocyterna. Däremot såg vi att HMGB1 hämmade den aktiva infektionen i makrofagerna. Dessa fynd visar att HMGB1 reglerar HIV infektionen i den viktiga celltypen monocyter på olika sätt beroende på om infektionen är sovande eller inte.
Efter att ha gjort dessa provrörsförsök, studerade vi även blod från HIV-1 infekterade patienter med olika grader av immundefekt. I de patienter med sämst immunförsvar och allvarliga infektioner och tumörer, fann vi en ökad mängd HMGB1 i blodet, jämfört med personer utan HIV och HIV-patienter med bättre immunförsvar. Efter att ha gjort dessa fynd på patienter ville vi försöka förstå på vilket vis HMGB1 släpps ut i blodet när en cell dör. Vi kunde påvisa att HGMB1 släpps ut på de båda sätten som cellen dör på, d.v.s. vid nekros och apoptos. Vi tror att detta kan vara av stor vikt för immunaktiveringen vid HIV-1 infektion.

Sammanfattningsvis, bidrar våra fynd till ökad förståelse för hur immunaktiveringen sker under HIV´s sjukdomsförlopp, och särskilt hur HMGB1 verkar. Vi anser att HMGB1 är ett potentiellt mycket viktigt protein för utvecklandet av immunbrist vid HIV-1 infektion.

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